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Determination of the partition coefficient of 1-naphthol, an excited state acid, in DMPC membrane

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Abstract

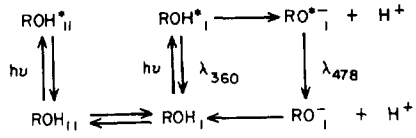
1-Naphthol, an excited state acid, undergoes partial dissociation in the excited state in liposome membranes, giving rise to two peaks, as opposed to a single peak in water. The excited state prototropic equilibrium is affected by perturbations in the membrane system. This property can be used to probe the properties of biomembranes. In this respect, as a preliminary study, the partition coefficient of 1-naphthol in liposomes has been calculated.

Keywords: Cholesterol effect; DMPC liposomes; Excited state proton transfer; Excited state prototropic equilibrium; Phase transitions

1. Introduction

Aromatic hydroxy compounds are excited state acids [1]. For 1-naphthol in water, the light-absorbing species is the neutral form, but rapid proton transfer follows excitation, and fluorescence is usually observed from the anionic species formed in the excited state. Thus 1-naphthol has a pK_a value of 9.2 and a pK_a^* value of 0.5 [2]. Such systems are easy to monitor fluorometrically since the bands due to the neutral and anionic forms are well separated. As the size of these aromatic hydroxy compounds is small, they are expected to perturb the membrane system minimally. The importance of thermal and light-induced proton transport in biomembranes has stimulated activity in the study of the dynamics of excited state proton transfer (ESPT) in biomembranes [3–6]. Strong retardation of excited state protolytic dissociation is observed in the case of naphthols in microemulsions and liposome suspensions [7–13]. Kinetic analysis of the excited state prototropism of some naphthols [12] leads to the kinetic scheme shown in Scheme 1, according to which two different sites with different lifetimes exist for the neutral form in the membranes, whereas the anionic form exists in a site with a single lifetime. Thermotropic phase changes lead to a retardation in the prototropic equilibrium [13].

The aims of the present work are to determine a method to obtain the true partition curve of naphthol in the membrane as opposed to the ratiometric method followed previously



Scheme 1. Kinetic scheme for the prototropism of naphthol (ROH) in liposome suspension [12].

[12] and to calculate the partition coefficient of 1-naphthol between the bulk and the liposome membrane (an essential parameter for a probe) by detailed kinetic analysis.

2. Experimental details

2.1. Materials

1-Naphthol was vacuum sublimated and used after checking its purity. DMPC (dimyristoylphosphatidylcholine) was purchased from Sigma Chemical Co. (USA) and was used as received after checking for a single spot in thin layer chromatography (TLC).

2.2. Liposome preparation [14]

DMPC lipid (approximately 3 mM) stock in chloroform was prepared. The solvent was removed using a rotary evaporator; residual solvent, if any, was removed by leaving the RB (round bottomed) flask in vacuum for a sufficient time. Multilamellar vesicles (MLVs) were prepared by adding an

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appropriate volume of phosphate-buffered saline (PBS) at pH 7 to the lipid film, with vigorous stirring and warming at 45–50 °C, to yield a final lipid concentration of 0.1 mM. Freshly prepared liposomes were used for all the experiments.

2.3. Labelling

A stock solution of 1-naphthol was prepared in PBS at pH 7. Partition experiments were performed by the titration method by adding, to 1 ml of various concentrations of liposome in PBS, 1 ml of the same concentration of 1-naphthol in PBS (optical density, 0.122); this yielded solutions of various lipid/probe (L/P) ratios in the range 1–30. After adding the probe, the solutions were allowed to equilibrate for 1 h at 30 °C before the experiment. For all the experiments, a control solution containing the same concentration of liposome, but no probe, was prepared and used as a blank.

2.4. Fluorescence measurements

Fluorescence measurements were recorded using a Hitachi F-4500 spectrofluorometer. The excitation wavelength was 300 nm, and the emission spectrum was recorded in the wavelength range 300–600 nm with the excitation and emission slit widths having a bandpass of 5 nm. The fluorescence intensity of the blank was subtracted from that of the experimental solution (contribution of blank intensity value is less than 0.5%).

3. Results and discussion

3.1. Fluorescence

A typical set of fluorescence spectra, demonstrating the incorporation of 1-naphthol in DMPC lipid membranes, is shown in Fig. 1. This figure shows a progressive increase in the fluorescence of neutral 1-naphthol (360 nm), with a concomitant decrease in the fluorescence of the anionic form (478 nm), with an increase in lipid concentration; the intensity of both peaks shows a saturation effect. The absorption spectra of 1-naphthol in the presence and absence of vesicles show no change in the peak positions, discounting the possibility of a specific interaction between 1-naphthol and the lipid in the ground state. The emission maximum of the anionic form remains unchanged in the presence and absence of lipid.

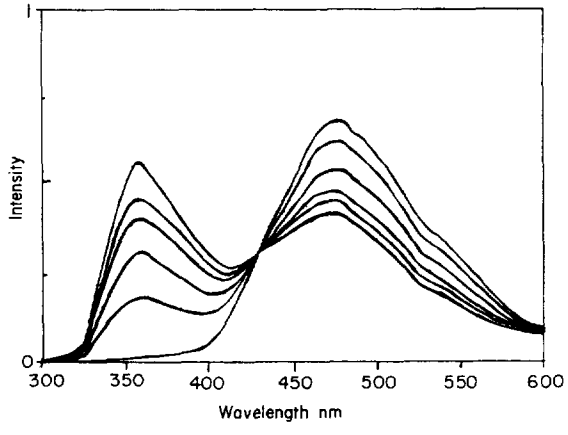


Fig. 1. Emission spectra of 1-naphthol in DMPC liposome for a concentration of lipid in the range (0–35) $\times 10^{-5}$ M at 30 °C. Concentration of 1-naphthol, 1.25×10^{-6} M.

3.2. Determination of the true partition coefficient

The intensities of the neutral and anionic forms and the neutral/anionic ratio were plotted against the lipid concentration (Fig. 2) to determine the distribution of 1-naphthol in DMPC vesicles. A saturation effect is seen much earlier for the neutral species (Fig. 2(a)) than for the anionic form (Fig. 2(b)), while the ratiometric plot (Fig. 2(c)) corresponds more or less with that of the anionic form.

The fluorescence quantum efficiency is defined by $\Phi_f = k_f \tau_f$. Assuming that k_f, the rate constant of fluorescence, remains constant in various environments, Φ_f is directly proportional to τ_f, the radiative lifetime. Thus the fluorescence intensity is proportional to the concentration only when the quantum yield (or lifetime) remains constant. Since the anionic and neutral forms have different lifetimes in the liposome [12], a simple ratiometric method involving the fluorescence intensity is clearly not suitable for determining the true partition coefficient K_p. Therefore K_p can be obtained by

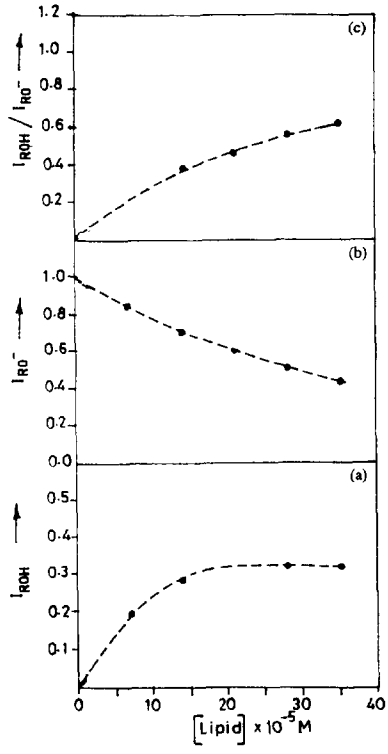
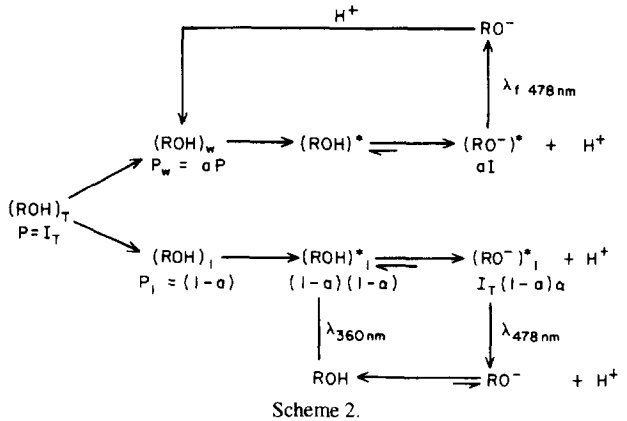


Fig. 2. Plot of intensity of emission at 360 nm of neutral species (I_{ROH}) (a), intensity of emission at 478 nm of anionic species (I_{RO^-}) (b) and ratio of intensity of emission of neutral to anionic form of 1-naphthol (c) vs. concentration of lipid at 30 °C.



Scheme 2.

following the intensity of either the neutral form or anionic form alone.

If the neutral form emits with a single lifetime in the liposome membrane, the partition coefficient may be calculated very easily from its intensity in the same way as for other standard probes, such as DPH (1,6-diphenyl hexatrienyl), ANS (1-anilino-8-naphthalene sulphonate), etc. [15]. However, the neutral form exists in two different sites with two different fluorescence lifetimes in the liposome membrane [12]. In such a case, the total quantum yield is given by

$$\Phi_T = lk_f\tau_1 + mk_f\tau_2 \quad (1)$$

where τ_1 and τ_2 are the two lifetimes in the two different sites and l and m are the fractions of intensity contributed by the two sites to the total intensity. An estimation of the distribution of the neutral emitting species between these two sites with a change in lipid concentration is required in order to explain the earlier saturation effect. Work on this is in progress.

Since the partition coefficient cannot be calculated using the fluorescence intensity of the neutral form, the same method can be used with the anionic form as it emits as a single lifetime component in water (7.5 ns) as well as in liposomes (12.5 ns) [12]. The emission intensity obtained for the anionic form (Fig. 2(b)) originates from two species: the anionic form in water and that in the liposome. These two components can be separated as shown in Scheme 2, where P is the total concentration of 1-naphthol, P_w is the fraction remaining in water and P_l is the fraction partitioned into the liposome.

At a lipid concentration before saturation, a fraction a of the total 1-naphthol remains in water and the remaining fraction $(1-a)$ partitions to the liposome. The fraction in water undergoes complete photodissociation emitting as RO^-*. However, the fraction in the liposome undergoes excited state dissociation partly according to the degree of dissociation α in the liposome, and emits as both ROH* and RO^-*. The observed intensity of the anionic form comprises

$$I(RO^-*)_{obsd} = I_w(RO^-*) + I_l(RO^-*) \quad (2)$$

Taking the intensity of RO^- in the absence of lipid as I_T

$$I_{obsd} = aI_T + (1-a)\alpha I_T \quad (3)$$

For normalized intensities $I_T = 1$. Hence by knowing α, a can be calculated.

α in liposomes is obtained indirectly from the lifetime of RO^- [12] in water (7.5 ns) and in liposomes (12.5 ns). Assuming the radiative rate constant k_f to be the same in water and in the membrane, the quantum yield of RO^- fluorescence from the lipid membrane is expected to be 12.5/7.5, i.e. 1.66, times larger than the corresponding quantum yield in water. Taking the fluorescence intensity maximum to be unity for the anionic fluorescence in the absence of lipid, it is expected that the RO^- fluorescence will increase to 1.66 at the saturation point (all naphthol transferred to liposome) if all the emission is due to RO^-* in the liposome. The observed RO^- intensity of 0.42 at the saturation point must therefore be due to the incomplete dissociation of 1-naphthol in the membrane. Therefore the degree of dissociation of 1-naphthol in the liposome is 0.4/1.6, i.e. 0.26.

From a knowledge of α, a is calculated from Eq. (3) for every L/P ratio. The fraction of intensity due to the anionic form in water is then determined from the term $aI_T(I_w)$ for each lipid concentration. $(1-I_w)$ is plotted vs. the lipid concentration in Fig. 3. In this way, from the complicated overall emission involving more than one species with multiple fluorescence lifetimes, the intensity proportional to only the ROH fraction present in water (emitting as RO^-*) can be extracted. The curve in Fig. 3 is the normally expected saturation curve for a simple species. The partition coefficient is calculated from the expression derived below.

The partition coefficient K_p is defined as

$$K_p = \frac{P_l}{P_w} \times \frac{W}{L} \quad (4)$$

where P_l and P_w are the concentrations of 1-naphthol in the liposome and water respectively and W and L are the weights of water phase and lipid phase respectively. The quantum yield of fluorescence of 1-naphthol is related to the concentration as

$$\Phi_l = xP_l; \quad \Phi_w = yP_w \quad (5)$$

where Φ_w and Φ_l are the overall quantum yields of the species present in water and in liposome respectively and x and y are proportionality constants. Substituting for P_l and P_w from Eq. (5) into Eq. (4), we obtain

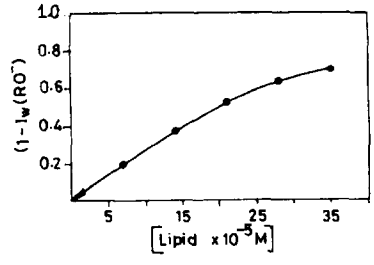


Fig. 3. Plot of $1-I_w(RO^-)$ (intensity component of anionic form emitting from water) vs. concentration of lipid at 30 °C.

$$K_p = \frac{\Phi_1}{\Phi_w}\frac{y}{x}\frac{W}{L} \quad (6)$$

An expression for K_p from fluorometric measurements is usually obtained by monitoring either the increase or decrease in intensity when the probe partitions into the liposome membrane. In the present case, the appearance of neutral peak fluorescence cannot be used since the emission has two lifetime components. The fluorescence intensity of the anionic species (at 478 nm) also contains contributions from the anionic species in water and the anionic species in the liposome with different lifetimes. However, it is possible to separate the contributions from the two components by using the anionic intensity and lifetime in pure water in the absence of liposomes. An expression for K_p is derived as follows by monitoring the decrease in the fluorescence intensity of the anionic form in water

$$P = P_l + P_w = \Phi_w/y + \Phi_l/x \quad (7)$$

where P is the total concentration of 1-naphthol taken. At [lipid] = 0, $\Phi_l = 0$. Therefore $P = \Phi_w^0/y$; substituting for y in Eq. (7) and rearranging, we obtain

$$y/x = (\Phi_w^0/\Phi_w - 1)\,\Phi_w/\Phi_l \quad (8)$$

Substituting for y/x in Eq. (6) and simplification yields

$$\frac{1}{\Phi_w} = \frac{K_p L}{\Phi_w^0 W} + \frac{1}{\Phi_w^0} \quad (9)$$

As the quantum yield is proportional to the intensity

$$\frac{1}{I_w} = \frac{K_p L}{I_w^0 W} + \frac{1}{I_w^0} \quad (10)$$

From a plot of $1/I_w$ vs. L (Fig. 4), K_p is calculated from the slope and intercept values using Eq. (10).

K_p for 1-naphthol in DMPC liposomes is found to be 5.0×10^6 at 30 °C, which is comparable with that of a standard probe such as DPH [15].

3.3. Effect of temperature

DMPC liposomes undergo a phase transition from the solid gel to the liquid crystalline phase at 23 °C (T_c) [16–18]. Partition coefficient values were calculated at 10 °C (when the vesicle is in the solid gel phase), at 23 °C (the phase

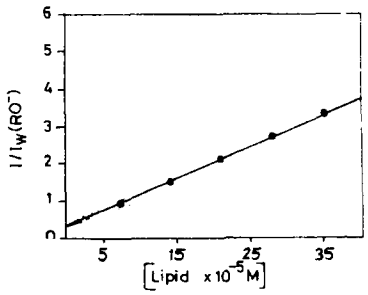


Fig. 4. Plot of $1/I_w(RO^-)$ vs. concentration of lipid at 30 °C.

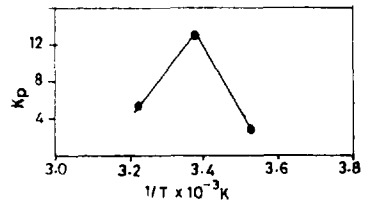


Fig. 5. Plot showing variation of K_p with temperature.

transition temperature of DMPC liposomes) and at 35 °C (the liquid crystalline phase). The values are 2.5×10^6 at 10 °C, 13.2×10^6 at 23 °C and 5.3×10^6 at 35 °C. The partition coefficient is low in the solid gel phase, increases with increasing temperature and exhibits a dramatic increase (Fig. 5) near the phase transition temperature. These changes can be attributed to the permeability changes accompanying the temperature changes [18,19]. In the solid gel phase, the membrane fluidity is low (low permeability) and partitioning is low. In the liquid crystalline phase, partitioning increases due to an increase in the fluidity (hence permeability) of the membrane. The dramatic increase in K_p near the phase transition temperature can be attributed to the coexistence of both solid and liquid domains at T_c, leading to a very large increase in the permeability of the membrane [19].

4. Conclusions

As the excited state proton transfer of 1-naphthol is retarded in liposome suspensions, a new peak appears at 360 nm, which may serve as a useful tool to mark the presence of intact membranes. A high partition coefficient ensures its validity as a good fluorescence probe for biomembranes. Thus the above results encourage the use of 1-naphthol as an excited state proton transfer probe for biomembranes.

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