Effect of Phase Transition on the Kinetics of Dye Transport in Phospholipid Bilayer Structures†

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ABSTRACT: Binding of 8-anilino-1-naphthalenesulfonate to dimyristoyl-L-α-lecithin bilayers enhances the fluorescence quantum yield of the dye molecule by 100-fold. By following the generation of fluorescence after a rapid mixing in a stopped-flow apparatus (mixing time 2 msec), kinetics of the binding of the fluorescence probe to the phospholipid vesicles has been investigated in the temperature range where the crystal-liquid crystal phase transition of the bilayer structures occurs. No reactions depending on the dye or the vesicle concentrations were detected. This suggests that the initial adsorption of the dye was very rapid. Two kinetic phases which appear in the 50 msec and the second time ranges are unimolecular. The faster one has a small amplitude and is observable in the entire temperature range studied. In the phase transition region the slower reaction becomes the major kinetic phase. It also increases the apparent concentration of bound dye by a factor of 2. These observations suggest that the 50-msec reaction has detected a reorientation of the probe molecule after the initial binding, and that the slow reaction represents a transport of the dye molecule into the inner layer of the lipid vesicles. The transport reaction is extremely temperature sensitive and exhibits a maximum rate at the midpoint of the bilayer phase transition (T_m = 24.1°). The Arrhenius plot of the transport reaction shows a maximum at the T_m. The same temperature dependence was also observed for the bromothymol blue transport reaction. However, no such effects were detected for less amphiphilic molecules such as tetracycline, chlortetracycline, and pyrene. In the latter systems only a slight bending of the Arrhenius plots were seen at the phase transition temperature. Since the kinetics of the transport of 8-anilino-1-naphthalenesulfonate is sensitive to the physical state of the phospholipid bilayers this reaction may be used for probing membrane structures.

Optical probe techniques have been applied to obtain information on the microviscosity (Shinitzky et al., 1971; Cogan et al., 1973; Lentz et al., 1975), fluidity (Träuble, 1972; Overath and Träuble, 1973; Haynes and Staerk, 1974), polarity (Turner and Brand, 1968; Radda, 1972), surface potential (Haynes, 1974), and the energy state (Azzi et al., 1971) of phospholipid bilayers and biological membranes. The most widely used optical probe is 8-anilino-1-naphthalenesulfonate (ANS).[1] The fluorescence quantum yield of ANS increases nearly 100-fold when it is bound to phospholipid bilayers. This fluorescence enhancement is sensitive to the physical state of phospholipids and has been used to monitor the crystal-liquid crystal phase transitions of the bilayer structures (Träuble, 1972; Overath and Träuble, 1973). In this communication we report a study of a transport reaction of ANS molecule across the lipid bilayers in the phase transition region. The permeation of ANS in the bilayer structures is directly observable by fluorescence spectroscopy. Thus it can serve as an excellent model system for studying molecular transport in biological membranes.

Prior to this study equilibrium measurements of ANS binding to lecithin vesicles have been done (Haynes and Staerk, 1974; Jacobson and Papahadjopoulos, 1975a). A temperature-jump relaxation study of ANS binding to the surface of single layer lecithin vesicles has also been reported by Haynes (1972). Haynes observed that the bimolecular reaction of ANS binding to the monolayer vesicle of egg lecithin occurred in the 100-μsec time range. No unimolecular reaction of ANS interaction with the bilayer structures has been reported. It will be shown that the initial binding of the dye accounts for only a small fraction of the total fluorescence change. In the phase transition region more than 80% of the fluorescence enhancement takes place in a slow kinetic phase which is independent of the dye and the vesicle concentrations. The rate of this reaction depends on the physical state of the bilayer structures. In the following paper (Tsong, 1975) we report a study of the effect of cholesterol on the phospholipid bilayers by comparing the ANS transport reaction in aqueous dispersions of pure lipid and lipid–cholesterol mixtures.

Materials and Methods

Phospholipid Vesicle. DML and DPL were purchased from Calbiochem. Appropriate amount of phospholipid was dispersed in 0.1 *N* NaCl and 0.05 *M* phosphate buffer at pH 7.0. After a thorough degassing, the dispersion was transferred into a water-jacked glass container for ultrasonic irradiations in a Biosonic IV sonicator. The sonication was done under a constant nitrogen stream at 30° for DML dispersions and at 45° for DPL dispersions. A standard size titanium probe was employed at a power level of 100 W. The extent of the vesicle formation depends on the shape of the container and also on the ionic strength of the dispersions.

Usually the sonication was terminated within 15 min. The dispersions so obtained can be easily forced through a 0.1 μ pore size Sartorius membrane. A very small fraction of the vesicles was found to be at the limiting size (Huang, 1966), as checked by Sepharose chromatography. Prolonged sonication can cause a degradation of phospholipid

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[1] Abbreviations used are: DML, dimyristoyl-L-α-lecithin; DPL, dipamitoyl-L-α-lecithin; ANS, 8-anilino-1-naphthalenesulfonate; BTB, bromothymol blue.

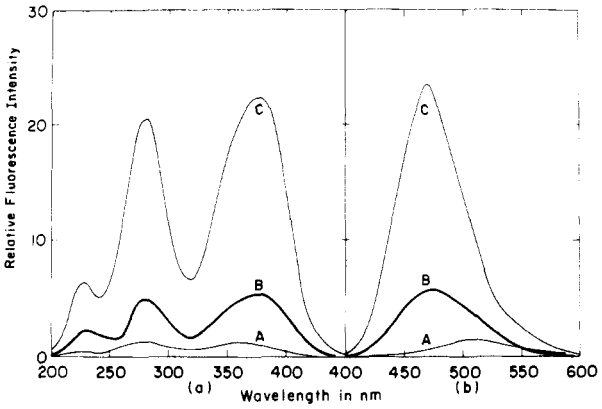

FIGURE 1: Fluorescence excitation and emission spectra of ANS in different solvent conditions. (a) Excitation spectra of 40 μM ANS in water (A), 1.6 μM ANS in a 35 μM DML-H_2O dispersion (B), and 1.6 μM ANS in absolute ethanol (C). Fluorescence measured at 480 nm. (b) Emission spectra of ANS. Conditions as given in the curves A, B, and C of (a). Excitation at 380 nm.

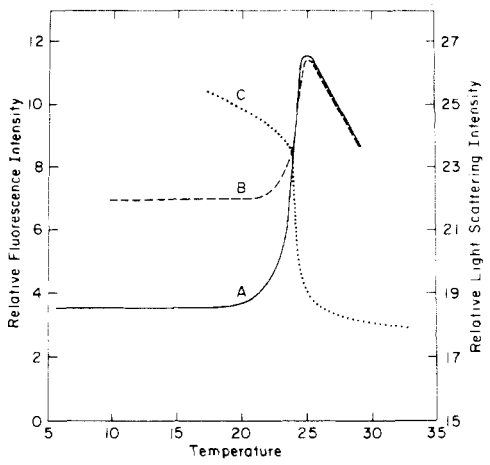

FIGURE 2: Equilibrium binding of ANS in the DML bilayers as a function of temperature. 5 μM of ANS was mixed with a 20 μM DML dispersion at low temperature. The relative fluorescence intensities of the solution were followed as a function of the temperature in curve A (the first heating). The solution was slowly cooled down and the second heating curve (curve B) was taken. The third and the consequent heating curves coincide with curve B. When the 90° light scattering of the suspension was followed curve C was obtained for the first, second, and the subsequent heatings. Conditions: 0.1 N NaCl–0.05 M phosphate buffer at pH 7.0. Heating rate, 0.2°/min. See text for details.

and was avoided. Before the membrane filtration, the dispersions were centrifuged in a Sorvall RC2B at 30000g for 15 min to remove solid metallic contaminations in the sonication process.

In most cases the vesicles prepared after the membrane filtration were used directly for the stopped-flow experiment. In order to examine the kinetics of ANS interaction in the small vesicle systems, in some cases prolonged sonications were done with the dispersions and the vesicles obtained after the membrane filtration were further fractionated by a Sepharose 4B column. The fractions corresponding to the large and the small vesicles reported by Huang were separated (Huang, 1969). The freshly prepared small vesicles can be easily forced through a Sartorius membrane of 0.05 μ pore size. However, in the presence of NaCl the vesicles slowly aggregate in the phase transition region (Sheetz and Chan, 1972; Tsong, 1974). Because of the fusion of the small vesicles precise kinetic data were difficult to obtain. Since kinetics of the dye transport was found to be qualitatively similar for the large and the small vesicle systems we have reported here only the result obtained for the large vesicle system (500 Å < diameter < 1000 Å).

Kinetic Measurements Stopped-flow kinetic measurements were done in a Durrum D-110 stopped-flow spectrophotometer equipped with a fluorescence kinetic attachment. In the fluorescence kinetic experiments excitation wavelength was set at 380 nm and a Corning Filter No. 3-72, which cut off wavelengths below 440 nm, was placed before the photodetector to eliminate signal due to the Rayleigh light scattering of the suspension. The reactions were recorded on a Tektronix 5103N storage oscilloscope for kinetic analysis.

Equilibrium Measurements Equilibrium melting curves of phospholipid vesicles were taken with an Aminco-Bowman spectrofluorometer which can monitor either the fluorescent or the 90° light-scattering intensities of the ANS-lipid dispersions. The heating rate was set at 0.2°/min with a Neslab TP-2 temperature programmer attached to a Lauda water circulator. The solution temperature was monitored directly with a calibrated YSI thermistor probe. The fluorescence and light-scattering intensity changes were recorded with a Sargent recorder.

Experimental Results

Fluorescence Enhancement of ANS in Phospholipid Bilayers. Several equilibrium studies of the binding of ANS to phospholipid vesicles have been reported (Träuble, 1972; Overath and Träuble, 1973; Haynes and Staerk, 1974; Haynes, 1974; Jacobson and Papahadjopoulos, 1975a). Träuble (1972) observed that the fluorescence quantum yield of ANS when bound to DPL bilayers was 0.08 compared to 0.004 in water. Haynes and Staerk (1974) have reported a higher value for ANS in DML monolayers (0.37) and bilayers (0.29). Our result agrees with the observations of these authors that the quantum yield of bound ANS increases nearly 100-fold in the DML bilayers. Figure 1a gives a fluorescence excitation spectrum of ANS in the presence of excess DML vesicles in curve B, to be compared with the spectra of a 25 times more concentrated aqueous solution in curve A, and the same concentration of dye in ethanol in curve C. The fluorescence emission spectra in the same solvent conditions are given in Figure 1b.

There is also a blue shift of the ANS emission maximum from 515 nm in aqueous solutions to 480 nm in DML dispersions. This change in the emission maximum of ANS is a sensitive measure of the polarity of the probe environment (Turner and Brand, 1968; Stryer, 1968). By comparing the emission maxima of the dye in a series of solvents with different Kosower (1958a) polarity values (Z) we have estimated that the ANS binds to the DML bilayers in a region of moderate polarity (Z = 83). This Z value corresponds to the polarity of pure methanol or a 90% ethanol solution (Kosower, 1958b). The result suggests that ANS molecules do not bind to the hydrophobic domain of the bilayer structures, but that they bind close to the surface of the DML vesicles, where the phospholipid molecules exhibit some amphiphilic properties. The same conclusions have also been reached by different authors for the binding of ANS and other dye molecules to phospholipid bilayers (Träuble, 1971; Hsu and Chan, 1973).

Transport of ANS Molecules across the Bilayers. At 480 nm the fluorescence intensity of the bound ANS is at least 150-fold higher than the free dye in aqueous solution (Figure 1). Thus fluorescence due to the free dye in solu-

Table I: Stopped-Flow Kinetic Measurements of ANS Transport in the DML Dispersions.[a]

Temp (°C)	Signal Occurred in 2 msec (V)	Fast Kinetic Phase		Slow Kinetic Phase	
		Signal (V)	$t_{1/2}$ (msec)	Signal (V)	$t_{1/2}$ (sec)
11.0	0.055	0.036	45	Small	
19.5	0.050	0.035	28	0.063	122
21.7	0.060	0.030	36	0.270	16
22.7	0.055	0.030	43	0.330	5.0
23.4	0.055	0.036	50	0.480	2.3
23.9	0.060	0.060	61	0.645	2.0
24.5	0.070	0.130	54	0.630	1.4
24.8	0.090	0.125	50	0.555	2.0
25.5	0.090	0.075	46	0.547	2.6
26.4	0.066	0.075	43	0.405	3.5
28.1	0.075	0.045	36	0.375	9.0
30.5	0.060	0.039	39	0.405	15.0
34.0	0.050	0.039	35	0.210	120

[a] Final conditions: 4 μM ANS, 20 μM DML, 0.1 N NaCl, and 0.05 M phosphate buffer (pH 7.0). Excitation at 380 nm.

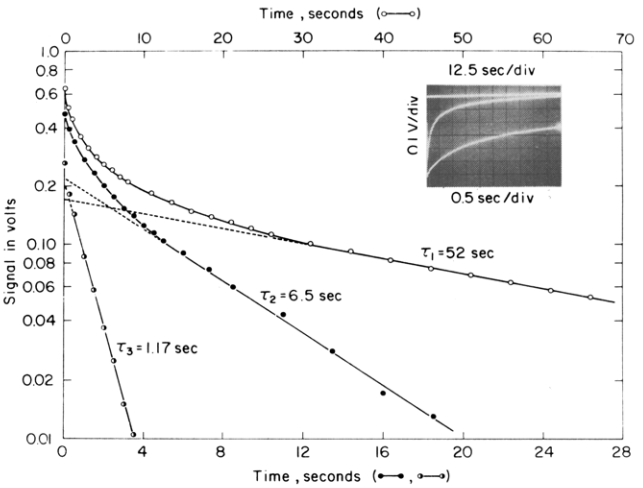

FIGURE 3: An oscillograph record of the slow kinetic phase. The semi-log plot of the reaction is shown by the open circles. The curve can be resolved at least into three close exponential decay terms, with the relaxation time of 52, 6.5, and 1.17 sec. Final conditions: 0.1 N NaCl–0.05 N phosphate buffer, pH 7.0, 24.1°. ANS concentration, 4 μM; DML concentration, 20 μM.

tions can be ignored. In Figure 2, curve A, excess ANS was mixed with DML vesicles at low temperature and the fluorescence intensity of the solution was measured following a slow elevation of temperature. As can be seen the fluorescence intensity of the solution increases abruptly at the crystal–liquid crystal phase transition temperature (T_m = 24.1°) of the lipid bilayers. This fluorescence enhancement may come from two sources: changes in the polarity of the dye environment or an increase in the amount of dye bound to the lipid in the phase transition region.

The same solution was cooled down to 10°. As shown in curve B the fluorescent intensity of the solution is now maintained at the level roughly twice that of the initial value, suggesting that approximately twice as much of ANS molecules is retained in the lipid bilayers. Second heating shows that there is also a fluorescence enhancement due to the structural changes of the lipid in the phase transition region. Subsequent heatings coincide with the second heating curve.

To ensure that ANS binding did not cause serious structure alteration of the bilayers we have taken a melting curve by monitoring the 90° light scattering of the DML–ANS mixture (Träuble, 1972; Tsong, 1974; Yi and MacDonald, 1973). This is shown in curve C. Curve C was reproducible for the first, second, and the subsequent heatings.

These experiments indicate that at low temperature binding of ANS to the DML bilayers is limited to the outer layer of the vesicles. And that a transport of ANS across the bilayer occurs at the phase transition temperature. In fact, permeation of ANS to the inner layer can also be achieved by a sonication of ANS–phospholipid mixtures at low temperatures. By such a treatment twice as much dye was found to bind to the vesicles without going beyond the phase transition temperature. Jacobson and Papahadjopoulos (1975a,b) have drawn the same conclusion in their equilibrium studies of ANS binding in a wide range of temperatures.

Kinetics of the ANS Binding at Low Temperatures. At low temperature binding of ANS to DML bilayers is limited to the outer layer of the vesicles. When ANS was mixed with a DML dispersion at 10° about 60% of the intensity change was completed within the 2-msec mixing time of the stopped-flow instrument. This intensity loss is attributed to the rapid, bimolecular reaction reported by Haynes (1974). Subsequent to the initial adsorption of ANS a kinetic phase in the 50-msec time range was detected. This reaction was found to be independent of the ANS and the vesicle concentrations (in the ranges of 1×10^{-6}–5×10^{-5} M for ANS, and 1×10^{-5}–2×10^{-4} M for DML). Since the ANS does not penetrate deep into the hydrophobic domain of the DML structures under these conditions the unimolecular reaction observed here must represent a reorientation of the bound dye in the lipid bilayers. In the phase transition region the amplitude of this reaction becomes negligibly small (Table I). It would be interesting to follow the anisotropy change of ANS binding at this time scale. Such a measurement would indicate whether the reaction actually detected a reorientation of the dye molecule. This reaction is only slightly influenced by the phase transition of the DML bilayers.

Kinetics of the ANS Transport in the Phase Transition Region. As can be seen from Figure 2 mixing of ANS with the DML bilayers in the phase transition region generates a large fluorescence change. More than 80% of the intensity changes occur in the second time range. A typical oscillograph record of the reaction is given in Figure 3. The kinetics is complex and can be resolved at least into three exponential decay terms. None of the three time constants depend on the vesicle and the ANS concentrations, indicating that they are the unimolecular transport reactions of the ANS in the bilayer structures. Since the resolution of the kinetic curves into three exponential terms is arbitrary and is subjected to a larger statistical uncertainty (Johnson and Schuster, 1974), we have chosen the half-time of the reactions, $t_{1/2}$, for the discussion of our kinetic data. The half-time of the ANS transport is a marked function of temperature, and exhibits a minimum at the T_m of the lipid phase transition. This is shown in Figure 4. The minimum in the half-time corresponds to a maximum for the rate of the reactions. In the same figure the Arrhenius plot of the reaction is shown. As expected there is a maximum point at the T_m of the lipid phase transition. The apparent activation energy of the ANS transport is 240 kcal/mol in the first half of the transition and becomes negative at the second half of the transition. These results contrast sharply to our

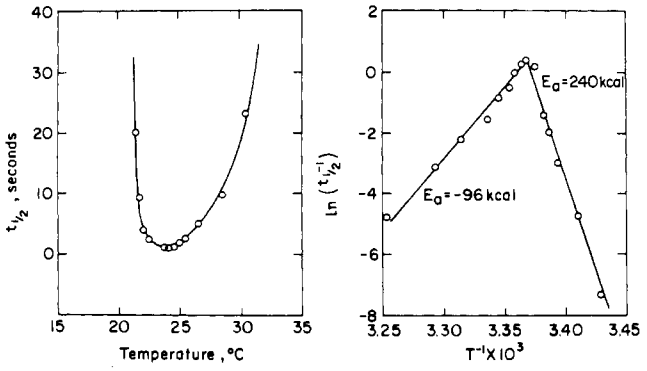

FIGURE 4: The temperature dependence of the ANS transport reaction. The half-time of the slow reaction given in Figure 3 is plotted against the temperature in the left-hand panel. The minimum of the curve is found at 24° which is the crystal-liquid crystal phase transition temperature of the DML dispersion. The minimum in $t_{1/2}$ corresponds to the maximum in the rate of the reaction. In the right-hand panel ln ($t_{1/2}^{-1}$) is plotted against T^{-1}. This is an equivalent of the Arrhenius plot. The apparent activation energy of the transport reaction is 240 kcal mol^{-1} in the first half of the phase transition and becomes negative in the second half of the transition. The activation energy is zero at the critical temperature of the phase transition. Conditions as given in Table I.

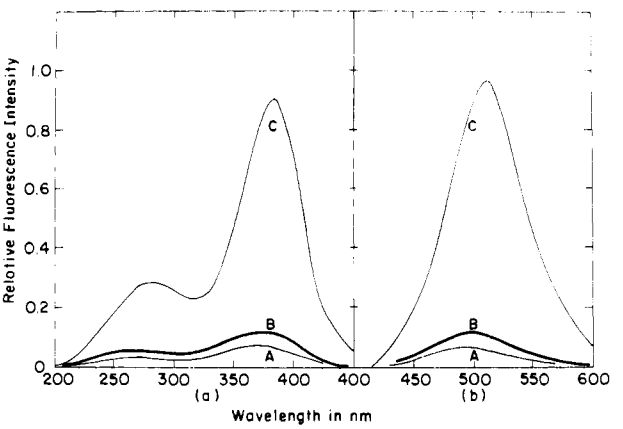

FIGURE 5: Fluorescence excitation and emission spectra of tetracycline in different solvent conditions. (a) Emission spectra; (b) fluorescence spectra. Curves A, B, and C are the spectra of 5 μM tetracycline in water, in 70 μM DML–H_2O dispersions, and in absolute alcohol, respectively. Excitation at 380 nm; emission measured at 500 nm.

temperature-jump experiments of the kinetics of the phase transition itself. In the latter experiment a maximum relaxation time or a minimum rate of the phase transition was observed at the critical temperature.[2]

The rate of the ANS transport depends also on the vesicle preparation. In an experiment done with the limiting size (Huang, 1969) of DML vesicles the reaction was found to be uniformly faster. However, the rate of the reaction showed a similar temperature dependence and exhibited a maximum at 23° compared to 24° for the reaction in the large vesicle system. Our result with the small vesicle system has not been very reproducible because of the slow fusion of vesicles under our experimental conditions.

The effect reported here is extremely large. The rate of the ANS transport decreases 100-fold just a few degrees away from the transition temperature. The same effect was also observed in the transport of bromothymol blue in the dipalmitoyllecithin bilayer system (Tsong and Shortle, 1975). The results obtained for the ANS-DML system are summarized in Table I.

Bindings of Tetracycline, Chlortetracycline, and Pyrene. In order to examine whether the effect discussed in the previous section is a general phenomenon of molecular transport in membrane system or is limited to certain classes of molecule, we have extended our stopped-flow experiment to include tetracycline, chlortetracycline, and pyrene.

In Figure 5 the excitation and emission spectra of tetracycline in an aqueous solution (curve A) are given. Binding of tetracycline to the DML bilayers causes only a slight increase in the fluorescence quantum yield of the dye. Surprisingly, kinetics of the binding reaction was simple exponential in the entire temperature range studied. The Arrhenius plots of the binding of tetracycline (in open circles) and chlortetracycline (in filled circles) to the DML bilayers are given in Figure 6. As is clear from the plots there is no maximum at the phase transition temperature. Although a slight bending of the plots occurs at the T_m of the transition the effect is very small compared to the effects observed in ANS-DML and BTB-DPL systems. The activation energies of the tetracycline and chlortetracycline binding were also one order of magnitude smaller. Some numerical values of the binding of the two classes of molecules to DML and DPL bilayers are given in Table II.

Binding of pyrene to the DML bilayers induces a sizable change in the fluorescence intensity of the suspension. Kinetics of the pyrene binding was complex. However, the half-time of the binding shows no abnormality in the phase transition temperature of the phospholipid vesicles.

Discussion

A Kinetic Model for the Dye Transport. The fact that the kinetics of the ANS transport is extremely complex indicates that there are different types of binding sites in the phospholipid bilayer structures. At low temperature, where the binding occurs only in the outer layer of the lipid vesicle, the generation of fluorescence signals after a rapid mixing in the stopped-flow apparatus can be represented by

$$F(\infty) - F(t) = \alpha + \beta e^{-t/\tau_1} \qquad (1)$$

In the equation $F(\infty)$ and $F(t)$ stand for the final fluorescence intensity and the intensity at time t of the solution, respectively. α denotes an instantaneous appearance of signal within the 2-msec mixing time of the instrument. β is the amplitude and τ_1 is the relaxation time of the 50-msec reaction. A simplest kinetic scheme consistent with the observed signals will be

$$F + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F_1 \xrightarrow{k_2} F_2 \qquad (2)$$

In eq 2, L denotes phospholipid vesicle, and F, F_1, and F_2 represent free ANS and ANS bound to type 1 and to type 2 binding sites, respectively. The first step is a rapid bimolecular adsorption of dye on the vesicle surface (Haynes, 1974) and the second step may be a reorientation of the bound dye in the head group region of the bilayer structures (Figure 7a).

At the phase transition region an additional, and a major kinetic phase appears in the second time range. This kinetic phase is complex (Figure 3) and can be resolved into three close exponential terms. The total fluorescence signal generated in the reaction is now described by

$$F(\infty) - F(t) = \alpha + \beta e^{-t/\tau_1} + \sum_{i=2}^{4} \gamma_i e^{-t/\tau_i} \qquad (3)$$

[2] To be published. See also Maxfield et al. (1975) and Tsong (1974).

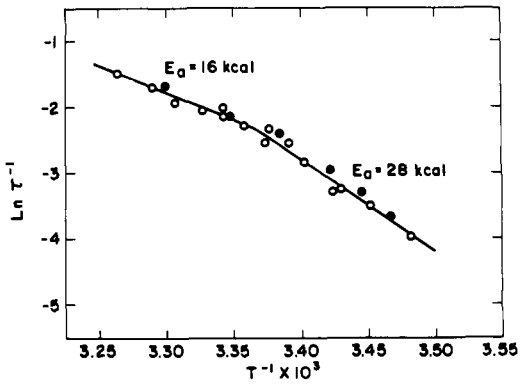

FIGURE 6: Temperature dependence of tetracycline and chlortetracycline bindings to DML bilayers. The Arrhenius plots of the binding reaction shown in Figure 2. (○) data for the tetracycline binding; (●) data for the chlortetracycline binding. Experimental conditions: 25 μM DML, 25 μM tetracycline or chlortetracycline, 0.1 N NaCl, 0.05 M phosphate buffer at pH 7.0. The break of the plots occurs at 24° which is the critical temperature of the phase transition of DML bilayers.

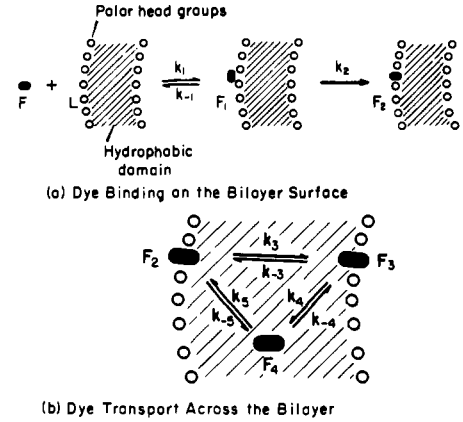

FIGURE 7: A schematic representation of a kinetic model (eq 4a and 4b) for dye transport in the phospholipid bilayers. In (a) the fluorescence probe F is absorbed onto the bilayer surface followed by a reorientation reaction. In the phase transition temperature, transport of dye occurs through two different paths. $F_2 \rightleftarrows F_3$ reaction is interpreted as the carrier mediated "flip-flop" of the probe across the bilayers. $F_2 \rightleftarrows F_4 \rightleftarrows F_3$ reaction is a normal diffusion of the probe into the inner layer of the membrane. For details see text.

Table II: Some Numerical Values for Dye Transport in Phospholipid Bilayers.[a]

Dye	Lipid	T_m (°C)	$t_{1/2}$ at T_m (sec)	Arrhinius plots at T_m	E_a[b] $T <$ T_m (kcal/mol)	E_a[b] $T >$ T_m (kcal/(mol)
ANS	DML	24.1	1.4	A maximum	240	−96
ANS	DPL	41.2	0.60	A maximum	125	−110
BTB[c]	DPL	38.2[d]	0.75	A maximum	90	−104
Tetracycline	DML	24.0	17	A break	28	16
Chlortetracycline	DML	24.0	16	A break	28	16

[a] The slow kinetic phase as shown in Figure 3. [b] The apparent activation energy E_a = is defined by $E_a = -R(\mathrm{d} \ln (t_{1/2}^{-1})/\mathrm{d}(1/T))$. [c] Data from Tsong and Shortle (1975). [d] T_m of BTB–DPL mixtures is a strong function of BTB concentration; 10 mol% of BTB was used in this case.

A simple kinetic scheme consistent with such signal changes is

$$F + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F_1 \xrightarrow{k_2} F_2 \qquad (4a)$$

$$F_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} F_3, \quad F_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} F_4, \quad F_4 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} F_3 \qquad (4b)$$

Equation 4b, in which F_2, F_3, and F_4 denote ANS bound to the outer layer, the inner layer, and the hydrophobic domain of the lipid structures, represents a transport of ANS via different paths across the lipid bilayers (Figure 7b) This model assumes that at temperatures removed from the phase transition region all the rate constants in (4b) become negligibly small, and that the concentration of F_4 is also nonsignificant. However, at the phase transition region the rate constants abruptly increase. At the same time, the concentration of F_4 is significantly increased. Assuming that F_4 has the same fluorescence intensity as F_2 and F_3, from curve B of Figure 2 one can estimate that in the phase transition region about 40% of the total bound dye is dissolved in the hydrophobic domain of the bilayer structures. Indeed, Jacobson and Papahadjopoulos (1975a,b) have recently reported that there is a maximum in the ANS binding to the phospholipid vesicles in the phase transition region, as revealed by the equilibrium dialysis technique. The kinetic model presented here must be regarded as tentative and subjected to further experimental proof.

In eq 4b, the physical difference between the pathways $F_2 \rightleftharpoons F_3$ and $F_2 \rightleftharpoons F_4 \rightleftharpoons F_3$ is not yet clear. Toyoshima and Thompson (1975a,b) have suggested that the Cl^- transport in the bilayers may proceed through a carrier mediated diffusion process in which phospholipid molecule acts as the carrier species. This process may be associated with the trans membrane "flip-flop" of lecithin (Kornberg and McConnell, 1971). One may concede that the transport of ANS may also follow such a flip-flop mechanism, i.e., $F_2 \rightleftharpoons F_3$ step in eq 4b. And since ANS is amphiphilic and is not totally immiscible with the hydrophobic domain of the lipid bilayers the diffusion of ANS across the bilayer may also occur through a regular permeation pathway, i.e., $F_2 \rightleftharpoons F_4 \rightleftharpoons F_3$ shown in the scheme.

To account for the differences in the kinetic behavior of ANS and BTB compared to that of tetracycline, chlortetracycline, and pyrene the following assumptions have to be made. In the former class of molecules binding to the bilayers is limited by the transport reaction (4b) and is expected to be greatly influenced by the lipid phase transitions. On the other hand, in the latter class of molecules the binding may be limited by the initial adsorption step, k_1, or the reorientation step, k_2. In such cases the phase transition would have a much smaller effect on the transport reaction.

It is not yet clear what is causing this difference in the transport property of the two classes of molecules. Papahadjopoulos et al. (1973) have reported that the self-diffusion of $^{22}Na^+$ through dipalmitoylphosphatidylglycerol vesicles shows a local maximum at the phase transition temperature. ANS and BTB molecules are ionic and carry a negative charge at pH 7. In contrast, tetracycline, chlortetracycline, and pyrene are noncharged species at the same solvent conditions. We have done the ANS transport experiment at acidic pH, in which the sulfonic group of the molecule is neutralized, and still observed the same temperature

dependence of the rate of the transport. It appears that the peculiar behavior of ANS and BTB transport may be due to the amphiphilic nature of the molecules.

Biological Significance. The crystal–liquid crystal phase transition of membrane lipids has been found to have profound influence on membrane oriented transport and enzyme activities. Overath and Träuble (1973) have reported that the Arrhenius plots of in vivo *o*-nitrophenyl galactoside hydrolysis and other transport parameters in *Escherichia coli* cells show breaks at the phase transition temperatures of the corresponding lipids. Linden et al. (1973) have studied the transport of β-glucoside in *E. coli* cells. They have also observed breaks of the Arrhenius plots at the temperatures where the phospholipid constituents underwent phase changes. Van Deenen and his co-workers (Op Den Kamp, et al., 1974) have reported that the hydrolysis of lecithin liposomes by pancreatic phospholipase A_2 occurred only in the phase transition region of the liposomes. All these experiments suggest that molecular interactions and transport in the membrane systems depend strongly on the physical state of the lipid bilayers. Such being the case direct measurement of kinetics of the molecular transport is difficult to undertake since no suitable physical properties can be monitored to follow the time course of the transport processes. Because of this limitation study of kinetics of the transport has to rely on model systems such as the optical probe transport reported in this communication.

The kinetics data reported here indicate that transport of amphiphilic molecules or ions differ from that of other classes of molecules in that there is a maximum transport at the critical temperature of lipid phase transition. The apparent activation energy of the reaction is zero at the phase transition temperature, but becomes extremely large at both ends of the phase transition. These observations suggest that transport of different classes of molecules in cellular membranes may also be subjected to different physical constraints. Transport of ions and amphiphilic molecules may be regulated by the physical state of the bilayer structures.

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