Micropolarities of Lipid Bilayers and Micelles. 3. Effect of Monovalent Ions on the Dielectric Constant of the Water Membrane Interface of Unilamellar Phosphatidylcholine Vesicles

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to the values of $[A]_s°$ for each donor, demonstrating that there is long-range energy transfer. In each case and especially with pyrene $[A]°$ is less than $[A]_s°$, implying that the energy transfer is more efficient than predicted. Similar differences are evident in the data reported by Lisovskaya et al.[27] for doublet quenching of singlet excited states. This difference was not noticed in the work done in acetonitrile with TCNQ⁻·[4] and is in opposition to what Faulkner noted with fluoranthene and TMPD⁺· in acetonitrile where they report lower efficiencies than were expected but blamed this to an artifact due to the data analysis.[1] Inasmuch as Förster's predictions are only an approximation, using dipole–dipole interactions to represent the total Coulombic interaction, we perhaps should not expect better agreement. Still, to explain experimentally the differences in the case of pyrene, for example, our value for the product $\phi_D J$ would have to be in error by 100%. Fortunately critical distances, $R°$, which are the most commonly used parameters for the measuring of energy-transfer efficiency depend on the cube root of $[A]°$ and therefore the error introduced by using energy transfer as a ruler should not be too

great. We have evaluated $R°$ and $R_s°$ using the equation[10]

$$R° = 7.66[A]°^{-1/3} \tag{7}$$

where $R°$ is in angstroms and the concentration in moles per liter. The values are presented in Table I for comparison with other published data.

In conclusion, this study demonstrates with certainty, whereas low viscosity experiments have only implied, that long-range energy-transfer quenching of aromatic hydrocarbons by ion radicals does occur. It shows that the fluorescence quenching can be predicted approximately from spectral parameters and demonstrates the approximate nature of the relationship between the values predicted and those actually measured.

Registry No. TCNQ⁻·, 34507-61-4; 9,10-DPA, 1499-10-1; fluoranthene, 206-44-0; pyrene, 129-00-0.

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Micropolarities of Lipid Bilayers and Micelles. 3. Effect of Monovalent Ions on the Dielectric Constant of the Water–Membrane Interface of Unilamellar Phosphatidylcholine Vesicles

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A study was undertaken of the effect of monovalent cations (Li^+, Na^+, K^+) on the dielectric constant (ϵ) of the water–lipid interface of unilamellar phosphatidylcholine (PC) vesicles, i.e., the ester carbonyl oxygen region of the PC molecules or the neighborhood of the oxygen atoms of the phosphorylcholine moiety. ϵ was determined according to a method developed previously by Fragata and Bellemare where the free radical 1,1-diphenyl-2-picrylhydrazyl is reacted with α-tocopherol incorporated in the lipid vesicles. The results are consistent with a decrease of ϵ (LiCl: 35.5 to 29.5; NaCl: 34 to 29; KCl: 33 to 29) as the concentration of the salts in the solvent media increases from 0.025 to 0.5 M. These effects can be rationalized in terms of dielectric saturation at the water–lipid interface brought about by ion-induced local electric fields. We observed, furthermore, that in the unilamellar PC vesicles the effect of the ions on ϵ follows the sequence $K^+ > Na^+ > Li^+$ which contrasts strikingly with what happens in the liquid state where this is $Li^+ > Na^+ > K^+$ instead. Although there is not as yet any convincing explanation of these differences, we hypothesize tentatively that they are related to the degree of hydration of the dissociated ions at the water–lipid interface which must differ in a considerable way from that in the liquid state.

Introduction

Monovalent ions are known to modify the dielectric constant (ϵ) of aqueous solutions.[1-4] It was observed that ϵ decreases with increasing concentrations of the ionic species in solution. This

effect is explained[2] by a reorientation of the dipole moment of the water molecules in the neighborhood of the ions up to a distance of about 2 Å from their atomic centers. In this region the dielectric constant of water is thereby much smaller than 78 (at 298 K), say a value between 4 and 6 (cf., e.g., Figure 1 of ref 2). This is interesting since on the one hand the dielectric constant of the solvent medium has an effect on the yield of biological oxidation–reduction reactions,[5-9] and on the other monovalent ions

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are able to adsorb at the water–lipid interfaces of bilayer membranes (see, e.g., ref 10). A simple working hypothesis is that the ions affect the polarity (ϵ) of the interfacial region and thereby the rates of electron transfer of surface and intrinsic membrane components. As a step toward the elucidation of these questions we report here a study of the effect of monovalent cations (Li^+, Na^+, K^+) on the dielectric constant of the water–lipid interface of unilamellar phosphatidylcholine (PC) vesicles.

The dielectric constant of the interface headgroup-hydrocarbon core of the PC vesicles was determined previously[11,12] by a reaction between the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) and α-tocopherol (αT) incorporated in lipid vesicles. This same method was used in the present work (see Experimental Procedures) to investigate the ionic effects on ϵ at the site of reaction of αT with DPPH, i.e., the ester carbonyl oxygen region of the PC molecule[13] or the neighborhood of the oxygen atoms of the phosphorylcholine moiety.[14] We observed that the results obtained with the three ionic species (Li^+, Na^+, K^+) are in general consistent with a decrease of ϵ at the water–lipid interface as the ionic strength of the solvent medium increases. It will be seen that this effect can be rationalized in terms of dielectric saturation at the water–lipid interface brought about by ion-induced local electric fields if the transition-state complex of the αT–DPPH reaction has a dipole moment equal to or greater than about 6 D (which corresponds to a dipole length of ~ 1.2 Å), or else the dielectric constant of the interface where the αT-DPPH reaction takes place is lower than the values determined in the present work. The latter alternative is clearly related to dehydration of the interfacial region[15,16] near the site of the αT-DPPH reaction. This is discussed hereunder.

Experimental Procedures

Preparation of Phosphatidylcholine–α-Tocopherol Vesicles. Egg yolk phosphatidylcholine was isolated and purified according to the method of Singleton et al.[17] The purity of the lipid was checked by thin-layer chromatography (TLC) on silica gel G (Sigma Chemical Co.) (Chloroform:methanol:water, 65:25:4) using the Dragendorf reagent or iodine vapor. α-Tocopherol was a product of Eastman Kodak Co. Its purity was verified by TLC on silica gel G (benzene:ethanol, 99:1) using a $K_3Fe(CN)_6$ reaction followed by $FeCl_3$.

The PC–αT vesicles were prepared as follows. Approximately 150 mg of PC was mixed with αT in $CHCl_3$ (AR grade). The initial molar ratio PC:αT was 20:1. After solvent evaporation under a current of nitrogen, the dried lipids were dispersed in 10 mL of Tris-HCl buffer (0.01 M, pH 8.0) containing appropriate amounts of salts (see Results). The mixture was then sonicated 15–20 min at 20 °C in a sonifier cell disrupter (Heat Systems-Ultrasonics) set at about 20-W output, having nitrogen bubbling into the solution. The mixture was then centrifuged at 100 000g for 1 h and the supernatant was concentrated to 1–2 mL in a Amicon cell. This was followed by fractionation of the lipid dispersion in a Sepharose-4B column (Pharmacia Fine Chemicals, 2.5 × 60 cm) and elution with the above described buffer containing appropriate amounts of salts to keep iso-osmotic conditions.
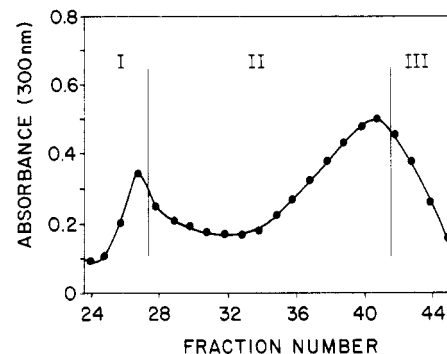

Figure 1. Typical elution profile of phosphatidylcholine–α-tocopherol vesicles prepared by ultrasonic dispersion in 0.01 M Tris-HCl buffer (pH 8.0) followed by centrifugation (100 000g, 1 h) and gel filtration in a Sepharose-4B column.

Then, 5-mL fractions were collected. Only homogeneous fractions (class III vesicles; see Figure 1) constituted of unilamellar vesicles were retained for reaction kinetics studies.

All salts (Fisher Scientific Co. Certified ACS) were purified by washing successively with three different organic solvents (methanol, benzene, chloroform) to remove organic impurities. The salts were then dried at 150–160 °C for 24 h to remove any traces of adsorbed solvent. The degree of purification of the salts obtained by this procedure was checked by surface tension experiments. It was observed that the surface tension values of the purified salts dissolved in water (demineralized, distilled) were in the range of values published in the literature (e.g., "Handbook of Chemistry and Physics", 61st ed., CRC Press, Boca Raton, FL, 1980–81, p F-43).

αT–DPPH Reactions in Ethanol–Water and Dioxane–Buffer Mixtures. For reactions in ethanol–water, DPPH (BDH Chemicals, Lot 58625300 and 60035100) was dissolved in ethanol (2 mg/mL) and an aliquot of the solution added to different binary mixtures of ethanol with H_2O (75, 90, 93, 95, and 100% ethanol, v/v). Note that the degree of purity of DPPH may differ from lot to lot. The removal of residual impurities eventually present in the bulk product is however a very difficult task since on the one hand this free radical adsorbs strongly to the chromatography substrate and on the other its recrystallization yield is extremely low. Therefore, each set of experiments was always performed with DPPH originating from the same lot in order to minimize the effect of side reactions, if any, on the statistical dispersion of the data. αT was dissolved in ethanol (0.2 mg/mL) and 0.1 mL of this solution was reacted with 3 mL of the DPPH–ethanol–water mixtures. The final concentration of DPPH was at least 10 times greater than that of αT. The mixtures were incubated at about 22 °C, and the course of the reaction was followed at 516 nm (cf. Figure 2 of ref 11) in a Perkin-Elmer fast scan spectrophotometer, Model 553. Pseudo-first-order and second-order reaction rate constants were then computed in a Perkin-Elmer 3600 Data Station.

For reactions in dioxane–buffer, DPPH was added to a mixture of dioxane and a Tris-HCl, 0.01 M, pH 8.0 (61% v/v) solution (2 mg/mL) containing different amounts of NaCl. All other conditions were identical with those described above for αT–DPPH reactions in ethanol–water solutions.

αT–DPPH Reactions in Phosphatidylcholine Vesicles. DPPH was dissolved in ethanol (2 mg/mL) and a 0.6-mL aliquot of the solution was added to 100 mL of Tris-HCl buffer, 0.01 M, pH 8.0. It was observed that in general the absorbance of the above DPPH–ethanol–buffer solution is about 0.250. This condition is important for determinations of pseudo-first-order rate constants of the αT-DPPH reaction in the αT–PC unilamellar vesicles. To measure this reaction a 350-μL aliquot of vesicles suspensions (class III vesicles; cf Figure 1) was reacted with 3 mL of the DPPH–ethanol solution. Note, in this respect, that the ethanol concentration in the final reaction mixture never exceeded 1% (v/v). This low ethanol concentration was shown previously[11] not to affect the vesicles' integrity. It is remarked in addition that

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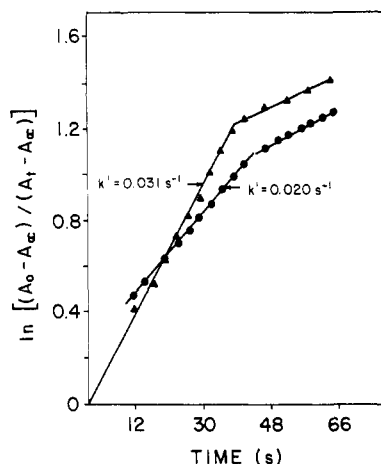
Figure 2. Time dependence of the reaction of 1,1-diphenyl-2-picryl-hydrazyl with α-tocopherol in ethanol–water (90% v/v) (▲) and in phosphatidylcholine vesicles (●) in presence of 0.3 M NaCl. k', the pseudo-first-order rate constant, is given by $k't = \ln\,[(A_0 - A_\infty)/(A_t - A_\infty)]$, where A_t is the absorbance at time t, A_0 the initial absorbance, and A_∞ the absorbance at reaction completion.

the concentration of DPPH in the reaction mixture was at least 10 times greater than the αT concentration. All other conditions were identical with those described above for αT–DPPH reactions in ethanol–water solutions.

Results

Characterization of Phosphatidylcholine–α-Tocopherol Vesicles. A typical elution diagram of a dispersion containing single- and multilayered PC–αT vesicles is shown in Figure 1. The vesicles were obtained upon sonication of binary mixtures of the lipids followed by centrifugation (100000g, 1 h) and gel filtration in a Sepharose-4B column. The diagram consists of three main classes of fractions constituted of large multilamellar vesicles (class I), single lamellar vesicles about 200–250 Å in diameter (class III; fractions 42–45), and a nonhomogeneous population constituted partly of type I and type III vesicles (class II). The diameter of the single lamellar vesicles in the range 200–250 Å was obtained on the basis of K_{av} (gel filtration) and trapped volume determinations. This range of values is in good agreement with results published in the literature (cf., e.g., ref 24 and 36). Nevertheless, as we are interested in small interfacial volume variations (see Discussion) that cannot be detected by electron microscopy or light scattering experiments, we pursue at present hydrodynamic studies of the vesicles' sedimentation coefficients corrected for the specific volume of the polar headgroup of the lipids in aqueous solutions of differing ionic concentrations (C_i). We expect that this will enable us, in conjunction with trapped volume determinations referred to above, to ascertain with reasonable precision predicted variations of volume of the lipid–water interfacial region as a function of C_i (F. Bellemare, N. Lacroix, and M. Fragata, work in progress).

Note in addition that the αT concentration of class III vesicles is proportional to the PC content of the membranes (cf. Figure 3 of ref 11 and Figure 3 of ref 18). Only these vesicles were used for αT–DPPH reaction kinetics studies.

Polarity Determinations in Solution and in PC–αT Vesicles. This was done according to a method described in detail elsewhere.[11] In brief, one makes use first of the reaction of αT with DPPH in pure solvents or in their mixtures with H_2O, and then compares the results with data obtained from reactions of DPPH with αT included in lipid bilayer vesicles (see Experimental Procedures). It has been shown[19] that the αT–DPPH reaction is essentially biphasic (cf. Figure 2, and also Figure 4 of ref 11).
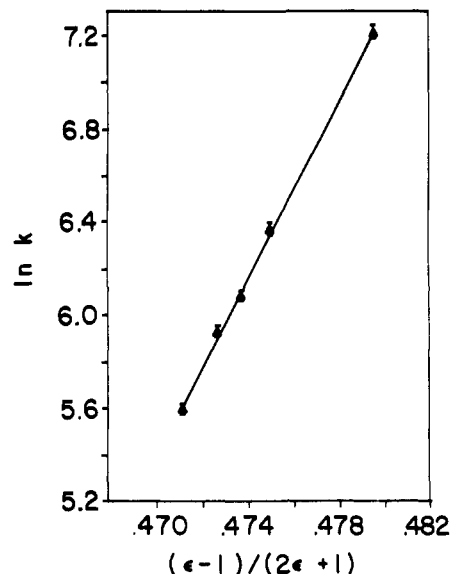

Figure 3. Natural logarithm of second-order rate constant k vs. $(\epsilon - 1)/(2\epsilon + 1)$ for reactions of 1,1-diphenyl-2-picrylhydrazyl with α-tocopherol in methanol and binary mixtures of ethanol and H_2O. The straight line was obtained by linear least-squares curve-fitting program, and is represented by eq 3 given in the text. The vertical bars are ±SD.

It starts with a fast reaction step that lasts for about 30–40 s and is the result of the formation of a chromanoxy radical,[19] and is then followed by a slower reaction where there is rearrangement of the radical and dimerizations or trimerizations.[20] To calculate the pseudo-first-order rate constant (k') of the αT–DPPH reaction in ethanol–H_2O mixtures we used the expression (cf. Figure 2)

$$k't = \ln\,[(A_0 - A_\infty)/(A_t - A_\infty)] \qquad (1)$$

where A_t is the absorbance at time t, A_0 the initial absorbance, and A_∞ the absorbance at reaction completion. The second-order rate constant (k) is then given by

$$k' = k[\text{DPPH}]_i \qquad (2)$$

where $[\text{DPPH}]_i$ is the initial concentration of DPPH present in the reaction medium. The molar extinction coefficients at 516 nm of DPPH in aqueous solutions with ethanol were determined previously (cf. Figure 1 of ref 18).

Note, in addition, that the application of the present method to lipid bilayers is possible on account of two main reasons: first, observation that the fast step of αT–DPPH reaction is sensitive to polarity variations of the solvent media (cf. Figure 3) provided that ϵ is greater than about 15–20 (for a recent discussion on this matter see ref 12); second, the recognition that the OH group of the chromanol ring of αT, which incidentally is the site of its reaction with DPPH during the fast step,[11,19] is also a site of hydrogen-bond formation with either the ester carbonyl oxygen of the PC molecule[13] or the oxygen atoms of its phosphorylcholine moiety.[14] These properties assure that one measures the polarity of the water–lipid interface in the vicinity of the hydrogen-belt region[22] of the bilayer membranes.

Figure 3 displays the dependence of $\ln k$ on $(\epsilon - 1)/(2\epsilon + 1)$, i.e., the Onsager reaction field parameter.[23] $(\epsilon - 1)/(2\epsilon + 1)$ is a measure of the polarity of the surroundings of the site of the αT–DPPH reaction. Extensive discussions of this matter were given before (see ref 7, 8, 11). The straight line of Figure 3 was obtained by linear least-squares curve-fitting program, and is represented by the equation

$$\ln k = -85.19 + 192.72[(\epsilon - 1)/(2\epsilon + 1)] \qquad (3)$$

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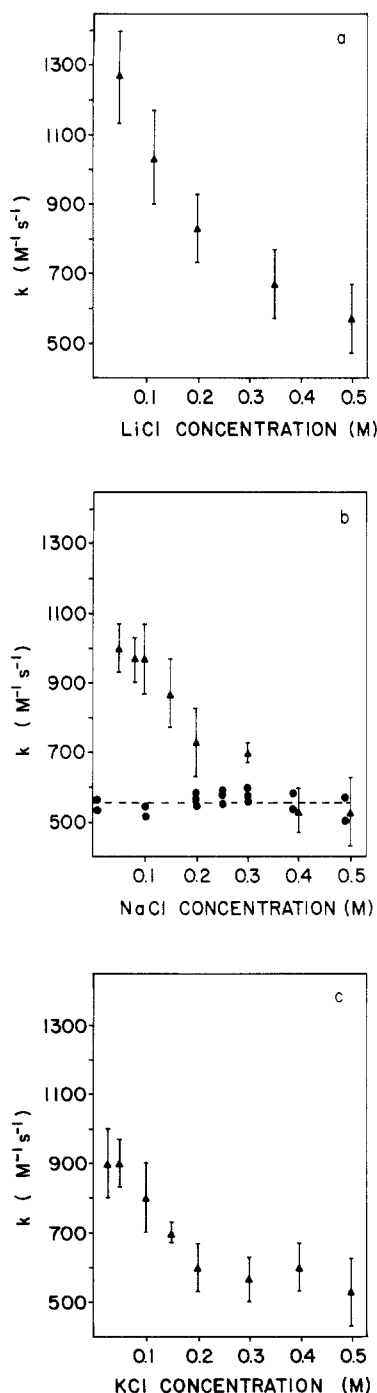
Figure 4. Effect of concentration of LiCl (a), NaCl (b), and KCl (c) on the reaction of 1,1-diphenyl-2-picrylhydrazyl with α-tocopherol in phosphatidylcholine vesicles (▲) and in a mixture of dioxane and Tris-HCl (0.01 M, pH 8.0) buffer (●; the straight line represents the average of all data points; i.e., $k \sim 555$ M^{-1} s^{-1}). The vertical bars are ±SD.

The linear regression correlation coefficient was calculated to be greater than 0.99. Note that these results differ slightly from those obtained previously by Bellemare and Fragata.[11] This is possibly because of the presence in DPPH of residual impurities which are not easily removed (see comments in Experimental Procedures). Equation 3 is used in the next section to determine the polarity of the water–lipid interface of the unilamellar PC vesicles. To this end, k for the reaction between DPPH and αT incorporated in the lipid vesicle was first obtained. Then, ϵ is calculated from eq 3.

It is worth noting at this point that in lipid bilayer vesicles containing αT one observes a very fast step during the first 5–10 s of the DPPH reaction (Figure 2). There is not as yet any plausible explanation of the phenomenon. One possible hypothesis is that the lipid–water interface of the unilamellar PC vesicles
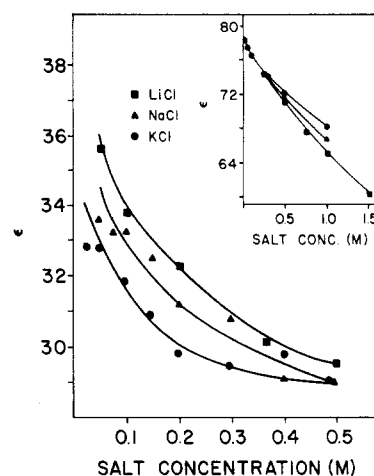

Figure 5. Effect of salt concentration on the dielectric constant (ϵ) of the water–lipid interface of unilamellar phosphatidylcholine vesicles. Inset: Dielectric constants of aqueous solutions of monovalent ions (data from Hasted et al.[1]).

is active as a heterogeneous catalyst, thus favoring reactions of DPPH with either αT or other chemical species present therein. It is interesting in this connection that a work of Misra[21] established that DPPH reacts easily with H_2O molecules adsorbed on hydroxylated semiconducting surfaces, thereby undergoing a number of intermolecular oxidation and reduction processes. These questions will be studied in a future work.

Ionic Strength Effects on the Polarity of the Water–Lipid Interface. Figure 4a–c represents the variation of k of DPPH reactions with αT incorporated in unilamellar PC vesicles as a function of LiCl, NaCl, and KCl concentrations in the aqueous bulk solvent. Let us remark first that the reaction rate constant in the absence of salt could not be determined precisely in the conditions of our experiments on account of the great rapidity of the αT–DPPH reaction. An estimated value is $k \sim 1500$ M^{-1} s^{-1}. However, we feel that the lack of a correct k at zero ionic strength is not sufficient to preclude the discussions of the data outlined hereafter.

Figure 4 shows that the three ionic species (Li^+, Na^+, K^+) have similar effects on the second-order rate constant of the αT–DPPH reaction; that is to say k decreases steadily with increasing salt concentration. To obtain the interface polarity (ϵ) at the nearness of the site of the αT–DPPH reaction as a function of the salt concentration we apply eq 3 to the data of Figure 4a–c. The result is displayed in Figure 5. Note that the curves shown in the figure are empirical and were drawn only to enhance the visualization of the ionic strength effects on ϵ. In addition, it should be emphasized immediately that in studies with lipid bilayer vesicles one is limited experimentally by salt concentrations of the order of 0.5 M or higher. This is because at such concentrations a phase separation occurs that causes the lipid vesicles to float at the top of the aqueous buffer. This phenomenon (vesicle's floatation[24]), that is probably the result of salt-induced changes of the solution density, was reported previously (see, e.g., ref 24) and has been observed currently with our preparations of vesicles kept at salt concentrations > 0.5 M (F. Bellemare, J. Lessard, and M. Fragata, unpublished). As a consequence, we cannot assure whether the curves of Figure 5 converge to a common ϵ, or whether they are characterized by different minimal ϵ values. This deserves to be investigated in future works since the data suggest that transmembrane diffusion of salts, e.g. Na^+ and K^+, may well give rise to sustained polarity fluctuations in lipid membranes in vitro as well as in vivo.

In spite of the aforediscussed difficulties, Figure 5 illustrates the following points. First, the dielectric constant of the water–lipid interface of unilamellar PC vesicles decreases with increasing salt concentration in the bulk water. This finding is corroborated by

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TABLE I: Dielectric Constants (ϵ) of Water–Lipid Interfaces of Lipid Micelles and Model Membranes[a]

exptl system	site of ϵ determination	method	ϵ	ref
unilamellar vesicles (PC,αT)	polar head/hydrocarbon core interface	chemical reaction: αT–DPPH	26	11
multilayers (PC)	polar head/bulk water interface	fluorescence polarization (ANS)	32	26
multilayers (PC)	polar head/hydrocarbon core interface	fluorescence polarization (N,N'-DOC)	25	26
micelles (CTAB, SDS, Triton X-100)	aqueous surface	fluorescence (HC,AC)	32	27
micelles (various types)	aqueous surface	fluorescence (P-CHO)	35–45	28
unilamellar vesicles (PC, DPPC)	polar head/hydrocarbon core interface	fluorescence depolarization (DSHA)	~32	29
GMO bilayers	polar head/hydrocarbon core interface	electrical time const	30–37	30
micelles (SDES, SDS, STS)	aqueous surface	absorbance wavelength maximum	29–33	31
unilamellar vesicles (PC, αT)	polar head/hydrocarbon core interface	chemical reaction: αT–DPPH	29–36	this work

[a] Abbreviations: AC, aminocoumarin; ANS, 1-anilino-8-naphthalenesulfonic acid; CTAB, cetyltrimethylammonium bromide; DPPC, dipalmitoylphosphatidylcholine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DSHA, N-dansylhexadecylamine; GMO, glycerylmonooleate; HC, hydrocoumarin; N,N'-DOC, N,N'-di(octadecyl)oxacarbocyanine; PC, phosphatidylcholine; P-CHO, pyrenecarboxaldehyde; SDES, sodium decyl sulfate; SDS, sodium dodecyl sulfate; STS, sodium tetradecyl sulfate; αT, α-tocopherol.

the observation (Figure 4b) that αT–DPPH reactions in dioxane–Tris buffer (0.01 M, pH 8.0) solutions (61% v/v) are not, or only slightly, affected by the presence of salts (NaCl in the particular case of Figure 4b) in the reaction media. This guarantees that the ion effect is not directly on the αT–DPPH reaction but on the properties of its environment instead, namely on ϵ.

Figure 5 shows, in addition, that the dielectric constant of the bilayer outer surfaces is affected by monovalent ions according to the sequence $K^+ > Na^+ > Li^+$. This effect bears a striking contrast to what happens in the liquid state where it is known (see, e.g., ref 1) that the dielectric constant of aqueous salt solutions of monovalent ions varies according to the sequence $Li^+ > Na^+ > K^+$ (cf. Figure 5 inset). While the latter set of data is explicable by the relative size of the hydrated Li^+, Na^+, and K^+ ions (radii equal to approximately 3.40, 2.76, and 2.32 Å, respectively; cf. Table 7-2 of ref 25) on account of dielectric saturation effects at the neighborhood of the ions' surface,[1–4] other considerations will have to be put forward to interpret the observed ionic effects at the water–lipid interface of the bilayer vesicles.

Discussion

Table I illustrates ϵ data of aqueous interfaces of different lipid systems (micelles, bilayers) obtained by us and by other investigators. The table gives good evidence to support the view that the dielectric constant of the interfacial region is lower (between 25 and 45) than that of pure water (~78 at 298 K). There is also a decrease of the interfacial polarity with increasing ionic strength (data not shown in Table I). For instance, Thomas[28] showed that in the vicinity of the surface of sodium lauryl sulfate micelles $\epsilon = 45$ in the absence of NaCl. This value decreases however to 35 when the NaCl concentration is increased to 0.2 M. A more recent work of Hashimoto and Thomas[32] with the fluorescent probe pyrene included in sodium taurocholate micelles demonstrates that the presence of NaCl (0.3 M, 1.0 M) in the aqueous bulk solution affects in a considerable way the dielectric constant of the micelles. Figure 1 of Hashimoto and Thomas[32] shows that the ratio III/I of the emission bands of pyrene, which incidentally is a measure of ϵ at the pyrene environment (see, e.g., ref 32), is affected by the salt concentration. Our results represented in Figures 4 and 5 are consistent with the aforediscussed data.[28,32]

The decrement of ϵ observed in the presence of monovalent ions in the bulk solvent has been explained in terms of either elec-

trostriction of H_2O molecules located at the water–lipid interface,[28] or dielectric saturation effects owing to the large electric field which the ions may create near the surface of the lipid micelles and bilayers.[33,34] We will investigate hereinafter whether the presence of monovalent ions at the water–lipid interface of the unilamellar PC vesicles is sufficient to give origin to dielectric saturation in the conditions of our experiments. To this end, we use Gauss's Law

$$E = \sigma/\epsilon\epsilon_0 \qquad (4)$$

or the simplification (see discussion in ref 33)

$$E = \sigma/\epsilon_0 \qquad (5)$$

to relate the electric field intensity (E) at the bilayer surface of the unilamellar vesicles to the surface charge density (σ) and the dielectric constant (ϵ) of the interfacial region. In eq 4 and 5, σ_0 is the permittivity of free space and ϵ is obtained from eq 3 as described in the text (see above). Moreover, σ is determined first by a method developed below that takes into account the thickness of the hydration shell of the lipid vesicles and a surface charge density equation given by Altenbach and Seelig.[35] The results are then compared to calculations performed directly according to the transition field approximation of Gouy–Chapman theory.[33]

Surface Charge Density Calculations. (a) Hydration Shell Method. The surface charge density is given by the expression[35]

$$\sigma = eC_{int}/S \qquad (6)$$

corrected for monovalent ions (cf. eq 3 of ref 35). $C_{int} = N_a/N_b$ is the number of interfacial ions per PC molecule, N_a the number of dissociated ions present in the water–lipid interface, N_b the number of PC molecules incorporated in the outer monolayer surface of the unilamellar vesicles (~1660 molecules; see ref 36 for an example calculation), S the area per PC molecule, and e the electronic charge. N_a is given by the expression

$$N_a = C_{eff}V_iN_A/1000 \qquad (7)$$

where C_{eff} is the effective concentration of dissociated ions, N_A the Avogadro's number, and V_i the volume of the water–lipid interfacial region. To obtain V_i we use the model described by Huang and Mason[36] for the cross section of a unilamellar lipid vesicle (cf. Figures 1 and 2 of ref 36); that is

$$V_i = (4\pi/3)(R_2^3 - R_1^3) \qquad (8)$$

R_1 is the outer vesicle's radius, and $R_2 = R_1 + S_{oh}$, where S_{oh} is the thickness of the outer hydration shell. Usually,[36] R_1 is taken equal to 99 Å and R_2 equal to 105 Å, so that $S_{oh} = 6$ Å (see, however, above comments in Characterization of Phosphatidyl-

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TABLE II: Effect of KCl on the Surface Charge Density (σ) and Electric Field Intensity (E) of the Outer Surface of Unilamellar Phosphatidylcholine Vesicles[a]

| | | Calculation of σ by Gouy–Chapman[f] Method | | | |
| | | σ,[d] 10^{-3} C m^{-2} | | E,[e] 10^7 V m^{-1} | |
C_{bulk},[b] M	C_{eff},[c] M	C_{bulk}	C_{eff}	C_{bulk}	C_{eff}
0.025	0.019	6.0	5.2	2.1	1.8
0.1	0.051	11.8	8.4	4.2	3.2
0.2	0.074	16.1	9.8	6.2	3.6
0.5	0.122	25.0	12.4	9.7	4.9

| | | Calculation of σ by Hydration Shell[g] Method | | | | | |
| | | σ,[d] 10^{-3} C m^{-2} | | | E,[e] 10^7 V m^{-1} | | |
C_{bulk},[b] M	C_{eff},[c] M	6 Å[h]	10 Å	15 Å	6 Å	10 Å	15 Å
0.025	0.019	1.2	2.0	3.4	0.42	0.62	1.20
0.1	0.051	3.3	5.4	9.2	1.20	1.90	3.30
0.2	0.074	4.8	7.7	13.3	1.80	2.90	5.10
0.5	0.122	7.9	12.5	22.0	3.10	4.90	8.50

[a] The dielectric constants used in these calculations are those obtained from eq 3 (see text) and the k's given by Figure 4c. [b] C_{bulk}, KCl concentration in the bulk solvent. [c] C_{eff}, effective KCl concentration calculated according to eq 12 (see text). [d] Calculated according to eq 17 (see text). [e] E was obtained from Gauss's law (eq 4, see text). [f] Transition field approximation of Wang and Bruner.[33] [g] See Altenbach and Seelig.[35] [h] Thickness of the outer hydration shell (S_{oh} = 6, 10, and 15 Å; the corresponding interfacial volumes are V_i = 0.78 × 10^6, 1.36 × 10^6, and 2.14 × 10^6 Å^3, respectively).

choline–α-Tocopherol Vesicles).

Now, to evaluate C_{eff}, and thereby C_{int}, one has to take into account the formation of ion pairs as a result of a decrease of ϵ at the water–lipid interface. The analysis of this phenomenon made the object of a number of works (see, e.g., ref 37). An expression to relate the association constant, K_a, of the ions and the dielectric constant of the medium is[38]

$$\log K_a = A + \frac{0.43 Z_1 Z_2 e^2}{a k_B T} \frac{1}{\epsilon} \tag{9}$$

where A is a constant, Z_1 and Z_2 the ions' valence, a the radius of the ion pair, k_B Boltzmann's constant, and T the temperature in Kelvin, and the other parameters have the meaning defined above. A simplified form of eq 9 is

$$\log K_a = -0.02 + 44.48(1/\epsilon) \tag{10}$$

which is easily obtained from data published in the literature.[38,39]

Now, to obtain C_{eff} one assumes first the equilibrium
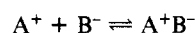
$$A^+ + B^- \rightleftharpoons A^+B^-$$

where A^+B^- represents an ion pair in a low polarity environment. Hence,

$$K_a = \frac{[A^+B^-]}{[A^+][B^-]}$$

Then putting

$$[A^+] = [B^-] = C_{eff}$$

and

$$[A^+B^-] = C_0 - [I^\pm]$$

where C_0 is the bulk salt concentration and $[I^\pm]$ represents either $[A^+]$ or $[B^-]$, one gets the quadratic equation

$$K_a C_{eff}^2 + C_{eff} - C_0 = 0 \tag{11}$$

Equation 11 has two solutions of which only

$$C_{eff} = \frac{-1 + (1 + 4K_a C_0)^{1/2}}{2K_a} \tag{12}$$

is applicable to the lipid systems described here. Some values of C_{bulk} and C_{eff} (obtained from eq 12) are given in Table II together

with the corresponding surface charge densities calculated for S_{oh} = 6, 10, and 15 Å. The reasons for using these values will be discussed later.

We wish to point out that the interfacial volumes corresponding to S_{oh} = 6, 10, and 15 Å are V_i = 0.78 × 10^6, 1.36 × 10^6, and 2.14 × 10^6 Å^3, respectively. We note that the ratios 10/6 (=1.67) and 15/6 (=2.50) are almost identical with 1.36 × 10^6/0.78 × 10^6 (=1.74) and 2.14 × 10^6/0.78 × 10^6 (=2.74). This is because the ratio of the interfacial volumes, say V_{i1} and V_{i2}, at S_{oh1} and S_{oh2}, where the subscripts 1 and 2 have the same meaning as in eq 8, is given by the general expression

$$\frac{V_{i2}}{V_{i1}} = k_0 \frac{S_{oh2}}{S_{oh1}} \tag{13}$$

$$k_0 = \frac{3R_1^2 + 3R_1 S_{oh2} + S_{oh2}^2}{3R_1^2 + 3R_1 S_{oh1} + S_{oh1}^2} \tag{14}$$

where R_1 is the outer vesicle's radius (see eq 8). When $R_1^2 \gg S_{oh}^2$, k_0 is close to 1 or slightly higher. For example, for S_{oh1} = 6 Å, S_{oh2} = 15 Å, and R_1 = 99 Å, one has k_0 = 1.09. Hence, as a first approximation

$$\frac{V_{i2}}{V_{i1}} \cong \frac{S_{oh2}}{S_{oh1}} \tag{15}$$

Now, the result expressed by eq 15 is essential for interpretations of the data presented in Table II (see following sections).

(b) *Transition Field Approximation.* This model is a variant of Gouy–Chapman theory that was developed by Wang and Bruner[33] for such cases where ϵ is lower than the dielectric constant of water at 298 K, i.e., 78. As a first approximation, this applies to our calculations collected in Table II as we used the dielectric constant of the water–lipid interface of the unilamellar PC vesicles, i.e. $\epsilon \sim 29$–36. One of the purposes pursued was to compare the results of calculations with data obtained by the hydration shell method (see previous section).

A comprehensive outline of classical Gouy–Chapman theory is given, for instance, by Aveyard and Haydon.[40] In brief, the theory states that the electrostatic potential, Ψ_s, at a plane with a surface charge density σ_s is given by

$$\sigma_s = 2\sigma_i \sinh\left(\frac{Ze\Psi_s}{2k_B T}\right) \tag{16}$$

where

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$$\sigma_i = (2\epsilon\epsilon_0 k_B T C_i)^{1/2} \tag{17}$$

is a "characteristic surface charge density".[33] C_i is the ionic concentration (bulk or effective), and the other parameters have the usual meaning. The results of calculations (σ_i) are displayed in Table II.

Surface Charge Densities and Electric Field Effects. Before undertaking the discussion of Table II first we wish to point out that while applying Gouy–Chapman theory (eq 17) to the determination of surface charge densities, we may take C_i as the bulk (C_{bulk}) or the effective salt concentration (C_{eff}). But, from what was said above it is obvious that C_{eff} leads to more reliable approximations of σ and E. Second, according to the hydration shell method the surface charge density is dependent on the thickness of the hydration shells of the lipid vesicles (outer shell, S_{oh}, in the present experiments). The reasons for using $S_{oh} = 6$, 10, and 15 Å in Table II are as follows (see also above comments in Characterization of Phosphatidylcholine–α-Tocopherol Vesicles): (i) 6 Å is the S_{oh} estimated by Huang and Mason[36] for minimal size vesicle's radius, i.e., 105 Å, which is currently used in the literature; (ii) 10 Å is the S_{oh} of vesicles having a 109-Å radius (see, e.g., ref 24), a value that proved in the present work to be instrumental in conjunction with $S_{oh} = 15$ Å to compare σ and E data obtained from Gouy–Chapman theory and the hydration shell method. Note in addition that Table II concerns only the experiments performed in presence of KCl, that is to say the ionic species which affects most the second-order rate constant of the σT–DPPH reaction and therefore ϵ (cf. Figures 4 and 5). We verified that consideration of LiCl and NaCl data does not affect significantly our discussion on dielectric saturation.

Table II gives σ computed according to eq 17 (Gouy–Chapman) for effective KCl concentrations between 0.019 and 0.122 M. The results range from 5.2 to 12 mC m^{-2}. The respective electric field intensities, E, obtained from eq 4 are 1.8×10^7 and 4.9×10^7 V m^{-1}. It is remarked at once that the data are not comparable to those obtained by the hydration shell method for $S_{oh} = 6$ Å. This puts the interesting question of the validity of the 105 Å radius (cf. ref 36) often reported for minimal size vesicles. A comparison of data obtained by Gouy–Chapman theory and the hydration shell method is nevertheless possible provided that one takes into account the effect of ionic strength on the thickness of S_{oh} that we equate to the Debye electrical double layer, $d = 1/\kappa$ where κ is Debye–Hückel's parameter (see, e.g., ref 40). Thus, we see for example that the electric field intensities obtained from eq 4 at C_{eff} equal to 0.019 and 0.051 M, i.e., 1.8×10^7 and 3.2×10^7 V m^{-1}, are comparable to the E values obtained by the hydration shell method with $S_{oh} = 15$ Å, that is, 1.2×10^7 and 3.3×10^7 V m^{-1}, respectively. Similarly, one gets $E = 3.6 \times 10^7$ and 4.9×10^7 V m^{-1} (Gouy–Chapman theory) for $C_{eff} = 0.074$ and 0.122 M, which correspond to $E = 2.9 \times 10^7$ and 4.9×10^7 V m^{-1} (hydration shell method) obtained with $S_{oh} = 10$ Å. This is most interesting, since it indicates definitely that the size of unilamellar lipid vesicles is rather sensitive to variations of ionic concentration. Some preliminary hydrodynamic data from our laboratory would seem to confirm this view (F. Bellemare, N. Lacroix, M. Fragata, unpublished results). These conclusions are also compatible with the work of Söderman et al.[16] on the effects of monovalent ions on the thickness of the water layer, here referred to as S_{oh}, of lipid bilayers formed with various phosphatidylcholines.

To ascertain the extent of dielectric saturation effects on the lipid bilayers we use the ratio, R_{ds}, of electrostatic energy to thermal energy

$$R_{ds} = pE/k_B T \tag{18}$$

where p is taken as the dipole moment of the transition-state complex of the αT–DPPH reaction, and the other parameters have

the usual significance. The onset of dielectric saturation is observed at $R_{ds} > 1$, say equal to 1.2 (see discussion in ref 33).

The application of eq 18 to the data of Table II supposes the knowledge of p. Such data, however, are not available. An acceptable estimation is $p \sim 6$ D,[41] or 9.6×10^{-29} C m, corresponding to a dipole length (transition state of αT–DPPH reaction) of ca. 1.2 Å. Hence, for $E = 4.9 \times 10^7$ V m^{-1} at 295 K ($C_{eff} = 0.122$ M), but not for lower values of E, eq 18 yields $R_{ds} = 1.15$ which is consistent with the onset of dielectric saturation. Thus, if this effect is proved to function at the water–lipid interfaces of the lipid vesicles at $C_{eff} < 0.122$ M then, either p is greater than 6 D or, alternatively, the dielectric constant of the aqueous interface is substantially lower than the values obtained from the curves of Figure 5. If so, one may use eq 5 instead of Gauss's law (eq 4) as was advocated by Wang and Bruner.[33] In that case, the calculations show that we may expect dielectric saturation at C_{eff} as low as 0.019 M which is probably doubtful. These matters deserve some clarification.

Equation 5 implies that in Gauss's law (eq 4) one has $\epsilon = 1$. This means that the local field at the site of the αT–DPPH reaction would not be attenuated by dielectric screening as the result, for instance, of the presence of H_2O molecules. However, this is only possible if H_2O is excluded from the interfacial region by a "salting-out-like effect" initiated by ion-induced electrostatic attractions. The salting-out hypothesis applied to lipid bilayers was described previously (see, e.g., ref 15) as a redistribution of water molecules between the lipid phase and the adjacent aqueous layers driven by the ionic species in solution with concomitant dehydration of the interface and drastic decrease of ϵ. It is worth noting, in this same connection, that the ^{7}Li NMR quadrupole splitting study of Söderman et al.[16] gives evidence for a squeezing of H_2O molecules out of the lamellar phase of phosphatidylcholine bilayers upon addition of LiCl to the aqueous bulk solution. Nonetheless, this is not yet sufficient to provide precise information on the hydration gradient which most probably prevails across the polar head/near hydrocarbon core of the lipid bilayers, and, by the same token, on the state of hydration of the interfacial dissociated ions and ion pairs.

Conclusion

The preceding discussion gives evidence for a possible dielectric saturation effect acting on the water–lipid interface of unilamellar bilayer vesicles as a result of the presence of the monovalent ions Li$^+$, Na$^+$, and K$^+$ that would be closely related to the decrement of ϵ observed in our experiments (cf. Figure 5). One of the points that remains unexplained is the question of the true hydration degree of the αT–DPPH reaction site. Another important unsettled point is the differential effect of the ions on ϵ which follows the sequence Li$^+$ > Na$^+$ > K$^+$ in the liquid state (cf. Figure 5, inset) but K$^+$ > Na$^+$ > Li$^+$ so far as the water–lipid interface is concerned (cf. Figure 5). This is probably related to the size of the dissociated ions whose degree of hydration at the water–lipid interface is certainly controlled by the interface itself. Our work is being continued with these aims in view.

Registry No. Li, 7439-93-2; Na, 7440-23-5; K, 7440-09-7.

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