EFFECT OF A PHASE TRANSITION ON THE BINDING OF 1-ANILINO-8-NAPHTHALENESULFONATE TO PHOSPHOLIPID MEMBRANES

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ABSTRACT   The binding of 1-anilino-8-naphthalenesulfonate (ANS) to dipalmitoyl-phosphatidylcholine (DPPC)-sonicated vesicles was measured by a fluorimetric method in the vicinity of the gel-to-liquid crystalline phase transition temperature (T_m). A similar measurement was performed on large multimellar DPPC vesicles through equilibrium dialysis. Both measurements demonstrated anomalous dye binding in the temperature region of the T_m and slightly above (prefreezing region). The amount of ANS bound at this temperature region was in excess of what would be expected based on extrapolation of the high temperature binding data; just below the T_m, the amount of bound dye decreased abruptly.   The fluorimetric studies on vesicles also indicated that inner monolayer binding of ANS was markedly inhibited below the T_m. The possibility that the increase in bound dye in the prefreezing region was caused by enhanced lateral compressibility, density fluctuations, or additional binding sites at the boundary of transient gel-like clusters is discussed and the general topic of anomalous increases in various membrane processes in the vicinity of a phase transition is briefly reviewed.

INTRODUCTION

There have been several recent reports of enhanced kinetics of a variety of membrane processes in the vicinity of a phase transition.   Pertaining to transmembrane transport phenomena, Papahadjopoulos et al. (1973) observed a local maximum in the $^{22}Na^+$ permeability of both dipalmitoylphosphatidylcholine (DPPC)[1] and dipalmitoylphosphatidylglycerol (DPPG) vesicles[2] at the phase transition temperature (T_m). Wu and McConnell (1973) reported enhanced valinomycin-mediated potassium conductivity at

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[1]*Abbreviations used in this paper:* ANS, 1-anilino-8-naphthalenesulfonate; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPOPC, dipalmitoleylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; I, total fluorescence intensity in a given emission wavelength band; I_{max}, total fluorescence when all dye is membrane bound; $\bar{\nu}$, moles dye bound per mole of lipid; τ, excited state lifetime; T_m, membrane gel to liquid crystalline phase transition temperature; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.
[2]Small unilamellar vesicles produced by sonication are referred to as "vesicles"; large multilamellar vesicles produced by suspending the phospholipids in buffer are referred to as "liposomes."

the T_m in DPPC bilayers formed with a fritted glass filter support. The transport of 1-anilino-8-naphthalenesulfonate (ANS) across dimyristoylphosphatidylcholine (DMPC) membranes proceeds much more rapidly in the vicinity of the T_m than either below or above this region (Tsong, 1975). Penetration of foreign molecules into bilayers also seems to be increased near a phase transition point. The surface pressure increase in DPPC monolayers at the air-water interface, induced by the penetration of the hydrophobic protein, β-casein, occurs in the temperature range where two-dimensional crystallization begins (Phillips et al., 1975); a high lateral compressibility of the monolayer is associated with the onset of crystallization. The hydrolysis of DMPC liposomes by phospholipase A_2 from pig pancreas shows an appreciable rate only at the T_m, indicative that substantial enzyme penetration occurs only at this temperature (Op Den Kamp et al., 1974). Finally, the growth of small DMPC vesicles to large structures when such vesicles incorporate fatty acids appears to proceed appreciably only near the T_m (Kantor and Prestegard, 1975).

Since under certain conditions the florescence of ANS in phospholipid dispersions shows an apparent local maximum at the phase transition temperature (Sackmann and Träuble, 1972), we decided to measure the amount of ANS bound to membranes undergoing a phase transition. Large density fluctuations and enhanced compressibility probably occur in the vicinity of a phase transition point, and we considered that such phenomena could give rise to altered dye binding in the region of the transition.

MATERIALS AND METHODS

Lipid Preparations

Dipalmitoylphosphatidylcholine (1,2-hexadecanoyl-sn-glycero-3-phosphoryl-choline; DPPC) and dipalmitoleylphosphatidylcholine (1,2-(9-hexadecenoyl)-sn-glycero-3-phosphorylcholine; DPOPC) were synthesized according to Robles and Van den Berg (1969). Palmitic acid and palmitoleic acid used for the synthesis of DPPC and DPOPC, respectively, were from Fluka AG, Switzerland (puriss, > 99%). The lipids were purified on a silicic acid column and gas liquid chromatography of the fatty acid esters indicated better than 99% purity (Papahadjopoulos et al., 1973).

Other Chemicals

ANS was obtained as the ammonium salt from Pierce Chemical Co., Rockford, Ill., and used without further purification. 5×10^{-4} M ANS stock solutions in buffer were stored at 4°C in the dark. L-Histidine (Sigma grade) and N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic-acid (TES) were obtained from Sigma Chemical Co., St. Louis, Mo. Silicic acid was from Mallinckrodt (A-R) (100 mesh). All solvents and chemicals were reagent grade. Water was twice distilled, the second time in an all glass apparatus.

Lipid Dispersions

Phospholipid vesicles were prepared as previously described, (Jacobson and Papahadjopoulos, 1975) always with the use of a buffer composed of 100 mM NaCl, 2 mM TES, 2 mM histidine, and 0.1 mM EDTA at pH 7.4. DPPC and DPOPC were suspended in buffer and sonicated (when required) at 45° and 25°C, respectively. Large multilamellar vesicles (liposomes) were removed from sonicated dispersions by centrifugation at 100,000 g for 1 h (Jacobson and

Papahadjopoulos, 1975). Concentrations of phospholipid were measured by assaying for inorganic phosphate.

Fluorescence Measurements

An Aminco Bowman spectrophotofluorimeter with temperature control (American Instrument Co., Inc., Silver Springs, Md.) was used for fluorescence measurements (Jacobson and Wobschall, 1974) and modified as described previously (Papahadjopoulos et al., 1975). ANS fluorescence was excited using the 365 nm mercury line isolated with the excitation monochromator (1-mm slits) and a 365 nm interference filter having 12 nm half band pass. Emission was observed at 480 nm through the emission monochromator with 1-mm slits (12 nm half band pass) and 3 mm of aqueous 2 M $NaNO_2$ as an ultraviolet cutoff filter. A 5 mm $\times$ 5 mm $\times$ 40 mm cuvette was employed. The exciting light was vertically polarized and emission intensity was sequentially measured first through a vertical polarizer (I_{vv}) and then through a horizontal polarizer (I_{vh}). The total intensity emitted at 460 nm is given by $I = I_{vv} + 2GI_{vh}$, where G is the grating transmission factor (Jacobson and Wobschall, 1974). The background contribution of the vesicle suspension before adding ANS was always measured and subtracted from the fluorescence of the ANS-labeled suspension. This contribution is significant at the lower concentrations of ANS employed (0.25–1 μM).

Fluorometric Determination of ANS Binding to DPPC and DPOPC Vesicles

Since ANS has negligible aqueous fluorescence, the amount bound to DPPC vesicles can be estimated by taking the ratio of the fluorescence (I) when a given concentration of ANS is added to a suspension of sonicated DPPC to the fluorescence (I_{max}) of that concentration of ANS when all of the dye is bound. As discussed in Results, it is important to equilibrate the samples properly after ANS addition because it appears that transport of ANS across the vesicle membrane is limited below the T_m. For measurements at $T \leq T_m$, ANS was added to the suspension at 50–55°C, incubated 10–15 min, and then equilibrated at the temperature of the measurement. I_{max} can be determined by titrating a given concentration of ANS with increasing concentrations of sonicated DPPC (or DPOPC) vesicles. Double reciprocal plots of I^{-1} vs. $[DPPC]^{-1}$ yield straight lines, meaning that such data obey the empirical equation, $I^{-1} = I_{max}^{-1} + (K) [DPPC]^{-1}$. The intercept on the ordinate is I_{max}^{-1} and is obtained by extrapolation of the least squares fitted straight line to $[DPPC]^{-1} = 0$. Since such extrapolations are subject to error, double reciprocal plots were made with data taken at various total concentrations of ANS (1, 2, and 5 μM) with lipid concentrations varying from 0.3 to 1.8 mM. The results were averaged to give a mean I_{max} for a bound ANS concentration of 1 μM.

Dye-binding measurements were performed in high ionic strength (100 mM NaCl) and molar ratios (typically, 1/4,000 to 1/100) in order to minimize both structural perturbations caused by bound dye (Lesslauer et al., 1972) and electrostatic repulsion effects between bound and free dye. With the use of approximately 1 mM lipid, the binding isotherms are linear, with added ANS from 0.25 to 1–2 μM indicating saturation effects were not occurring.

EQUILIBRIUM DIALYSIS

Binding of ANS to unsonicated DPPC vesicles (liposomes) was measured by equilibrium dialysis. DPPC was suspended at 45°C in 10 ml of the usual buffer containing 5 μM ANS. Final lipid concentration was approximately 1.5 mM. 1 ml of this suspension was placed in cleaned dialysis tubing of $\frac{1}{4}$-in diameter (Union Carbide Corp., New York). The loaded bags were placed in a screwtop test tube containing 4 ml of buffer with no ANS and immersed and rotated in a water bath at various temperatures controlled to ± 0.1°C for at least 18 h. The ANS con-

centration of small aliquots taken from inside and outside the bag was determined with the ANS fluorescence in $CHCl_3$-CH_3OH (4:3 vol/vol) as an assay.

RESULTS

Temperature Dependence of I_{max}

The temperature dependence of I_{max} for both DPPC and DPOPC vesicles suspensions is shown in Fig. 1 and is of crucial importance to this study. It should be proportional to the temperature dependence of the quantum yield and excited state lifetime of membrane-bound ANS. To our knowledge, such data have not been published. However, Haynes (personal communication) has measured the excited state lifetime (τ) of ANS bound to sonicated DMPC vesicles as a function of temperature. These data are also plotted in Fig. 1, and shifted along the abscissa so that the transition temperature of DMPC coincides with that of DPPC. Above the T_m, the agreement in the temperature dependence of I_{max} and τ is striking, although not completely convincing because the lipids are slightly different (DPPC vs. DMPC). Nevertheless, Haynes and Staerk (1974) report that the slopes of τ^{-1} vs. T plots above the T_m for both DMPC bilayer and monolayer vesicles as well as DPPC monolayer vesicles were all 0.0019 ±
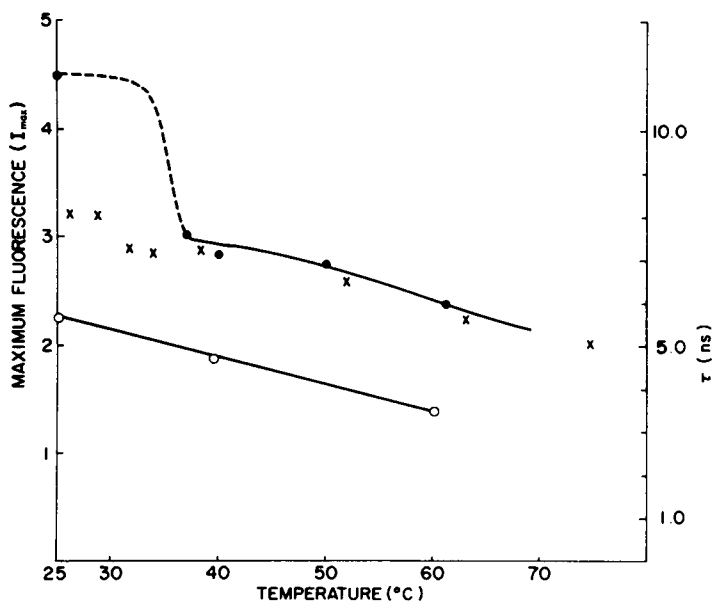

FIGURE 1   Right ordinate: maximum fluorescence (I_{max}) as a function of temperature when ANS at 1 μM is completely bound to lipid vesicles; (●—●), for DPPC vesicles; (o—o), for DPOPC vesicles. In the curve of I_{max} vs. T for DPPC (●—●), the broken portion is drawn on the assumption that an abrupt change in slope of the curve occurs at the minor transition (Haynes and Staerk, 1974). Left ordinate is excited state lifetime (τ) measured by Haynes (personal communication) for ANS bound to DMPC and is shown by X. *Note:* in order to compare the temperature dependence of τ near the phase transition, the DMPC lifetime data is shifted along the abscissa so that the transitions for DMPC (23°C) and DPPC (41°C) match.
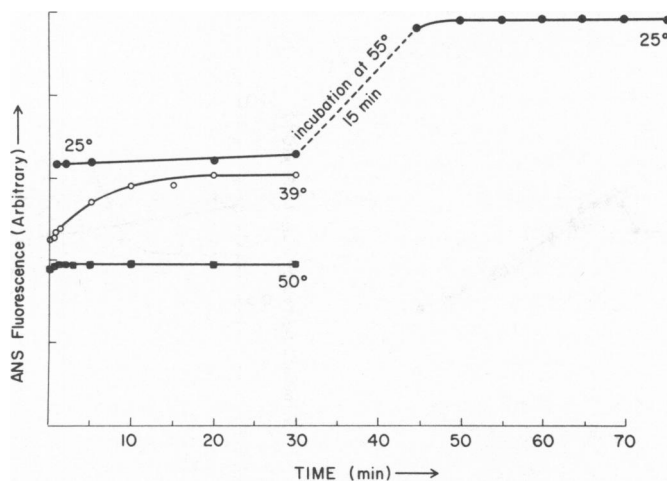
0.001 ns^{-1}·°K^{-1}. This fact suggests that the good agreement between I_{max} and τ in Fig. 2 is not fortuitous. Below the transition, however, I_{max} increases more than τ and could mean that a static quenching phenomenon is more effective above the transition than below it. Such an effect would cause I_{max} to increase more than τ as temperature was decreased through the T_m. In contrast, I_{max} monotonically decreases with the temperature in ANS-labeled DPOPC membranes which do not exhibit a phase transition in this temperature range.

KINETICS OF FLUORESCENCE ENHANCEMENT: TRANSPORT OF ANS ACROSS BILAYER MEMBRANES

In the course of this study, it was necessary to find how long after addition of dye to aqueous suspensions of sonicated DPPC vesicles at different temperatures an equilibrium fluorescence value was reached. Typical data are given in Fig. 2. Equilibration is fast at 50°C and slower at 39°C. At 25°C, however, a pseudo-equilibrium value is reached quickly which is increased by about 50% after a 15 min incubation at 55°C ($T > T_m$). The ratio of fluorescence before and after the incubation is about 2:3 which is similar to the ratio of outer monolayer surface area to the total surface area of sonicated vesicles (Bystrov, 1971, and reviewed recently by Johnson et al., 1975). This suggests that inner monolayer sites are inaccessible to ANS for $T < T_m$ because the dye is not transported across the hydrocarbon region when the membranes are in the gel state.
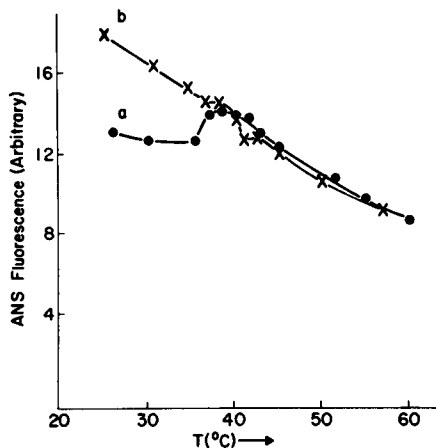
FIGURE 3   FIGURE 4

FIGURE 3   ANS fluorescence vs. temperature when dye is bound to sonicated DPPC vesicles.
(*a*) (●—●), 1.04 mM DPPC suspension is made 5 μM in ANS at 25°C, equilibrated 20 min at
25°C, and measurements made as temperature is increased; (*b*) (X—X), 0.85 mM DPPC suspen-
sion is made 5 μM in ANS at 25°C, then incubated 15 min at 55°C, followed by 15 min equi-
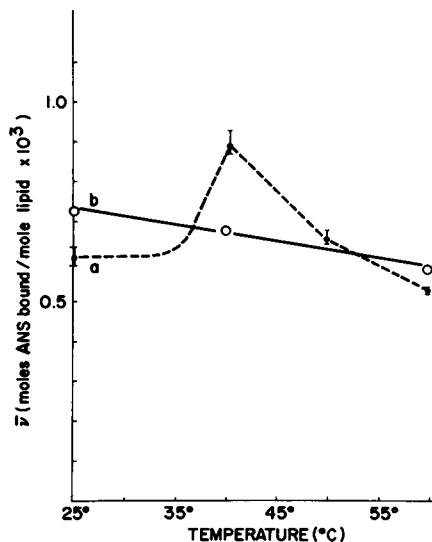libration to 25°C and then upward temperature scan is started.

FIGURE 4 $\bar{\nu}$, moles of membrane bound ANS per mole of phospholipid (sonicated vesicles) as
a function of temperature as determined by fluorimetric assay. Total concentration of ANS was
1 μM; concentration of phospholipid was 1 mM. (●- - -●), DPPC; (o—o), DPOPC. For DPPC,
bars represent maximum uncertainty in $\bar{\nu}$ caused by random dye addition errors. Independent ex-
periments gave similar temperature profiles with $\bar{\nu}$ values differing by less than 25%.

Temperature scans of ANS fluorescence from sonicated DPPC suspensions also
indicate that dye transport to inner monolayer sites is limited below T_m. In Fig. 3
(curve *a*), ANS is added to the vesicle suspension at 25°C so that, presumably, only
outer monolayer sites should be accessible to ANS. Increasing temperature very slowly
to approximate equilibrium in the range 25°–35°C does not alter fluorescence. At
about 36°C an abrupt increase in fluorescence begins, continuing to about 40°C when
fluorescence decreases with temperature in a monotonic fashion. In contrast, curve *b*
of Fig. 3 shows the temperature dependence of fluorescence when ANS is added to a
similar suspension but incubated at 55°C for 15 min before beginning the upward
temperature scan to insure accessibility of the dye to both inner and outer monolayers
of the vesicle. In this case the fluorescence drops almost linearly with temperature
except for anomalous behavior in the region of the transition. The fluorescence at 25°C
is about 1.25-fold greater than that measured when ANS is added outside, but the two
curves gradually come closer as the temperature is increased, reaching near coinci-
dence for temperatures greater than 43°C. Our interpretation of these data is that when
ANS is added at 25°C binding of the dye only occurs on the outer monolayer of the

vesicles, but as the temperature is increased to the phase transition region transport of ANS across the hydrocarbon region increases, allowing binding of the dye to the inner monolayer as well. Thus, curve *a* gradually approaches curve *b*, in which presumably ANS was available to both inner and outer monolayer binding sites from the outset. The data shown in curve *a* of Fig. 3 suggest that the onsets of phase transitions in model membranes might generally be detected by adding ANS to the vesicle suspension below its T_m. A sudden increase in fluorescence observed as the temperature is increased to the onset of the transition presumably will correspond to transport of ANS to inner monolayer binding sites.

ANS Binding to Phospholipid Vesicles at Different Temperatures

Fig. 4 shows the amount of ANS bound to both DPPC and DPOPC sonicated vesicles at various temperatures. DPOPC is identical to DPPC except for the presence of a cis double bond in the acyl chain between the 9 and 10 carbon atoms. ANS binding to DPOPC vesicles (solid line) decreases linearly with rising temperature. In contrast, the binding of ANS to sonicated DPPC vesicles (broken line) shows a local maximum in the region of the phase transition. This is reproduced in equilibrium dialysis experiments for DPPC liposomes as shown in Fig. 5. Below 41°C, the curve for DPPC in Fig. 4 is drawn with a sharp break on the basis of the following facts: points from independent experiments at temperatures slightly below 41°C (39.5°, 38.7°, 37°) follow such a curve; equilibrium dialysis with unsonicated DPPC vesicles (Fig. 5) shows an abrupt drop in $\bar{\nu}$ just below the T_m; the data of Haynes and Staerk (1974), obtained with DMPC monolayer vesicles, show $\bar{\nu}$ to be constant with increasing temperature until the minor transition is reached, when the linear decrease in $\bar{\nu}$ with temperature begins.

The area of more concern for this paper is dye binding in the region of $T \geq T_m$. The amount of ANS bound per mole of DPPC falls off in a nonlinear fashion for both sonicated (Fig. 4, broken line) and unsonicated (Fig. 5) DPPC vesicles. In the case of the unsonicated dispersions, $\bar{\nu}$ decreases in an approximately linear fashion from the T_m to 60°C, then decreases less rapidly with temperature from 60° to 70°C. Similar behavior occurs with the sonicated dispersions, except that the region of sharpest decrease in $\bar{\nu}$ occurs between 40° and 50°C with a less rapid decrease occurring between 50° and 60°C. The amount of dye bound obtained by the equilibrium dialysis experiments with liposomes and the amount obtained by the fluorimetric measurements with sonicated vesicles are in good agreement ($\lesssim 10\%$ discrepancy) when experiments at identical lipid and total dye concentrations are compared.

DISCUSSION

ANS Binding to Vesicles

Binding of ANS to DPPC vesicles, both unsonicated and sonicated, is strongly dependent on temperature. The amount of dye bound abruptly increases by about 20% in DPPC liposomes (Fig. 5) and by approximately 30% in sonicated DPPC vesicles (Fig. 4, curve *a*) in the region of the phase transition. This increase is not observed

with sonicated DPOPC vesicles (Fig. 4, curve *b*) which do not show a phase transition in this region (25°–60°C). It is interesting that ANS binding to fluid DPOPC membranes exceeds that for DPPC at the extreme temperatures (25°C, 60°C) in the range studied. This behavior is similar to that observed when the $^{22}Na^+$ membrane self diffusion in fluid dioleylphosphatidylglycerol membranes is compared to that for DPPG membranes which display a transition in the temperature range examined (Papahadjopoulos et al., 1973). The ANS binding data of Haynes and Staerk (1974) for monolayer DMPC vesicles do not show this increase in $\bar{\nu}$ at the T_m.

Approaching the transition from high temperatures, the amount of dye bound increases with decreasing temperature reaching a maximum at the phase transition temperature. The slopes of these curves ($\bar{\nu}$ vs. T) increase as the T_m is approached in a manner reminiscent of critical opalescence phenomena. Similar curves can be constructed from the data of Huang and Charlton (1972; Table I) for the binding ANS to egg phosphatidylcholine (PC)-sonicated vesicles; these binding curves ($\bar{\nu}$ vs. T) are biphasic exhibiting a greater slope at the lower temperatures examined and a change in slope in the range 25°–30°C.

We interpret these dye-binding measurements in terms of cluster formation in the prefreezing region proposed by Lee et al. (1974). These investigators suggested that 2,2,6,6-tetramethylpiperdine-1-oxyl (TEMPO) partitioning data for DOPC vesicles can be explained by postulating transient molecular clusters above the T_m having a gel-like character which exclude the probe, although other explanations are available (Wu and McConnell, 1975). According to the interpretation of Lee et al. (1974), clusters begin to form 50°C above the T_m (−22°C) for DOPC. The onset of cluster formation would be accompanied by enhanced lateral compressibility as compression in this temperature range would result in the conversion of molecules having expanded conformations characteristic of the liquid crystalline state to those having condensed conformations (in clusters) typical of the gel state. The binding of amphipatic ANS to DPPC bilayers is thought to involve insertion of the apolar naphthalene moiety into the hydrocarbon region while the charged sulfonate moiety remains in the plane of the polar headgroups (Lesslauer et al., 1971, 1972; Colley and Metcalfe, 1972). Thus, enhanced lateral compressibility in the liquid crystalline state near the T_m could result in increased dye binding in this region by allowing the insertion of ANS into its membrane binding site with minimal perturbation. It is also conceivable that additional dye-binding sites will be created in the boundary regions (Papahadjopoulos et al., 1973) caused by the coexistence of transient gel-like clusters with fluid membranes. In agreement with Wu and McConnell (1973), we believe that the two possibilities discussed above may be related since enhanced compressibility and density fluctuations may be characteristic of these boundary regions.

Our data indicate that the onset of increased dye binding (with decreasing temperature) occurs between 60° and 70°C for DPPC liposomes and between 50° and 60°C for sonicated DPPC vesicles. It is possible that the greater strain in the sonicated vesicles would shift the onset of cluster formation and increased compressibility to lower temperatures. Consistent with this observation, the onset of enhanced $^{22}Na^+$ perme-
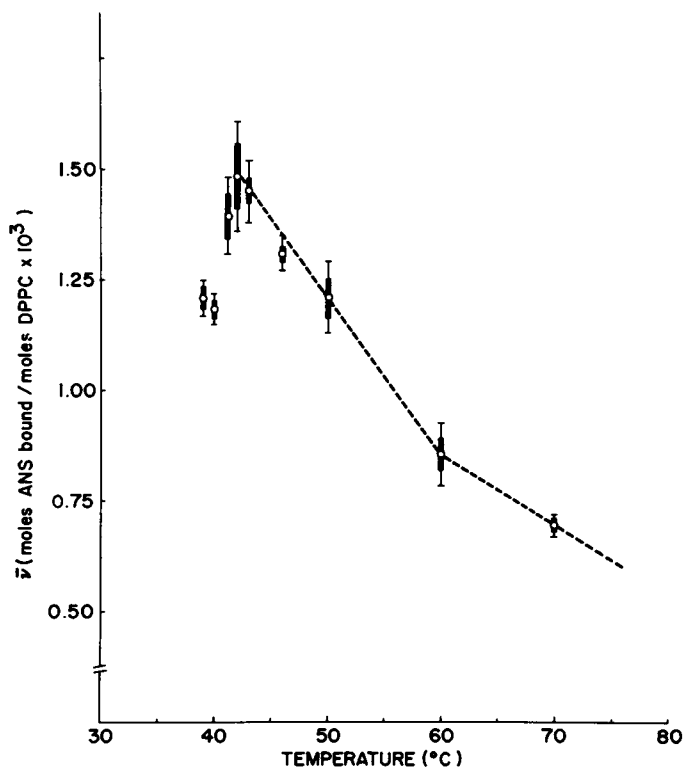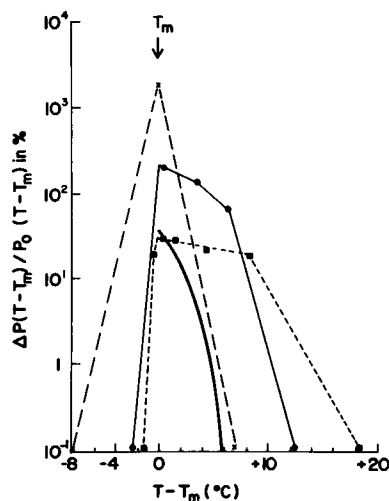
FIGURE 5

FIGURE 6

FIGURE 5 $\bar{v}$, moles ANS bound per mole of DPPC (unsonicated suspension with 1.5 μmol DPPC per sample) determined at various temperatures by equilibrium dialysis as described in the text. Narrow bar gives ± standard deviation; broad bar gives ± standard error of the mean.

FIGURE 6 $\Delta P(T - T_m)/P_o(T - T_m)$ vs. $(T - T_m)$ for various membrane related parameters in the region of a phase transition. $\Delta P(T - T_m)$ represents the difference $P(T - T_m) - P_o(T - T_m)$ where $P_o(T - T_m)$ represents the estimated value of the parameter in the absence of any enhancement by prefreezing effects and $P(T - T_m)$ represents the experimental value of a selected parameter at a temperature interval $(T - T_m)$ away from the phase transition. $P_o(T - T_m)$ is obtained by extrapolation to $T \simeq T_m$ of the $P(T - T_m)$ curve based on high values of $T - T_m$ where, presumably, prefreezing effects are negligible. (X----X), Extent of pig pancreatic phospholipase A_2 hydrolysis of DMPC liposomes in 15 min at indicated temperature (Op Den Kamp et al., 1974, Table I). Similar data were obtained for DPPC liposomes. (●—●), Self-diffusion rates of $^{22}Na^+$ through DPPC liposomes (Papahadjopoulos et al., 1973, Fig. 7). $P_o(T - T_m)$ was obtained by extrapolation of line defined by two highest temperature points to the T_m. (■—■), $\bar{v}$ for ANS binding to DPPC liposomes (this study, Fig. 5). $P_o(T - T_m)$ was obtained by extrapolation of line defined by two highest temperature points to T_m. (—), Valinomycin-mediated current in DPPC multilayers formed on fitted glass support in 0.1 M KCl (Wu and McConnell, 1973, Fig. 3 c). Data from this figure were plotted in the form ln (current) vs. T^{-1}. $P_o(T - T_m)$ was obtained by extrapolation of the straight line on the Arrhenius plot in the region 47°-49°C to T_m.

ability in both sonicated DPPC and DPPG vesicles is shifted closer to the T_m than in the respective unsonicated suspensions (Papahadjopoulos et al., 1973). It is somewhat surprising that changes in the slope of the $\bar{\nu}$ vs. T curves (Figs. 4 and 5) occur $10°-20°C$ and $20°-30°C$ above the T_m for sonicated and unsonicated DPPC suspensions, respectively. However, in studies of the binding of other dyes to egg PC vesicles (Huang and Charlton, 1972; Ting and Solomon, 1975), changes in slope of the association constant vs. T curves occur some $30°-40°C$ above T_m for egg PC (about $-10°C$; Williams and Chapman, 1970). It is likely that the onset temperature (T_o) of cluster formation and enhanced compressibility will depend, in part, on the transition point of the lipid bilayer. The higher the T_m, the smaller will be the separation ($\Delta T = T_o - T_m$) between T_m and the onset of cluster formation since such structures become entropically less favorable at higher temperatures.

Anomalous Behavior in the Vicinity of Phase Transitions in Other Membrane Systems

Prefreezing behavior in phospholipid bilayers close to their phase transition points appears to affect several membrane properties differently, both in regard to the extent to which the process is enhanced and in regard to how close to the T_m the enhancement begins. This is shown in Fig. 6 where a measure of process enhancement near T_m is plotted vs. temperature away from T_m for several literature examples. Fig. 6 shows that pig pancreatic phospholipase A_2 (Op Den Kamp et al., 1974) and $^{22}Na^+$ self-diffusion (Papahadjopoulos et al., 1973) undergo a considerably larger rate enhancement than is observed for either the increase in equilibrium binding of ANS or the increase in valinomycin-mediated K^+ current (Wu and McConnell, 1973). The last example may reflect an increase in the membrane partition coefficient for valinomycin near the T_m. It may therefore be that rate processes (enzyme activity, cation transport) are accelerated more than equilibrium membrane concentrations (ANS, valinomycin) are increased by prefreezing behavior. This is supported by the recent work of Tsong (1975), who has shown that the rate of ANS transport through DMPC membranes undergoes a very large increase in the vicinity of the T_m. As an exception, it should be noted that in studies of β-casein penetration into DPPC monolayers having high compressibility the amount of bound protein is increased, but not the rate of penetration (Phillips et al., 1975).

Fig. 6 also reveals that the range of temperature, ΔT, over which enhancement in membrane processes induced by prefreezing effects occurs, may depend on molecular size of the active species. Thus, activities associated with larger molecules such as the enzyme, pig pancreatic phospholipase A_2 ($\Delta T < 7°C$), or the transport peptide, valinomycin ($\Delta T \sim 5°C$), show enhancement at temperatures closer to the T_m compared with ANS binding ($\Delta T \sim 18°C$) or $^{22}Na^+$ self-diffusion ($\Delta T \sim 12°C$). It is plausible that the compressibility and fluctuation effects in the prefreezing region become large enough to affect membrane processes mediated by macromolecular species only at temperatures quite close to the T_m.

Not all membrane processes are enhanced by fluctuation and compressibility effects

in the prefreezing region. As noted above, the decreased affinity of membranes for TEMPO as the temperature is decreased can be interpreted simply in terms of cluster formation within the membrane (Lee et al., 1974). Below 20°C, the solubility of N-phenyl-naphthylamine in egg PC vesicles remains constant (Ting and Solomon, 1975). This probe, like TEMPO, is presumed to locate in the hydrocarbon interior of bilayers. Similarly, polar compounds such as the antibiotics, tetracycline and chlortetracycline do not show faster membrane adsorption kinetics in the vicinity of a phase transition (Tsong, 1975). Thus, it may be that small amphipathic molecules, such as ANS, which have membrane-binding sites near the interface and/or rate limiting steps for their function which occur in this region of the bilayer are most sensitive to prefreezing behavior.

While the activity of some reconstituted lipid dependent enzymes such as the $(Na^+ + K^+ + Mg^{2+})$-ATPase (Kimelberg and Papahadjopoulos, 1972) or β-hydroxybutyrate dehydrogenase (Houslay et al., 1975) are sensitive to the phase of the activating lipid, no maxima in activity at the transition temperature have been observed. In contrast, the $(Na^+ + K^+ + Mg^{2+})$-ATPase of isolated plasma membranes of LM cells (Wisnieski et al., 1974) and the β-galactoside transport (Linden et al., 1973) through membranes of a fatty acid auxotroph of *Escherichia coli* supplied with exogenous fatty acids do show maximal activity in narrow temperature regions thought to correspond to membrane phase separations. Although it is not possible to discuss the precise molecular mechanisms responsible for such regions of maximal activity, our results demonstrate that the binding of certain molecules to lipid bilayers can be increased in the vicinity of a phase transition. Thus, the availability of substrate, cofactor, or effector molecules to membrane enzymes could be enhanced near a phase transition point.

REFERENCES

BYSTROV, V., N. DUBROVINA, L. BARKSUKOV, and L. BERGELSON. 1971. NMR differentiation of the internal and external phospholipid membrane surfaces using paramagnetic Mn^{2+} and Eu^{2+} ions. *Chem. Phys. Lipids.* 6:343–350.

COLLEY, C., and J. METCALFE. 1972. The localization of small molecules in lipid bilayers. *FEBS (Fed. Eur. Biochem. Soc.). Lett.* 24:241–246.

HAYNES, D. H., and H. STAERK. 1974. 1-Anilino-8-napthalenesulfonate: a fluorescent probe of membrane surface structure, composition and mobility. *J. Membr. Biol.* 17:313–340.

HOUSLAY, M., G. WARREN, N. BIRDSALL, and J. METCALFE. 1975. Lipid phase transitions control β-hydroxybutyrate dehydrogenase activity in defined lipid protein complexes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 51:146–151.

HUANG, C., and J. CHARLTON. 1972. Interactions of phosphatidylcholine vesicles with 2-p-toluidinylnapthalene-6-sulfonate. *Biochemistry.* 11:735–740.

JACOBSON, K., and D. PAPAHADJOPOULOS. 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH and concentrations of bivalent cations. *Biochemistry.* 14:152–161.

JACOBSON, K., and D. WOBSCHALL. 1974. Rotation of fluorescent probes localized within lipid bilayer membranes. *Chem. Phys. Lipids.* **12**:117–131.

JOHNSON, L., M. HUGHES, and D. ZILVERSMIT. 1975. Use of phospholipid exchange protein to measure inside-outside transposition in phosphatidylcholine liposomes. *Biochim. Biophys. Acta.* **375**:176–185.

KANTOR, H., and J. PRESTEGARD. 1975. Fusion of fatty acid containing lecithin vesicles. *Biochemistry.* **14**:1790–1795.

KIMELBERG, H., and D. PAPAHADJOPOULOS. 1972. Phospholipid requirements for $(Na^+ + K^+)$-ATPase activity: head-group specificity and fatty acid fluidity. *Biochim. Biophys. Acta.* **282**:277–292.

LEE, A. G., N. J. M. BIRDSALL, J. C. METCALFE, P. A. TOON, and G. B. WARREN. 1974. Clusters in lipid bilayers and the interpretation of thermal effects in biological membranes. *Biochemistry.* **13**:3699–3705.

LESSLAUER, W., J. CAIN, and J. K. BLASIE. 1971. On the location of 1-anilino-8-napthalene-sulfonate in lipid model systems. An X-ray diffraction study. *Biochim. Biophys. Acta.* **241**:547–566.

LESSLAUER, W., J. E. CAIN, and J. K. BLASIE. 1972. X-ray diffraction studies of lecithin biomolecular leaflets with incorporated fluorescent probes. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1499–1503.

LINDEN, C., K. WRIGHT, H. McCONNELL, and C. FOX. 1973. Lateral phase separations in membrane lipids and the mechanism of sugar transport in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **70**:2271–2275.

OP DEN KAMP, J. A. F., J. DEGIER, and L. L. M. VANDEENEN. 1974. Hydrolysis of phosphatidylcholine liposomes by pancreatic phospholipase A_2 at the transition temperature. *Biochim. Biophys. Acta.* **345**:253–256.

PAPAHADJOPOULOS, D., K. JACOBSON, S. NIR, and T. ISAC. 1973. Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. *Biochim. Biophys. Acta.* **311**:330–348.

PAPAHADJOPOULOS, D., K. JACOBSON, G. POSTE, and G. SHEPHERD. 1975. Effects of local anaesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. *Biochim. Biophys. Acta.* **394**:504–519.

PAPAHADJOPOULOS, D., G. POSTE, B. SCHAEFFER, and W. VAIL. 1974. Membrane fusion and molecular segregation in phospholipid vesicles. *Biochim. Biophys. Acta.* **352**:1028.

PHILLIPS, M. C., D. E. GRAHAM, H. HAUSER. 1975. Lateral compressibility and penetration into phospholipid monolayers and bilayer membranes. *Nature (Lond.).* **254**:154–156.

ROBLES, E. C., and D. VAN DEN BERG. 1969. Synthesis of lecithins by acylation of 0-(sn-glycero-3-phosphoryl) choline with fatty acid anhydrides. *Biochim. Biophys. Acta.* **187**:520–526.

SACKMANN, E., and H. TRÄUBLE. 1972. Studies of the crystalline-liquid crystalline phase transition of lipid model membranes. I. Use of spin labels and optical probes as indications of the phase transition. *J. Am. Chem. Soc.* **94**:4482–4491.

TING, P., and A. K. SOLOMON. 1975. Temperature dependence of N-phenyl-l-napthylamine binding in egg lecithin vesicles. *Biochim. Biophys. Acta.* **406**:447–451.

TRAUBLE, H. 1971. Phasenumwandlungen in Lipiden Mögliche Schaltzprozesse in Biologischen Membranen. *Die Naturwissenschaften.* **58**:277–284.

TSONG, T. Y. 1975. Effect of a phase transition on the kinetics of dye transport in phospholipid bilayer structures. *Biochemistry.* **14**:5409–5414.

WILLIAMS, R., and D. CHAPMAN. 1970. Phospholipids, liquid crystals and cell membranes. *Prog. Chem. Fats Other Lipids.* **11**:1–79.

WISNIESKI, B., J. PARKES, Y. HUANG, and C. FOX. 1974. Physical and physiological evidence for two phase transitions in cytoplasmic membranes of animal cells. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4381–4385.

WU, S., and H. M. McCONNELL. 1973. Lateral phase separations and perpendicular transport in membranes. *Biochem. Biophys. Res. Commun.* **55**:484–491.

WU, S., and H. M. McCONNELL. 1975. Phase separations in phospholipid membranes. *Biochemistry.* **14**:847–854.