J. Membrane Biol. 34, 157–201 (1977)

Effect of Small Molecules on the Dipalmitoyl Lecithin Liposomal Bilayer:

III. Phase Transition in Lipid Bilayer

Mahendra Kumar Jain and Nora Min Wu

Department of Chemistry and Division of Health Sciences, University of Delaware, Newark, Delaware 19711

Received 19 March 1976; revised 28 September 1976

Summary. The effect of more than ninety lipid-soluble compounds on the phase transition behavior of DL-α-dipalmitoyl lecithin bilayer has been examined by differential scanning calorimetry. The type of effect on the phase transition profile depends on the nature of the additive, whereas the extent of the effect depends on the concentration. The compounds examined include uncouplers, alkanols, fatty acids, detergents, organic solvents, ionophores, inorganic ions, and some commonly used spin-labelled and fluorescent membrane probes. A qualitatively distinct effect of several of these additives on the phase transition behavior of bilayer provides a method of determining the nature of the perturbation they induce in the bilayer organization. The observations are consistent with the hypothesis that the type of effect induced by an additive on the phase transition profile of the bilayer is related to the position of localization of the additive along the thickness of the bilayer. At least four different types of modified transition profiles that are related to changes in bilayer fluidity can be distinguished. These correspond to the localization of the additive in phosphorylcholine (type D), glycerol backbone (type B), C_1–C_8 methylene (type A), C_9–C_{16} methylene (type C) region of the bilayer. A possible relationship between the type of phase transition profiles of modified liposomes and the physiological effects of drugs is also discussed.

Introduction

Biomembrane is a locus of action of a large variety of small molecules. Some of these compounds (transmitters, hormones) are known to interact with specific protein sites on membranes. However, a relatively large number of compounds seem to interact nonspecifically with biomembrane. Some of these compounds exhibit a variety of other biochemical activities in addition to the one primarily responsible for the pharmacological action. Such compounds include drugs like tranquillizers, antidepressants, narcotics and anesthetics. Their mode of action involves not only their passage through a hydrophobic barrier but also interaction with hydrophobic sites in target tissues. There is a growing body of

evidence that quite a few of these compounds presumably interact with biomembranes to elicit or modulate a variety of biological phenomena and activities, including catalytic functions, transport (action potential, active transport, passive diffusion, synaptic transmission), recognition and morphology (adhesion, aggregation, fusion and immunogenic response). The effect of small molecules on bilayers is also important to understand the potential-perturbing effect of membrane probes (spin label, fluorescent probes) and to develop modified liquid crystals. In general, the molecular mechanism of action seems to involve nonspecific hydrophobic interaction between these agents and lipid bilayer. However, the changes in the bilayer organization, consequent and subsequent to such interactions, remain to be characterized.

In spite of a widespread interest, little direct attention has been paid to the problem of interaction of small molecules to biomembrane and related model systems. Evidence to support the membrane as the site of action of the various types of molecules is derived from the observations that these agents partition into or bind to, and exert their effect on isolated membranes. For several of these agents their biological activity has been correlated to their lipid solubility and/or to their lipid-water (buffer) partition coefficient. However, there are some limitations to this approach. From the standpoint of their lipid solubility many of these substances may be lumped together as nonspecific drugs, but from the clinical standpoint they may be very specific indeed in their individual pharmacological effects. That is to say, all drugs that affect membrane functions nonspecifically are lipid soluble, but the converse is not true. Also, all lipid-soluble drugs that act on membranes nonspecifically do not necessarily elicit the same response. Moreover, the biomembrane differs from the bulk lipid or lipidic solvents not only in composition but also in organizational features. Biomembranes have an anisotropic interior separated from the aqueous medium by a layer of polar groups. Besides this, there is considerable interaction between polymethylene chains in the bilayer such that they show cooperativity in phase transition. These characteristics of a bilayer organization would make the "solubility" properties of additives in bilayer distinct from those in isotropic bulk solvents.

The cooperativity of interaction of acyl chains in bilayer show significant long-range (over several molecular diameters) order-disorder transitions. Such transitions are endothermic and can be detected by differential scanning calorimetry. Small molecules can be accommodated in between the acyl chains, and the disruptive effect of these additives is thus

expected to influence the mode of packing of the hydrocarbon chains and, therefore, the order-disorder transition. In a bilayer, in gel phase the motion in one fatty acid chain is to some extent transmitted to the chains next to it, and so on to several molecules that form a cooperative unit. Thus, the conformational changes can be transmitted over a substantial portion of the cooperative unit. Molecules of an additive, however, would be expected to block this transmitting system, so that the cooperative motion of the chains will be quickly damped out, ensuring that changes in the membrane remain relatively local instead of involving the cooperative unit as a whole. Thus, the additives may modify the phase transition profile of lipid. Indeed, they do so and the effect is dependent both on the structure and the concentration of the additive (Jain, Wu & Wray, 1975). These results suggest that in a certain temperature range the various additives may be used to induce a phase transition in lipid bilayer.

The purpose of the present study is to screen a variety of small molecules for their ability to modify the phase transition profile of dipalmitoyl lecithin bilayer. The information thus obtained is potentially useful for understanding the mechanism of action of various lipid-soluble solutes on the biomembrane. In this paper we describe the phenomenology of additive-induced phase change and phase separation in dipalmitoyl lecithin bilayer. These additives include *n*-alkanols, fatty acids, some organic solvents, detergents, ionophores, inorganic ions, and some commonly used spin-labelled and fluorescent membrane probes.

Materials and Methods

Most of the compounds used in this study were purchased from Sigma or Aldrich Chemical Co. Sources of the other compounds are indicated in the Results section. All these compounds were used as received. DL-1,2-dipalmitoyl lecithin was chromatographed on neutral alumina (Woelm) and the fraction that eluted with 1:4 (v/v) methanol+chloroform was used for the preparation of liposomes.

The liposome+additive samples for differential scanning calorimetry (DSC) studies were prepared by one of the following methods:

(a) If the additive is soluble in water, then appropriate volume of its solution was mixed with liposome dispersion.

(b) If the additive is nonvolatile and insoluble in water, then its methanol, or chloroform, or benzene (in that order) solution was evaporated under nitrogen in a small test tube. These samples were then dried in an evacuated (pressure ~ 10 mM) dessicator. Liposomes were transferred onto the dry film.

(c) If the additive is volatile and insoluble in water then a known amount of the additive was mixed with the liposomes directly. Whenever necessary, the concentration of the additive was adjusted by successive dilution with liposomes.

(d) For comparison, some samples were prepared by premixing the lipid and the additive in an organic solvent. The liposomes were prepared from the mixed film after removal of the solvent.

In any of the above situations, a final volume of 32 μl (liposome + additive) was mixed in a test tube with a 3 mm glass bead on a Vortex shaker at full setting under a nitrogen atmosphere at 45–50 °C (room temperature for volatile additives) for about 2–3 min. The sample was then allowed to equilibrate for 1–4 days at 4 °C and finally shaken again for several minutes at 45–50 °C (room temperature for volatile additives). These samples were used at least 2 hr after the final mixing.

All samples for scanning were 75 mM in dipalmitoyl lecithin, 50 mM in KCl, 5 mM in Tris buffer, and were prepared at pH 7.3. The samples (15 μl) were scanned in sealed sample holders on a Perkin-Elmer DSC-1B calorimeter operating at a sensitivity of 1 mcal and a scanning rate of 1.25 °K/min. The temperature range for all the scans reported in this paper was 295 to 320 °K. Although not shown in any of the profiles, the base line for all the profiles was horizontal. The slope setting required for this was between 680–700 for all the samples. The transition profile of unmodified liposomes was symmetrical. In agreement with the published data (Simon, Lis, Kauffman & Mac Donald, 1975) for unsonicated liposomes of dipalmitoyl lecithin, the beginning and midpoint transition temperatures (that is, the peak of profile) were found to be 314.6 and 315.2 °K, respectively. The enthalpy of this transition was found to be 9.2 kcal/mole. The transition width (HHW) at half-peak height of the heating cycle was 0.60 °K. More than 95% of the peak area was between 314.6 and 315.8 °K. These transition characteristics of the liposomes were the same for different preparations of liposomes carried through any of the mixing procedures (without additive) described above. Under the conditions these studies were carried out, we did not notice any significant pretransition peak as reported elsewhere (Ladbrooke & Chapman, 1969).

We are aware of the fact that the transition profiles of modified liposomes depend upon the history of the sample. For such reasons the protocol described in this paper was strictly adhered to. Except for such precautions, the reproducibility of various measurements described in this paper is better than ±10%. The transition profiles of the modified liposomes of the same composition prepared by either of the methods described above were identical. Interpretation of DSC curves has been discussed elsewhere (Ladbrooke & Chapman, 1969; Hinz & Sturtevant, 1972). For a discussion and interpretation of the phase transition data, we derived the following parameters from each of the profiles: half-height width (HHW), area, shift at the half height (HHW′), temperature at the beginning (T_c) and the end of the transition (T_e) for each peak. From this, we also derived the constants describing the concentrations of the additive at which half-height width is doubled (HHW_{100}), or the concentration at which the half-height width is shifted along the temperature axis by 100% (HHW'_{100}), or the concentration at which the area of the main peak is reduced by 50% (A_{50}), or the ratio HHW/HHW′ at HHW'_{100} (α), or the ratio HHW_{100}/HHW'_{100} (β). A full description of these arbitrary parameters is given in the following sections. The various lines connecting different points in a plot are to illustrate a trend in the data. These lines are not meant to imply a theoretical relationship.

Results

A large variety of solutes modify the gel-to-liquid crystalline transition profile of dipalmitoyl lecithin liposomes. In this section we will describe the characteristics and the concentration dependence of the phase transi-

tion profiles induced by various classes of additives. Although our initital choice of the various additives was somewhat random, some relationship between the structure of the additive and the type of profile it induces has been found. To illustrate our viewpoint the data is presented such that the structural features of the additives responsible for inducing a particular type of transition profile may be emphasized. At times the results presented in some subsections of this section may appear to be unrelated; however, an attempt has been made in the Discussion section to elaborate the underlying theme.

Effect of n-alkanols

The effect of alkanols on biological and model membranes is reflected in a variety of phenomena (Brink & Posternak, 1948; Seeman, 1972; Fourcans & Jain, 1975). These observations suggest that the *n*-alkanols interact with and perturb the organization of lipid bilayer (Paterson, Butler, Huang, Labelle, Smith & Schneider, 1972; Hill, 1974) and consequently modulate such diverse functions of membrane as permeability (Jain *et al.*, 1973) and their susceptibility to phospholipases (Jain & Cordes, 1973). The ability of alkanols to modify the lipid bilayer organization is probably best manifested in their effect on the phase transition characteristics. The effect on *n*-alkanols on the transition profile of liposomes is both concentration- and chain-length dependent. C_1 to C_4 alcohols induce profiles whose shape (height, area, and half-height width) is almost identical to that of the profile for unmodified bilayer; however, both the beginning (T_c) and the end of the transition (T_e) shift to lower temperature. We shall refer to such modified profiles as type C↓ profiles. Their characteristic features are described later. The C_5–C_{10} alcohols induce type A↓ profiles which are characteristically broad as compared to those of unmodified lipid bilayer transition profile (Fig. 1). If T_e and/or T_c are shifted towards lower temperature, such profiles will be referred to as type A↓; if T_e and/or T_c shift towards higher temperature, the profile will be called type A↑. This convention of referring to the direction of temperature shift in the profiles will be used for other types of profiles as well.

The type C↓ or A↓ profiles induced by the various alcohols show a dependence upon their concentration (Fig. 2). The enthalpy of both the type A or C transitions (area under the profiles) induced by the various alcohols is not appreciably influenced at the various concentra-

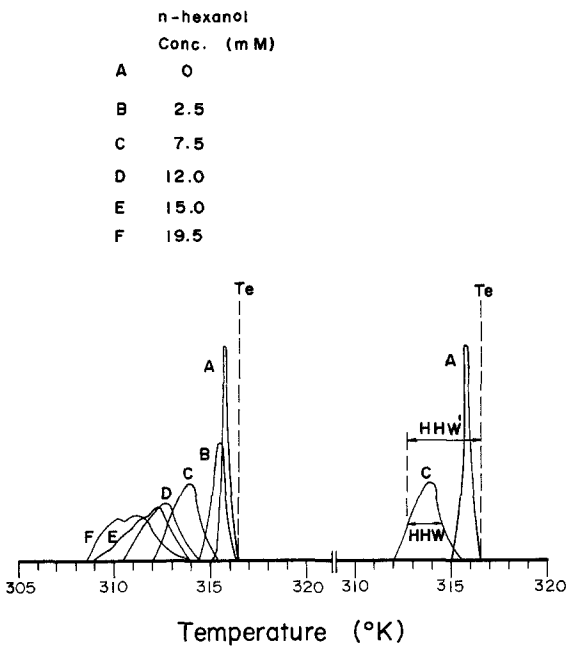

Fig. 1. A set of type A↓ phase transition profiles for dipalmitoyl lecithin liposomes modified with successively higher concentrations of *n*-hexanol. The profiles are broadened and both T_c and T_e shift towards lower temperature. Such profiles are referred to as type A↓. It may be noted that the broadening shows concentration dependence which can be measured either as half-height width (HHW) or as the gross shift at half-height width (HHW′). Values of HHW and HHW′ are measured in degrees Kelvin. The area of the various peaks is the same within error of the measurements of the area (±10%). The behavior illustrated by this set of profiles is qualitatively typical to those induced by C_5 through C_{10} alcohols

tions which we studied. HHW′ is more sensitive than HHW to alcohol concentration. HHW′ is a measure of the shift at half height of the transition peak. The procedure for the measurement of HHW and HHW′ is shown in Fig. 1. HHW′ will also be used to describe the concentration dependence of type C profiles for which HHW does not change significantly. Fig. 2 shows the concentration dependence of HHW′ for C_1 through C_{10} alcohols. Only the lower alcohols show a biphasic response; that is, at high concentrations one observes a shift in T_c and T_e towards higher temperature. Indeed, methanol above 4.5 M shows type C↑ profile; that is, both T_c and T_e in the presence of alcohol are higher than the corresponding values for liposomes in the absence of alcohol. Moreover, at these high alcohol concentrations one also notices a slight sharpening of the peak accompanied by an increase in the enthalpy of the transition.

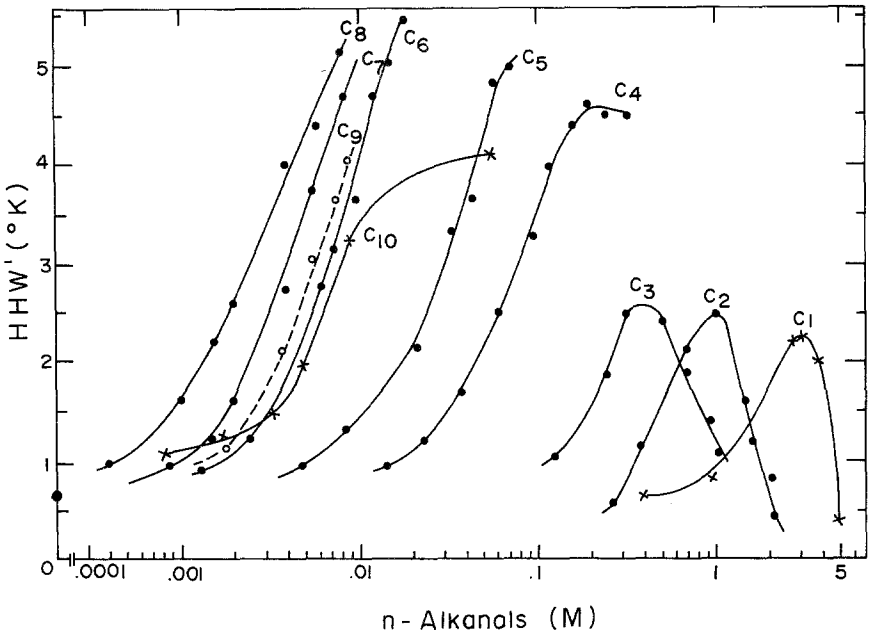

Fig. 2. Concentration dependence of HHW′ for methanol through *n*-decanol (C_1–C_{10} alkan-1-ols). Values of HHW′ were obtained from the transition profiles similar to those shown in Fig. 1. The concentration of dipalmitoyl lecithin in all the samples was 75 mM. The concentration (on the abcissa) is the alcohol concentration in the sample. It may be noted that the three smallest alcohols show a biphasic concentration dependence; for others the effect is monotonic. Plots like these are used to derive the equipotency concentrations, such as HHW'_{100} which is the concentration of a given alcohol at which it increases HHW′ by 100%. The samples were prepared by method *a*, that is, by mixing aqueous solutions. Higher alcohols (C_8 and above) were mixed by the successive dilution method *c*

The first eight alcohols show equal effect at successively lower alcohol concentrations as reflected in the values of HHW'_{100}; that is, the concentration at which the HHW′ for a given alcohol is increased by 100%. Other constants similar to HHW'_{100} can also be devised to compare the equipotency concentrations of the various alcohols. The values of HHW'_{100} (or some other similar equipotency constant) can be used to compare the potency of the various additives. C_9 and higher alcohols show a significant departure from the trend in the value of HHW'_{100} for *n*-octanol and the lower alcohols. The potency for nonanol and decanol is successively lower (i.e. higher HHW'_{100}) than that of *n*-octanol. Moreover, the C_{11} and higher alcohols do not seem to modify the transition profile significantly even at 15 mM concentrations.

For alcohols smaller than *n*-octanols one observes an inverse relationship between HHW'_{100} and the chain length. This is most likely related

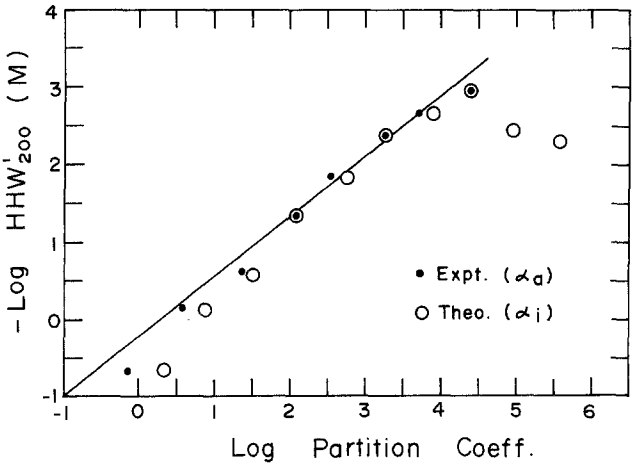

Fig. 3. Plot of log HHW'_{200} against log "experimental" (α_a, filled circles) and "theoretical" (α_i, open circles) partition coefficient values for C_1 through C_{10} alkanols. The slope of the line is about 0.75. The theoretical values are based on solubility data, and the "experimental" values are calculated from a lowering of the transition temperature as determined by the light-scattering changes (Hill, 1975). HHW'_{200} values were obtained from the data presented in Fig. 2

to the bilayer/buffer partition coefficient of the alcohols. Unfortunately, the experimental values of such partition coefficients are not available. However, Hill (1975) has reported the values of the partition coefficients ("experimental") of *n*-alkanols calculated from the shift in the transition temperature of dipalmitoyl lecithin induced by the alcohols. He has also reported the "theoretical" values of the partition coefficient, that are based on the solubility of alcohols in water. In Fig. 3 we have plotted HHW'_{200} values for C_1–C_{10} alcohols against these "experimental" (α_a) and "theoretical" (α_i) values of partition coefficient. Here one notices a good correlation between the "experimental" (α_a) values of partition coefficient and the reciprocal of HHW'_{200}. The relationship with the theoretical partition coefficient values (α_i) is not as good. In this plot one also observes that the even chain length (C_{even}) alcohols consistently show a better correlation than the odd chain length (C_{odd}) alcohols. This difference in the behavior of C_{odd} and C_{even} alcohols is probably best reflected in the relationship shown in Fig. 4. The free energy change per methylene residue, $\Delta F(\text{-}CH_2\text{-})$, for the even methylene residues is consistently higher than the values for the odd methylene residues. The ratio of ΔF for odd/even methylene residues is 0.80 ± 0.1. Moreover, the values of $-\Delta F$ for the first eight methylene groups is only slightly

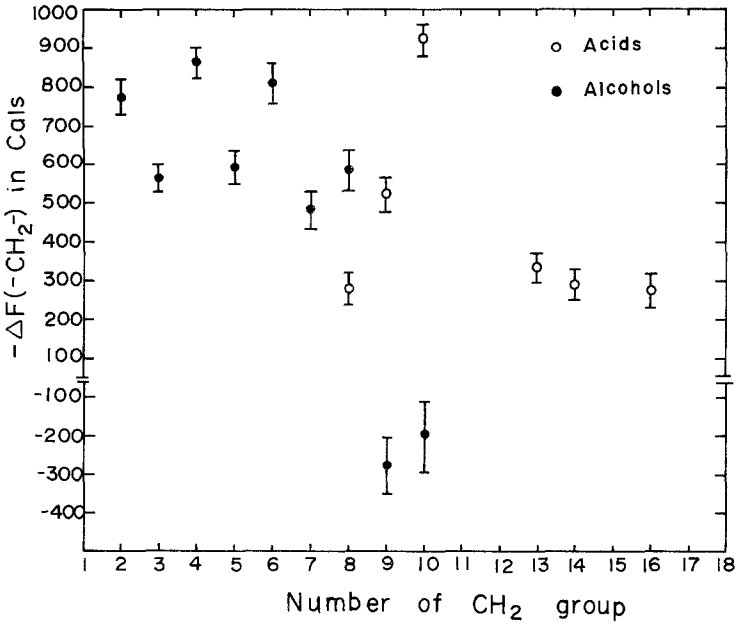

Fig. 4. Plot of incremental free energy per methylene group, ΔF(-CH_2-), against the chain length (N). The ΔF(-CH_2-) values were calculated as RT log A; where A is the ratio of HHW'_{200} for alkanols with chain length N and (N-1). The bar corresponds to uncertainty in the values of HHW'_{200}. The average value of ΔF(-CH_2-) for C_1–C_8 alcohols is calculated as −730 cal/mole. The data for alcohols (filled circles) was obtained from Fig. 2, and for acids (open circles) from Fig. 8

smaller than the expected value. However, a sudden change is observed for the ninth and higher residues.

The observations on the effect of alcohols on the phase transition behavior of DPC liposomes is consistent with their incorporation into bilayer. Their ability to do so is dependent upon their chain length. The potency of C_1–C_8 alcohols would be qualitatively consistent with their lipid/water partition coefficients. The behavior of C_9 and higher alcohols is, however, not consistent with their partition coefficients. To further examine the applicability of partition coefficient consideration, we studied the effect of four isomeric *n*-octanols (which should have almost the same lipid/water partition coefficient) on the phase transition of liposomes. The octanols induced type A profiles, but as shown in Fig. 5, the concentration dependence of HHW′ for these alcohols is quite different. The values of HHW'_{100} for these alcohols is:

$$n\text{-octan-1-ol} < n\text{-octan-2-ol} < n\text{-octan-3-ol} \leqq n\text{-octan-4-ol}.$$

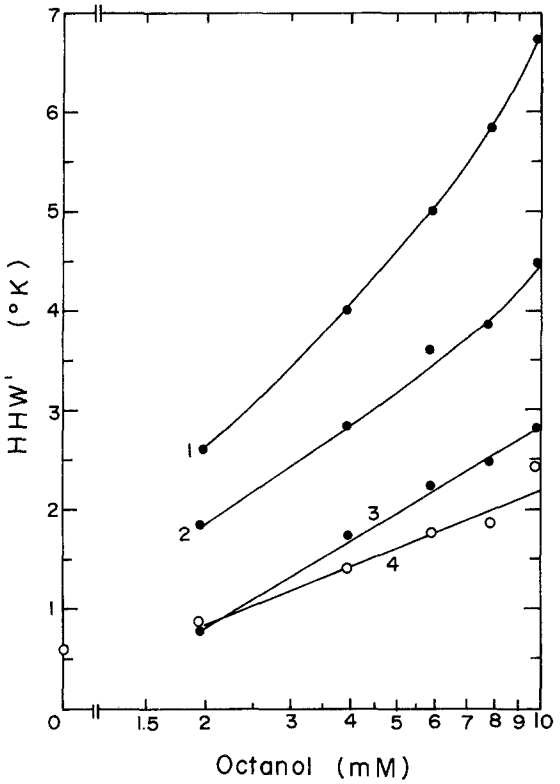

Fig. 5. Plots of HHW′ against the concentration of four isomeric *n*-octanols. The plots are for octan-1-ol (1), octane-2-ol (2), octane-3-ol (3), octane-4-ol (4). All these alcohols induced type A↓ profile. The samples were prepared by method *c*

One would not expect such a trend if only equal concentration of solute in the bilayer were to induce equal effect.

Effect of Some Small Organic Solutes

The behavior of lower alcohols prompted us to investigate the effect of some small solutes on the phase transition profiles. We investigated the effects of seven solvents. They all induced type C↓ profiles (Fig. 6). However, some of them induced type A↓ profiles at higher concentrations. The plots of HHW′ against their concentration is shown in Fig. 7, and the shift from type C↓ to A↓ profile is indicated by an arrow. There is no structure activity correlation apparent from this data. However, one may note the following features: all these additives are relatively

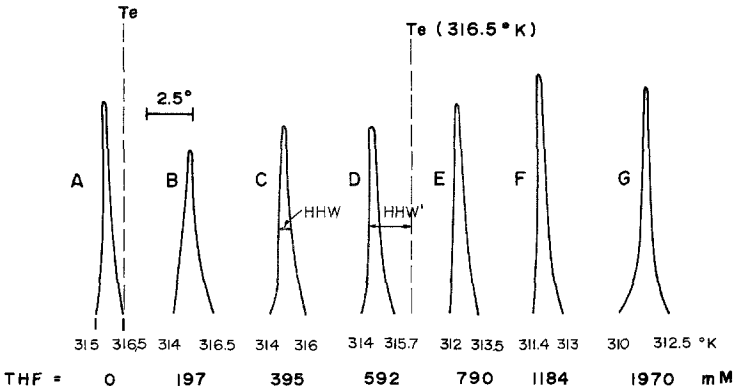

Fig. 6. A set of type C phase transition profiles for dipalmitoyl lecithin liposomes modified with successively higher concentrations of tetrahydrofuran. Such profiles do not show any change in the shape, but T_c and T_e is shifted towards lower temperature (type C↓). It may be noted that the shift in T_c and T_e or at half height (HHW′) shows a monotonic concentration dependence. The area of the peaks at various additive concentrations remains unchanged. Type C profiles are induced by small organic solutes of low polarity such as ether, chloroform, benzene (*see* Fig. 7). The horizontal bar corresponds to 2.5 °K

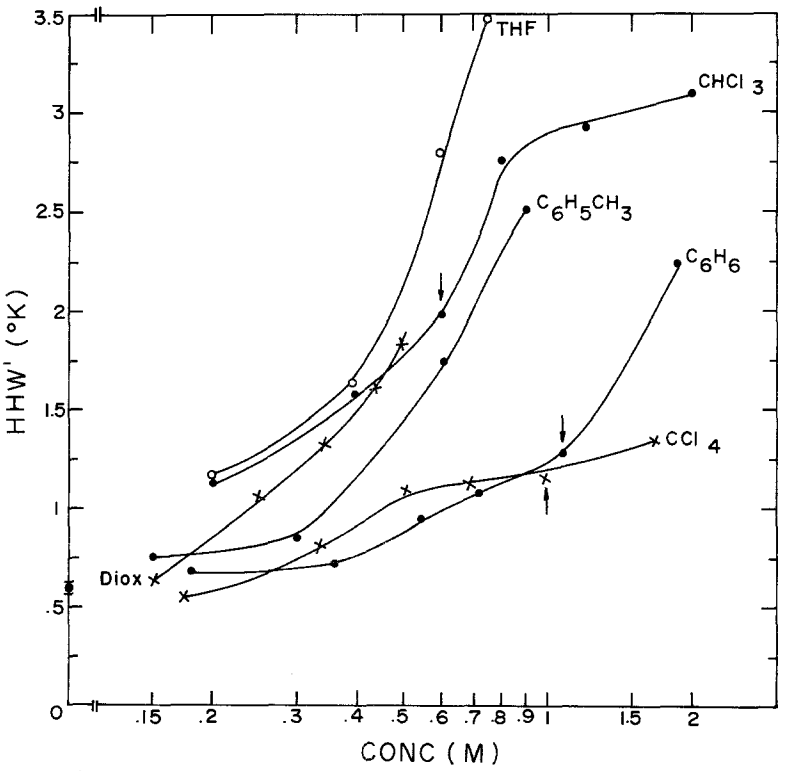

Fig. 7. Plot of HHW′ against their concentration for carbon tetrachloride, benzene, toluene, chloroform, dioxane, and tetrahydrofuran. They induced type C↓ profiles. At concentrations above that of the position of the arrow some solutes induce type A↓ profile. Samples were prepared by method *a* or *c*

small; the oxygenated molecules (tetrahydrofuran, dioxane) are relatively more effective than the other solutes; the least effective molecules (benzene and carbon tetrachloride) are also the most symmetrical and least polar of the small solutes examined. Further studies on the small solute-induced changes is in progress. However, for the present, the qualitative trend that the small solutes, at least at low concentrations, induce a type C↓ profile appears to be a general feature.

Effect of Fatty Acids

Free fatty acids have been implicated in a variety of membrane processes, including antimicrobial action (Freese, Sheu & Galliers, 1973), growth inhibition and morphologic changes in mammalian cells (Ginsburg, Salomon, Sreevalson & Freese, 1973), growth promotion in plants (Stowe & Dotts, 1971), and as inducer of membrane fluidity (Kosower, Kosower, Faltin, Diver, Saltoun & Frensdorf, 1974; Kosower, Kosower & Wegman, 1975) and membrane fusion (Ahkong, Fisher, Tampion & Lucy, 1973; Kantor & Prestegard, 1975; Maggio & Lucy, 1975). The various saturated fatty acids we examined induced the following response:

C_5 and C_6	alkanoic acids	- none
C_7–C_{10}	alkanoic acids	- type A↓
C_{12},C_{13},C_{14},C_{16}	alkanoic acids	- type A↑
C_{18},C_{20},C_{24}	alkanoic acids	- none

The concentration dependence of HHW′ of the profiles induced by the various alkanoic acids is shown in Fig. 8. Decanoic acid is most effective in lowering (type A↓) the transition temperature. The higher (C_{12} and up) acids broaden the profile towards higher temperature (type A↑). The inversion is represented in Fig. 8 by plotting HHW′ values in the opposite direction. For C_{12} and higher acids again the potency increases with chain length until palmitic acid (C_{16}). Here one notices some indication of specific interaction between the acid and phospholipid in the form of type B or D profiles (*see below*). In fact, there are several other indications of such specific interactions or molecular associations in such systems (Goddard & Kung, 1966). The data in Fig. 8 can also be used to calculate ΔF(-CH_2-) values presented in Fig. 4 for fatty acids.

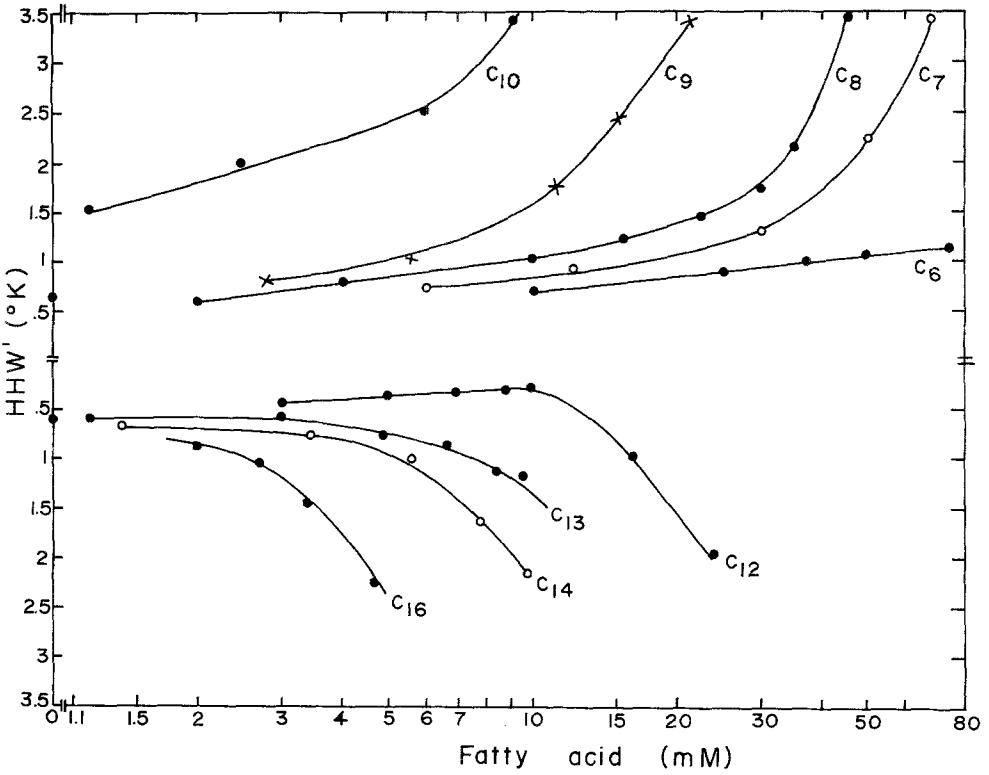

Fig. 8. Plot of HHW′ against their concentration for the various *n*-fatty acids. Fatty acids smaller than decanoic acid (C_{10}) showed type A↓ profile whereas the C_{12} and higher acids show type A↑ profile. This difference is presented as two sides, HHW′ values on the ordinate. Samples were prepared by method *b*. The labels represent the number of carbon atoms in the fatty acids

Here one observes inversion (change in sign) in the value of $\Delta F(\text{-}CH_2\text{-})$, between C_{10} and C_{12}.

We also examined the effect of the various unsaturated fatty acids: undecylenic (Δ^{10}), palmitoleic, oleic, linoleic, linolenic, arachidonic, ricinoleic, and ricinalaidic. They all showed type A↓ profile (data not presented here). In general, the more highly unsaturated and the less polar acids were more potent when one compares their equipotency concentration. However, they all show equal potency in a fairly narrow concentration range. For example, for all of the acids named above, the HHW'_{100} values are between 1.3 mM (for arachidonic acid) and 7.5 mM (for ricinalaidic acid).

Effect of the Uncouplers

So far we have described the effect of long amphipathic molecules. The behavior of other compounds with quite different molecular geometry was also examined. Some proton translocators, known as the uncouplers of oxidative phosphorylation, are of interest because of their aromatic skeleton, hydrophobicity, and their mode of action which implies that they interact with the lipid bilayer. All the uncouplers we examined (Table 1) showed type B↓ profile. A typical set of type B↓ profiles is shown in Fig. 9. These profiles are characterized by a shoulder towards lower temperature on the main peak. With increasing additive concentration only the area and height of the main peak decreases, the half-height width of the parent peak does not change significantly. Both the HHW and the area of the new peak or the shoulder increase with increasing additive concentration. The temperature at the beginning of the overall transition changes drastically at low additive concentrations, but it changes only slightly at higher additive concentrations. Generally speaking, the type B profiles represent a range of possibilities. On one extreme the new peak may be of the same shape as the parent peak (we will refer to such profiles as type D). On the other extreme the new peak may not show any noticeable phase transition (such profiles will be referred to as type E). The examples of these extreme cases are discussed in later subsections. However, all the cases where the new peak is discernible from the parent peak but has HHW greater than that of the parent peak, will be referred to as a type B↓ or B↑ depending upon the position

Table 1. A_{50} and HHW_{100} values for the uncouplers

Uncoupler	Type of profile	A_{50} (mM)	HHW'_{100}= HHW_{100} (mM)
2,4-Dinitrophenol	B↓	2.1	4.27
Picric acid	B↓	1.5	3.2
Carbonylcyanide phenylhydrazone (CCP)[a]	B↓	6.0	6.0
m-Cl CCP[a]	B↓	6.3	5.1
p-trifluoromethoxy-CCP (FCCP)[a]	B↓	3.7	4.4
Tetrachlorotrifluoromethylbenzimidazole (TTMB)[a]	B↓	3.6	3.4
Uncoupler 1799[a]	B↓	4	4
Phloretin	B↓	0.4	0.37

[a] These uncouplers were obtained from Dupont Chemical Co, through the courtesy of Dr. P. Heytler

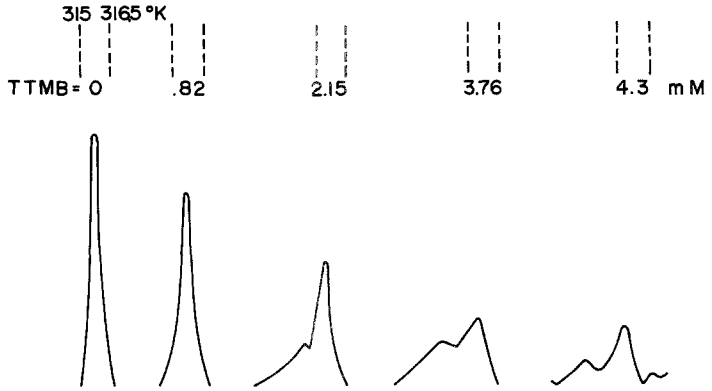

Fig. 9. A set of type B↓ phase transition profiles of dipalmitoyl lecithin liposomes modified with successively higher concentrations of TTMB. Such profiles show a shoulder, the area of which increases with increasing additive concentration. It appears that the parent peak decreases in area, whereas the area of the whole profiles remains the same. All the uncouplers (*cf.* Table 1) induced type B↓ profiles. The dotted lines show the values of T_c (=315.0 °K) and T_e (=316.5 °K)

of the new peak relative to the parent peak along the temperature axis.

In all type B profiles, the area of the main peak decreases as the concentration of the additive increases. However, the total area under the profile (enthalpy) remains unchanged at least at low uncoupler concentrations. Concentrations at which the area of the main peak decreases by 50% (A_{50}) for the uncoupler are given in Table 1. For most of these compounds there is a slight change in the half-height width of the parent peak. It may be noted that because of the shape of type B profiles, the area of the main transition peak can be determined only by assuming that its width at the base is not changed appreciably. This introduces a large uncertainty in the values of A_{50}. Within these limitations it is obvious that the uncouplers interact with lipid bilayer and they influence the phase transition characteristics in a qualitatively similar fashion. If there is any correlation between proton translocation ability (Cunarro & Weiner, 1975) and the ability to perturb the bilayer, that is not obvious from the data shown in Table 1.

Effect of Inorganic Ions

Biological membranes are affected by the ionic character of the surrounding media. This alteration has been attributed to direct ion-mem-

brane interaction (Shah & Schulman, 1965, 1967; Reynolds, 1972) and also to an indirect effect arising from an ion-induced structural change of the water surrounding the membrane (Hatefi & Hanstein, 1969). Although all ions may show both of these types of interactions to a certain extent, some ions seem to show more of one type of effect. Therefore, this subsection is divided into two parts that emphasize these two types of interactions.

Effect of Multivalent Cations

The effect of cations with lipids is thought to be electrostatic in nature, with ions interacting coulombically with the negative charge on the lipid head group structure. The most distinctive effect is exhibited by di- and trivalent cations. We studied the effect of five cations (Mg^{2+}, Ca^{2+}, Pr^{3+}, Eu^{3+}, Gd^{3+}) and they all induce type D profiles. A set of typical type D↑ profiles is shown in Fig. 10. In these profiles one observes appearance of a new peak and the disappearance of the parent transition peak at successively higher cation concentrations. For all these

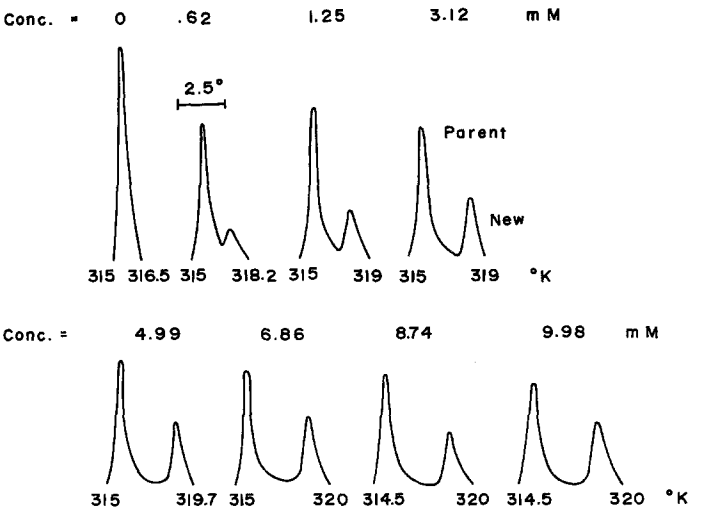

Fig. 10. A set of type D↑ phase transition profiles of dipalmitoyl lecithin liposomes modified with successively higher concentrations of gadolinium chloride ($GdCl_3$). Such profiles show a new transition peak with increasing additive concentrations. Several multivalent ions showed type D↑ profiles (data in Table 2). Samples were prepared by method *b*. The horizontal bar corresponds to 2.5 °K

Table 2. HHW_{100} and A_{50} for type D profiles induced by multivalent cations

Additive	Type of profile	HHW_{100}= HHW'_{100} (mM)	A_{50} (new)	(Parent) A_{50} (mM)	Characteristics of the new peak		
					T_c	T_e	HHW(°K)
$MgCl_2$	D↑→B↑	214	~100	~100	415.50°	417.2°	0.75 at 160.0 mM
$CaCl_2$	D↑	4.76	18	14	416.6	417.7	0.44 at 95 mM
$EuCl_3$	D↑	0.53	6	7	417.0	418.5	0.60 at 15 mM
$PrCl_3$	D↑	0.48	4.5	9	418.3	420.2	0.64 at 15 mM
$GdCl_3$	D↑	0.26	3	5.5	417.5	419.5	0.68 at 10 mM

cations the new peak always appears at a temperature higher than that of the parent peak. At low cation concentrations the new peak also shifts successively towards higher temperature. However, at higher cation concentrations one observes very little shift along the temperature axis. As the concentration of the cations increases the area of the parent peak decreases and that of the new peak increases. At very high solute concentrations the parent peak disappears completely. The new peak showing transition at higher temperature generally appears to be quite similar to the original peak. The area of the new peak or the area of the parent peak, when plotted as a function of the additive concentration, shows a saturation behavior. From these plots one can, at least in principle, calculate the apparent affinity constants or the concentration at which half of the maximally induced effect is observed. These values are given in Table 2. These apparent association constants are in the order:

$$Gd^{3+} \leqq Pr^{3+} \leqq Eu^{3+} < Ca^{2+} < Mg^{2+}.$$

This sequence strongly suggests that multivalent ions interact electrostatically, but specifically and stoichiometrically, with ionic sites on the lecithin bilayer, perhaps in the manner discussed by Sundaralingam and Jensen (1965). The monovalent cations (data presented in the next section) do not seem to do so appreciably. Such interactions of multivalent cations are expected to have significant effects on the packing of the lipid molecules in the bilayer (Trauble & Eibl, 1974) presumably due to some rearrangement in the zwitterionic head group region. Our results show that the complexed lipids apparently form a more condensed bilayer, albeit it exists as a separate phase with higher transition temperature.

Moreover, the data also suggest that the new modified phase has the same enthalpy of transition and the same size of the cooperative unit undergoing transition as the unmodified bilayer phase (*see* Discussion). It may be noted that the effects of multivalent ions on zwitterionic lipid bilayer, which we have reported here, are somewhat surprising. Calcium-induced phase separation has been reported in zwitterionic+ acidic phospholipid bilayers (Ohnishi & Ito, 1974; Galla & Sackman, 1975; Jacobson & Papahadjopoulos, 1975). Such effects have not been reported in the literature, even though related cation-water-lipid systems have been studied by DSC (Trauble & Eibl, 1974; Lis, Kauffman & Shriver, 1975; Simon *et al.*, 1975). Assuming that these authors did not observe such effects, it would imply some difference in the experimental protocol. This is being investigated.

Effect of Chaotropic Anions

Certain anions, known as chaotrops, perturb water structure (Hatefi & Hanstein, 1969). Among other manifestations this could destabilize biomembrane. Indeed, these ions have been used for solubilizing certain membrane-bound proteins. Chaotropic ions are thought to affect membrane structure by their influence on hydrogen bonding between the membrane and surrounding water structure. According to this model, these anions decrease the polarity of the surrounding water because the hydrogen atoms in the anion hydration shell are directed inwards, resulting in a water anion aggregate less polar than the OH..O bonds of water itself (Hatefi & Hanstein, 1969). Another possibility is highly specific ion pairing between head groups and anions.

All of the chaotropic anions except ClO_4^- at low concentrations induced type B↑ profiles; that is, appearance of a shoulder towards higher temperature side of the transition peak. It was particularly hard to determine the area of the parent peak. Therefore, as a first approximation, we measured HHW(=HHW′ in this case) as a function of the concentration of the various chaotrops and related salts. This is somewhat justified in view of the similarity of trends in the values of A_{50} and HHW'_{100} shown in Table 1. The data presented in Fig. 11 suggest several interesting trends. The additives like urea, $KClO_3$, NaCl, KBr, and KNO_3 perturb the transition profiles only slightly both in terms of equipotency concentrations (related to the affinity) and in terms of maximum effect (related to the efficacy) induced by these salts. In conjunction with the

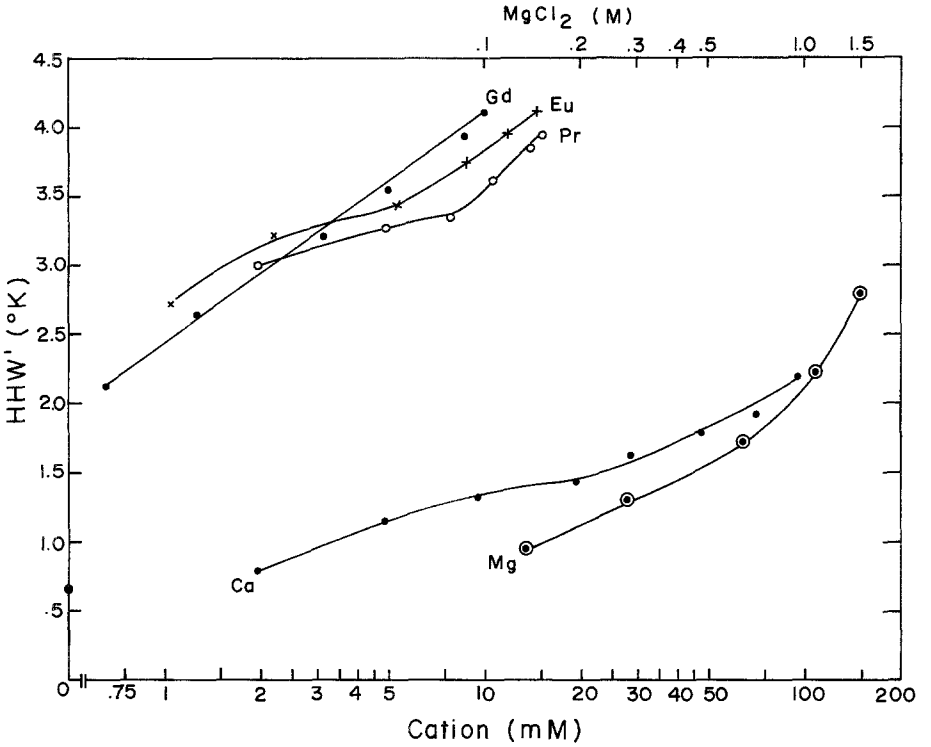

Fig. 11. Plot of HHW' (=HHW in this case) against the concentrations of several multivalent ions. Note that the axis for $MgCl_2$ is different (top) than that for the other ions (bottom). All the cations (Mg, Ca, Pr, Eu, Gd) induced type D↑ profiles. Samples were prepared by method *a*

data on multivalent cations these results suggest that the monovalent ions like Na^+, K^+, NO_3^-, Cl^- and Br^- do not affect the transition profiles significantly. Small changes induced by these ions, as shown in Fig. 12, confirm the effects reported in the literature (Trauble & Eibl, 1974; Simon *et al.*, 1975). However, the possibility that these effects are due to some trace impurity in the salts cannot be ruled out altogether.

The sequence in which the chaotrops induce broadening is:

$$CNS^- > I^- > \text{guanidine} > ClO_4^- > \text{urea}$$

on the basis of the maximum effect these ions can induce (related to their efficacy). However, if one considers equipotency concentration, the sequence of effectiveness (related to their affinity) is:

$$ClO_4^- > CNS^- > I^- > \text{guanidine} > \text{urea}.$$

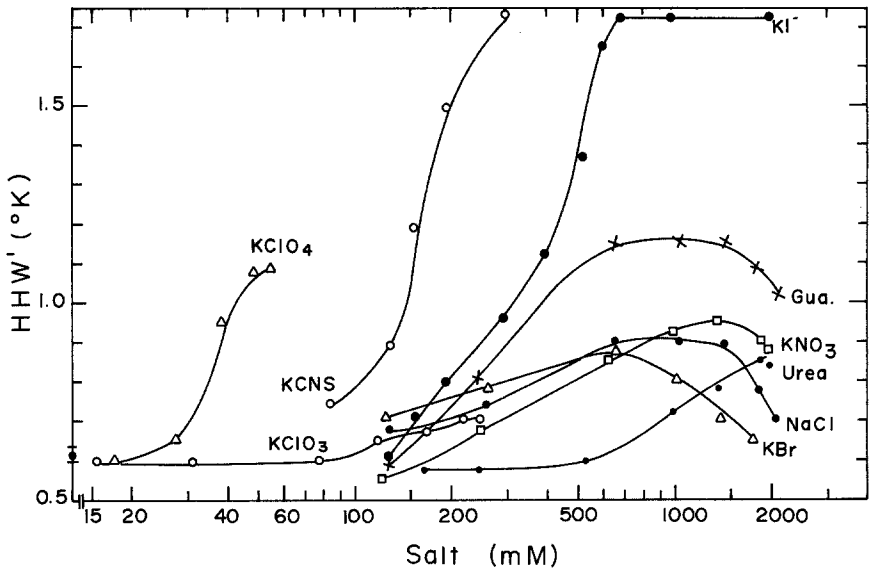

Fig. 12. Plot of HHW (=HHW′ in this case) against concentration of several chaotropic agents: potassium perchlorate ($KClO_4$), potassium thiocyanate (KCNS), potassium iodide (KI), guanidine (Gun), potassium nitrate (KNO_3), urea, sodium chloride (NaCl), potassium bromide (KBr), potassium chlorate ($KClO_3$). All these agents induced type B profiles except perchlorate which induced type D profiles. Samples were prepared by method *a*

Both of these sequences differ only with respect to the position of perchlorate whose behavior is even qualitatively different than the behavior of other chaotrops: perchlorate induces types D↑ profile, whereas all others induce type B↑. The latter sequence, related to the affinity of the cations, seems to be similar to that reported by Hatefi and Hanstein (1969). Further studies need be conducted to resolve the various geometric and electronic factors that may be involved in the interaction of ions with the membrane and the surrounding water. Our data suggest that both of these types of effects are involved in the mode of action of chaotrops.

Effect of Surfactants

Surfactants are widely used in the study of membranes, both to solubilize specific membrane components and to probe the structure of the membrane itself (Helenius & Simons, 1975). Some of the commonly used surfactants were examined for their effect on the phase transition

characteristics of bilayer. They all modify the transition profile, and qualitatively their effects show a pattern. Sodium dodecyl sulfate induces type E profile such that the area of the main peak decreases but no new peak appears in the profile. In contrast, some related ionic detergents such as dodecyl pyridinium chloride and dodecyl phosphate, show type B↓ profiles with a distinct shoulder. The weakly ionic detergents (such as dodecylamine, octadecylamine, octadecylammonium, cholic and deoxycholic acids and digitonin), however, show type D↓ profiles at low concentrations and some of these degenerate to type B↓ at high additive concentrations. All the nonionic detergents we examined induce type A↓ profiles. Plots of the area of the original peak (for type B, D and E profiles) or of the HHW′ (for type A profiles) against the surfactant concentration are shown in Figs. 13 and 14. The concentration dependence of the effects is monotonic in all cases, and the effects observed are fairly large even at low detergent concentrations. Type E profile can be considered a special case of type B profile such that the modified

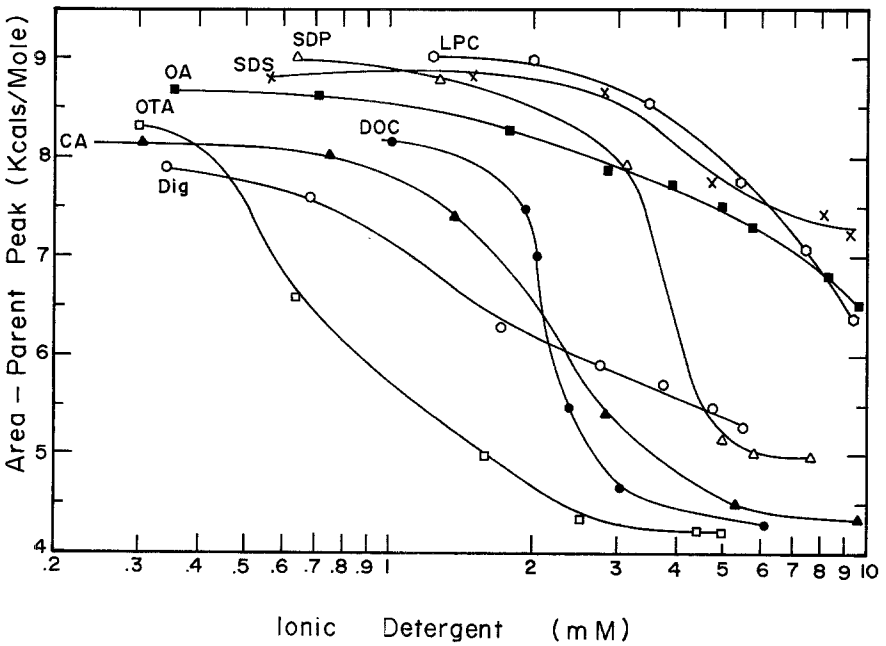

Fig. 13. Plots of the area of the parent peak against the concentration of ionic amphipaths: dodecyl pyridinium chloride (LPC), sodium dodecyl phosphate (SDP), sodium dodecyl sulfate (SDS), octadecyl amine (OA), octadecyltrimethylammonium chloride (OTA), deoxycholic acid (DOC), cholic acid (CA), digitonin (Dig). Type of profiles induced by these amphipaths is given in Table 4. Samples were prepared by method *a*

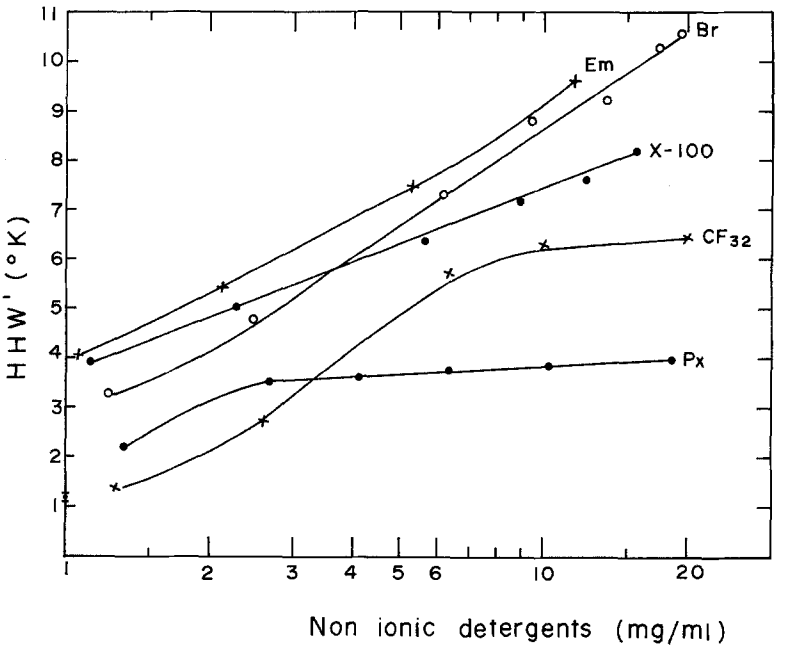

Fig. 14. Plots of HHW′ (=HHW in all these cases) against the concentration of nonionic amphipaths: Em, emulphogene BC-720; Br, Brig 36f; X-100, Triton X-100; CF_{32}, Triton CF-32; PX, Lubrol PX. All the nonionic detergents induced type A profiles. The broadening accompanied a lowering of T_c, with little or no shift in T_e. Samples were prepared by method *a*

phase is liquid-crystalline in the temperature range of the scan (above 295 °K). This sort of loose packing in the modified phase would result in the presence of a strongly polar group like sulfate in sodium dodecyl sulfate. N-dodecyl pyridinium chloride induces type B↓ profile. However, the new peak is very broad. Other less polar ionic surfactants with smaller head groups induce type B profiles with not so broad new peaks. The very weakly polar surfactants induce type D profiles at low concentrations. These observations suggest that the surfactants modify the packing characteristics of lipid bilayer and the type of effects on the transition profile are dependent upon the structure of the surfactant.

Effect of Ionophores

The effect of several ion-carriers on transition characteristics is of obvious interest. Most of the ionophores we examined showed little effect. Of the three related alkali metal ion ionophores, only nigericin showed

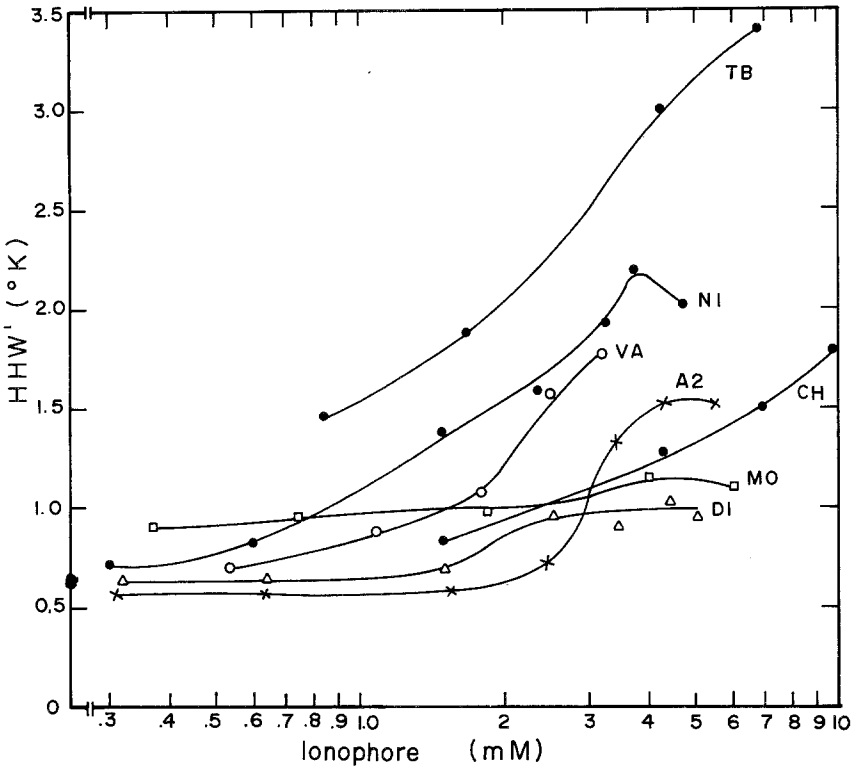

Fig. 15. Concentration dependence of the effect of some ionophores on the phase transition profile of dipalmitoyl lecithin. Nigericin (NI), dianemycin (DI), monensin (MO), and Ca^{2+}-ionophore A23187 (A2) induced type B↓ profiles, whereas the ionophores valinomycin (VA), di-*t*-butyl (TB) and di-cyclohexyl-18-crown-6 (CH) induce type A↓ profile. Samples were prepared by method *a*. NI, DI, MO and A_2 were obtained from Eli Lilly Co. through the courtesy of Dr. Hamill

any significant effect. In contrast, dianemycin and monensin showed no effect. Similarly, the Ca^{2+} ionophore A23187 induced type B↓ profile. Valinomycin, dicyclohexyl and di-*t*-butyl-18-crown-6 induced type A↓ profile. The concentration dependence of their effects is shown in Fig. 15. The channel-forming agents gramicidin, amphotericin and nystatin did not show any significant effect up to 7 mM concentration. It may be noted that most of the effects have been observed only at relatively high concentrations compared to the concentration at which they induce ion fluxes across lipid bilayer (*cf.* Jain, 1972). However, it may be noted that the ionophore to the lipid ratio at which these compounds induce the effects is less than 1 : 10. This small ratio may impart our observations with some mechanistic significance, for example, in predicting their position of localization in the lipid bilayer (*see* Discussion).

Effect of Spin-labelled Membrane Probes

The effect of some commonly used spin-labelled membrane probes (Keith, Sharnoff & Cohn, 1973) was examined. All the compounds examined induced type A↓ profiles except TEMPO which did not show any effect up to 15 mM. TEMPONE- and TEMPOL-induced broadening (Fig. 16) was small and only slightly concentration-dependent. The stearic acid derivatives (4-NS and 12-NS) showed quite a large quantitative difference in the profiles they induced. 4-NS showed only a slight change in the profile whereas 12-NS showed a large broadening, albeit only at higher concentration. Similar latency at low concentration is also observed with PCA. Such a sudden appearance of broadening at higher additive concentration would imply that at low concentrations the additive may be localized in a nongel region. A difference in the behavior of 4-NS and 12-NS would imply that 4-NS does not get into the gel

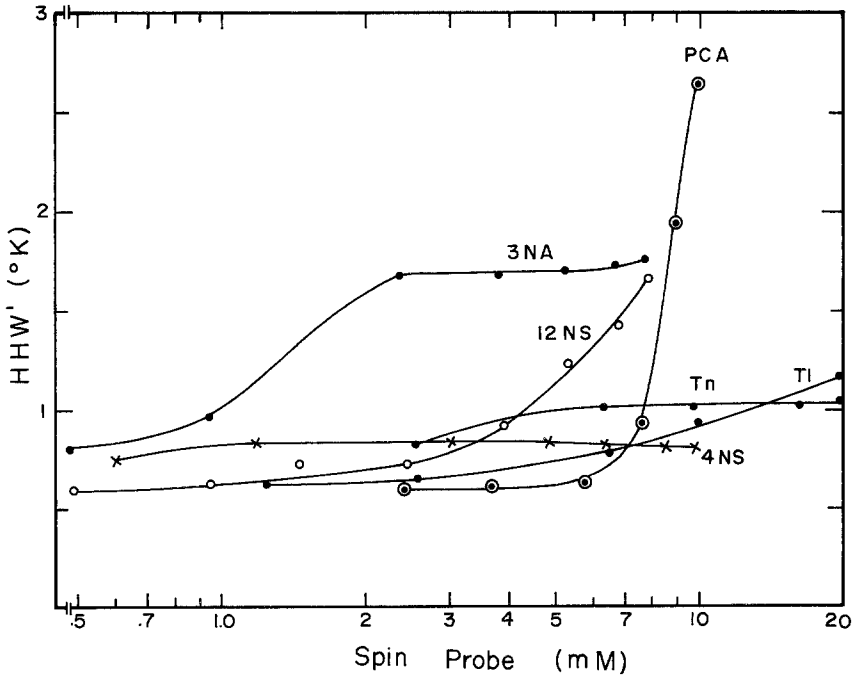

Fig. 16. Concentration dependence of the effect of some spin-labelled probes on the phase transition profile of dipalmitoyl lecithin. All the probes that had an effect induced type A profile. Samples were prepared by method *a*. All these spin-labelled probes were obtained through the courtesey of Dr. Alex Keith (Pennsylvania State University). N-oxyl-4′,4′-dimethyloxazolidine derivative of 4 and 12 ketostearic acid (4 NS and 12 NS), 3-N-oxyl-4′,4′-dimethyloxazolidine androstan (3-NA); Tempol (Tl), Tempone (Tn), cyclo pentyl carboxylic acid (PCA)

phase or if it does then it does not modify the packing characteristics of the bilayer. Indeed, it has been suggested that 4-NS is at least partially squeezed out of the bilayer (Tinoco, Ghosh & Keith, 1972; Cadenhead & Muller-Landau, 1973). Our data suggests that not only the various spin-labelled probes perturb the gel phase but also the concentration dependence of their effects is consistent with the view that these probes are selectively localized in nongel regions.

Effect of Fluorescent Probes

It has been argued that the fluorescent probes used for the study of membranes could perturb the bilayer organization (Krishnan & Balaram, 1975). The concentration dependence of the effects is shown in Fig. 17. Of the various probes examined only 8-aniline-naphthalene-1-sulfonic acid (ANS) and N-phenyl-naphthylamine (NPA) modified the transition profiles. They induced type B↓ and type A↓ profiles, respec-

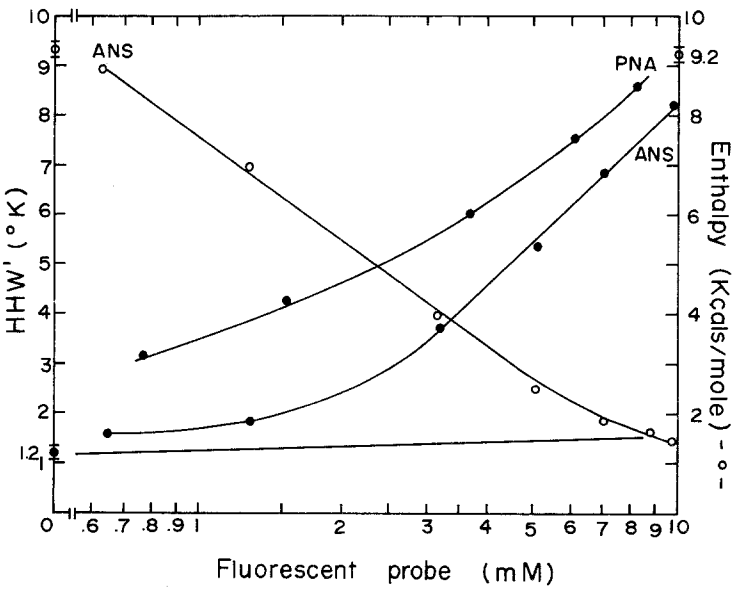

Fig. 17. Concentration dependence of the effect of some fluorescent probes on the phase transition profile of dipalmitoyl lecitin. ANS (8-anilino-1-naphthalene sulfonic acid) induced type B↓ profile, whereas PNA (N-phenyl-1-naphthylamine) induced type A profile. All the other probes did not induce any change (shown by the line without points) up to the concentration indicated: diphenylhexatriene (up to 9 mM), 9-vinyl-anthracene (up to 16 mM), 9,10-dimethylanthracene (up to 14 mM), 9,10-diphenylanthracene (up to 13 mM), perylene (up to 10 mM). All the samples were prepared by method *b*

tively. All the other probes, which are aromatic hydrocarbons, did not induce any change. This suggests that the hydrocarbon probes do not modify the gel phase. Qualitatively distinct effects of ANS and NPA indicate a difference in their environment, presumably due to a difference in their position of localization.

Discussion

Change in Gibb's free energy of a liquid containing a foreign molecule is related to the additive concentration. In the case of pure phospholipid bilayer as "solvent", this change in free energy can be measured, at least in principle, by following the gel-to-liquid crystalline transition. Following this line of argument, the additive-induced shift in transition temperature can be interpreted either as a case of depression in freezing point (Hill, 1974, 1975) or in terms of partition coefficient relationships (Leo, Hansch & Elkins, 1971; Hansch & Dunn, 1972). Both of these treatments rely on two basic assumptions: (a) equal molar amounts of different solutes induce equal effect, and (b) lipid bilayer and/or biomembrane can be approximated as a bulk solvent. Such a treatment cannot *a priori* explain qualitatively different behavior of the various additives, that is, the different types of transition profiles as observed in this study. Moreover, even a quantitative interpretation of our data in terms of these assumptions leads only to a limited success. For example, the value of free energy change per methylene residue, $\Delta F(\text{-CH}_2\text{-})$, for both *n*-alkanols and *n*-alkanoic acids (Table 3) show some dependence upon the chain length. Consistently, the values of $-\Delta F(\text{-CH}_2\text{-})$ are smaller for odd chain lengths than for the even chain lengths (Fig. 4). The ratio of C_{odd}/C_{even} observed in this study is 0.81 (for free energies) compared to 0.77 (for partition coefficient) observed by Hill (1975). This is also reflected in a good correlation (Fig. 3) of HHW'_{100} and "experimental" partition coefficient values (α_a) reported by Hill (1975). The values of $\Delta F(\text{-CH}_2\text{-})$ for lower alcohols is slightly higher than the values for the higher members in the series. The average value of $\Delta F(\text{-CH}_2\text{-})$ between C_1 and C_8 alcohols is -660 cal, which is similar to that expected if the bilayer had hydrocarbonlike environment for the localization of these alcohols (Hersh, 1971).

Other departures from the above assumptions are observed when one considers the effect of C_9 and higher alcohols and the C_{11} and higher acids on the phase transition characteristics. Both groups of addi-

Table 3. Values of free energy change per methylene residue, $\Delta F(\text{-CH}_2\text{-})$, for various systems

System	$\Delta F(\text{-CH}_2\text{-})$ cal/mole (°K)	Reference
n-alkanol/liposome		
C_1–C_8 average	−660 (314 °K)	This study
Even methylenes	−810 (314 °K)	This study
Odd methylenes	−560 (314 °K)	This study
n-fatty acids (C_7–C_{10})/ liposome	−625 (314 °K)	This study
n-fatty acids (C_{12}–C_{18})/ liposome	−300 (314 °K)	This study
oil/water phases		
n-alkanes	−850 (298 °K)	Molyneux, Rhodes & Swarbrick, 1965
n-alkanols	−810 (298 °K)	Butler, Ramchandrani & Thomson, 1935; Kinoshita, Ishikawa & Shinoda, 1958
Micelle/water		
n-alkyl amphipaths	−650 (298 °K)	Molyneux *et al.*, 1965
ΔCMC by *n*-alkanols	−680 (298 °K)	Herzfeld, Corrin & Harkin, 1950
n-alkanols		
petroleum ether/water	−820	Haydon & Taylor, 1960
air/water	−750	Posner, Anderson & Alexander, 1952
n-fatty acids (C_7–C_{15})		
n-heptane/water	−820	Tanford, 1972; Smith & Tanford, 1973

tives give positive values of $\Delta F(\text{-CH}_2\text{-})$. The average value of $\Delta F(\text{-CH}_2\text{-})$ for C_7 to C_{10} acids compares favorably to that expected on the basis of partitioning considerations (Table 3). However, the $-\Delta F$ values increase drastically from the 8th through 10th methylene group (Fig. 4). Lauric (C_{12}) and higher acids show type A↑ behavior, and here the free energy change per methylene residue is small (Table 3). The effect of higher acids is chain length dependent. However, only C_{12}–C_{16} acids show significant effect. Stearic acid induces type D↑ profiles, but the shift is only of about 1.5 °K. C_{20} and higher acids do not induce any change up to 5 mM.

The inadequacy of partition coefficient considerations is probably best illustrated in the concentration dependence of 1-, 2-, 3- and 4-*n*-octanols. These isomeric alcohols are expected to show almost the same partition coefficient (Leo *et al.*, 1971). As shown in Fig. 5, they show

about a fourfold difference in their ability to induce type A↑ profile. We have observed a similar lack of correlation between HHW_{100} and HHW'_{100} for a series of adamantane derivatives and their expected lipid/water partition coefficients (Jain *et al.*, 1976).

Thus, it is obvious that simple considerations based on organic solvent/water or membrane/buffer partition coefficient may not be adequate to account for additive-induced changes in the phase transition behavior of lipid bilayer. We wish to propose an alternative hypothesis that could not only account for the behavior of homologs in a given series, but it could also provide a unified account of solute-induced changes in phase transition characteristics of lipid bilayer. We wish to propose that both *the qualitative and quantitative differences in the transition characteristics arise from a difference in the position of localization of solutes in different regions of the bilayer along its thickness.* Elaboration of this hypothesis and consequent predictions are given below following a recapitulation of the observations that this hypothesis attempts to explain. In the Results section we have shown that:

(a) the bilayer organization can be perturbed by a variety of solutes;

(b) the effect (perturbation) induced by the various classes of additives on the phase transition profiles can be qualitatively different;

(c) the magnitude of the effect induced by an additive is dependent upon its concentration;

(d) the effect of additives that are structurally related (say long-chain alcohols, or uncouplers, etc.) is qualitatively the same, but the magnitude of the effects is different for different compounds in the same series at the same concentration.

It is now well established that the lecithin dispersions in aqueous medium almost exclusively contain lamellar bilayer stucture under a variety of conditions. However, the conformation and packing of lipid molecules in the bilayer is subject to significant reorganization. Such transitions, which can be observed by a variety of techniques, can be induced by a change in temperature, pH, membrane potential, and ionic and drug concentration. A rationale for the above observations can be derived from the concepts of organization of lipid molecules in bilayer.

Of the various transitions that a phospholipid bilayer can undergo, the temperature dependent gel-to-liquid crystalline phase change has been studied rather extensively by calorimetric techniques (Ladbrooke & Chap-

man, 1969; Hinz & Sturtevant, 1972; Phillips, 1972). The thermotropic transition profile contains information regarding not only the transition temperature but also provides quantitative information about enthalpy of transition, phase diagram, size of the cooperative unit undergoing the transition, range of temperatures in which phase transition and phase separation occurs, and if two or more phases coexist within the bilayer.

The molecular changes underlying the gel-to-liquid crystalline phase transition are best visualized from a synthesis of information obtained from a variety of physico-chemical techniques. According to the currently accepted model, in the gel phase the acyl chains are packed in a highly ordered hexagonal array. This packing requires that all carbon-to-carbon bonds in the chains be very near the *trans* conformation. In the liquid crystalline phase some of the C-C bonds assume *gauche* conformation. The following features (*see* Lee, 1975 for a review) of the bilayer organization are of particular interest in the present context:

(a) The probability of *gauche* conformation of C-C bonds increases as one moves away from carboxyl group towards methyl end. Both above and below the transition temperature, the portion of the hydrocarbon chain near the center of the bilayer is more "fluid" or disorganized than the portion of the chain near the head group (Phillips, Williams & Chapman, 1969).

(b) The average orientation and packing of the polymethylene chain changes abruptly at about the ninth carbon away from the carboxyl group. There is an indication that the acyl chain in the C_1 through C_8 region has a constant segmental order parameter. In the end region of the chains (C_{10} and up) there is considerable disorder (Hubbell & McConnell, 1971; Levine, 1973; Seelig & Seelig, 1974).

(c) The sharpness of the thermal phase transition is due to highly cooperative interaction among the lipid molecules. The features (a) and (b) would imply that the size of the cooperative unit that undergoes the transition would be largely regulated by the interaction in about 1 through 10 carbon regions of acyl chains (Trauble, 1971; Rothman, 1973).

(d) Several phases with distinct transition characteristics can coexist within a bilayer continuum (Shimshick & McConnell, 1973; for a review *see* Jain & White, 1977).

An explanation for the observations presented in this paper can be attempted in terms of these features of bilayer organization. The transition profile as measured by differential scanning calorimetry is a description of the phase properties of the lipid bilayer as modified by the

various additives. Since no other perturbation, except temperature, is used to monitor the changes in the bilayer, the information thus obtained is unambiguous. Indeed, the very fact that the phase transition profile of a lipid bilayer is modified by an additive provides a direct proof that the additive perturbs the bilayer organization. The type of perturbation is indicated by the shape of the transition profile. Thus, a broad transition profile (type A) would imply that the size of the cooperative unit undergoing transition in such a modified bilayer is small. The shape of type A profiles is also consistent with the explanation that the modified bilayer may consist of a range of phases which differ only slightly in their packing characteristics. A sharp transition profile shifted along the temperature axis (type C) would imply that the size of the cooperative unit in the modified bilayer remains unchanged, but the packing of these units can be perturbed at lower temperatures. Appearance of a new peak (type B or D) would in turn imply that a new modified phase is formed in the bilayer that coexists with the unmodified phase in the bilayer continuum. At present we cannot rule out the possibility that the new transition peak is due to the modified bilayer that exists as separate lamellae. However, the doped liposomes do not show any change in the phase transition behavior upon centrifugation at low speed. Such a treatment would remove liposomes with small radii of curvature and thus show profiles with lower transition temperature (*cf.* Sheetz & Chan, 1972; Suurkuusk, Lentz, Barenholz, Biltonen & Thompson, 1976). The properties of the new phase are indicated by the shape and position of the new peak. A broad peak or shoulder (type B profiles) would indicate that the new phase has smaller cooperative units, which do not coexist with pure phospholipid phase. As one would expect there are two extremes of the type B profiles. The new phase could be liquid (no cooperative units), and thus one would observe only the main transition peak whose area decreases with increasing additive concentration (type E profile). On the other extreme, appearance of a sharp new peak (type D profiles) would indicate that the modified phase has cooperative units comparable in size to that of the unmodified phase. Similarly, the position of the new peak along the temperature axis would indicate as to whether the shift is to a higher or lower temperature. In all these cases, the area under a given peak indicates the proportion of the lipid in that phase.

Thus from the type of transition profile one can not only determine whether or not an additive would "fluidize" or "solidify" the bilayer, but a modified profile also provides the information about the thermody-

namic properties of the new phase: the temperature range and enthalpy of the transition. Such data also indicates as to whether the whole bilayer undergoes a change or only a part of it does so. This is the kind of information that is lacking in the data obtained from the use of most other physico-chemical techniques applied to bilayer systems. Since the packing of lipid molecules is reflected in the position of the transition profile along the temperature axis, HHW′ should be related to a change in the solute-induced packing. Similarly, the size of the cooperative unit undergoing transition should be related to HHW of the profile since it is related to the midpoint slopes of the transition profile. In this paper we have presented only the HHW′ data since it is more sensitive to the additive concentration. However, the relationship between HHW′ and HHW is of interest. Plot of HHW and HHW′ as a function of concentration for various additive types is shown in Fig. 18. The ratio of HHW/HHW′ is characteristic for a given additive and varies significantly for different types of solutes (Fig. 18). From such relationships one can obtain not only HHW'_{100} and HHW_{100}, but also two different types of relationships between HHW and HHW′. These are defined as two arbitrary ratios α and β.

(a) α as the ratio HHW/HHW′ at the concentration at which HHW′ increases by 100% (HHW'_{100}). Thus the values of α will range between 1 (when HHW = HHW′) and 0.5 (when the profile is not broadened but only shifts along the temperature axis).

(b) β as the ratio of HHW_{100}/HHW'_{100}. Thus the values of β would range between 1 (when HHW = HHW′) and 0 (when the profile is not broadened).

The values of HHW_{100}, HHW'_{100}, α, β, and the type of profile induced are given in Table 4 for all the compounds discussed in this paper. Certain trends are obvious. The values of both α and β show the following decreasing order: Type D > Type B > Type A > Type C. As elaborated below this order is the same as the order for the localization of the various solutes away from the aqueous interface in the bilayer. Considering the definition of both α and β, it is obvious that these ratios should be related to the molecular parameters that characterize the size of and the packing within the cooperative unit that undergoes thermotropic transition. The data presented in Table 4 should also prove useful for a quantitative comparison of the effects described in this paper with other manifestations of these solutes in biological and model membranes.

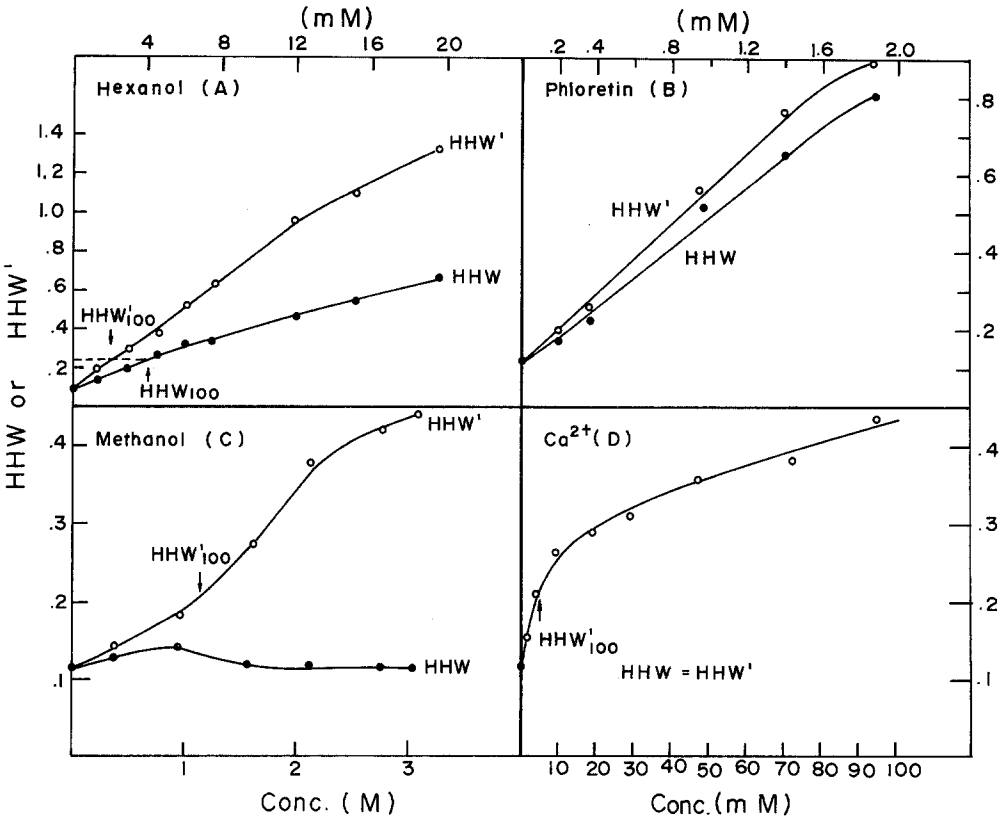

Fig. 18. Plots of HHW (closed circles) and HHW′ (open circles) as a function of concentration for additives of type A (hexanol), B (phloretin), C (methanol), and D (Ca^{2+}). For type B and D profiles HHW≅HWW′ at all concentrations. From the plots of this type were obtained the values of HHW'_{100} (concentration at which HHW′ is twice the original HHW), HHW_{100} (concentration HHW is twice the original HHW), α (ratio of HHW to HHW′ at HHW'_{100}) and β (ratio of HHW_{100} to HHW'_{100}). These values are given in Table 4 for all the additives discussed in this paper

The types of compounds which modify the phase transition profile are particularly interesting. The molecules which induce type A profile are generally long with one end polar and the other end nonpolar (C_5 and higher alkanols, alkanoic acids, some detergents). In contrast, the compounds that induce type C profile are small with a weak dipolar character. Compounds which induce type B profile are relatively large, disk-shaped, asymmetric, and reasonably polar. The solutes which induce type D profile are almost exclusively ionic or have an ionic end. Thus,

Table 4. Some constants characterizing the effect of the various solutes

Compound	Type of profile	HHW'_{100} (mM)	HHW_{100} (mM)	α	β
Alcohols					
Methanol	C↓	1400	–	0.521	~0
Ethanol	C↓	483	–	0.510	~0
n-Propan-1-ol	C↓	150	–	0.51	~0
n-Propan-2-ol	C↓	98	–	0.51	~0
n-Butan-1-ol	C↓	33.4	–	0.5	~0
n-Pentan-1-ol	A↓	11.5	32	0.642	0.278
n-Hexan-1-ol	A↓	2	5	0.731	0.405
n-Heptan-1-ol	A↓	1.4	2.76	0.75	0.507
n-Octan-1-ol	A↓	0.73	0.898	0.813	0.813
n-Octan-2-ol	A↓	0.833	1.167	0.714	0.714
n-Octan-3-ol	A↓	3.0	6.08	0.687	0.63
n-Octan-4-ol	A↓	3.8	7.0	0.666	0.543
n-Nona-1-ol	A↓	2.1	3.5	0.710	0.470
n-Decan-1-ol	A↓	2.1	3.5	0.710	0.470
n-Decan-1-ol	A↓	3.3	3.8	0.85	0.89
C_{11}–C_{16} alcohols no effect up to 5 mM; A↑ type of effect is observed at higher concentration					
1-Alkanoic Acid					
n-Pentanoic	No significant effect up to 250 mM				
n-Hexanoic	No significant effect up to 115 mM				
n-Heptanoic	A↓	31	55	0.57	0.564
n-Octanoic	A↓	17	22	0.788	0.773
n-Nonanoic	A	7	7	>0.95	1.0
n-Decanoic	A	1.5	1.5	>0.95	1.0
n-Dodecanoic	A↑	17.5	17.5	>0.95	1.0
n-Tridecanoic	A↑	8.1	8.1	>0.95	1.0
n-Tetradecanoic	A↑	6.2	6.2	>0.95	1.0
n-Hexadecanoic	A↑	3.1	3.1	>0.95	1.0
C_{18},C_{20} and C_{24}	Show no significant effect up to 10 mM				
n-Undecy-10-lenic	A↓	2.9	3.8	0.786	0.763
Palmitoleic	A↓	2.2	3.4	0.754	0.647
Oleic	A↓	2.5	2.8	0.80	0.82
Linolenic	A↓	1.9	3.2	0.76	0.594
Arachidonic	A↓	1.35	1.9	0.76	0.71
Ricinalaidic	A↓	7.3	7.3	>0.95	1.0
Ricinoleic	A↓	1.3	3.0	0.70	0.433
Small Organic Solutes					
Carbon tetrachloride	C↓	510	–	0.5	–
Benzene	C↓	720	–	0.5	–
Toluene	C↓	380	–	0.5	–
Dioxane	C↓	270	–	0.5	–
Chloroform	C↓	200	–	0.5	–
Tetrahydrofuran	C↓	180	–	0.5	–
Uncouplers	*See* data in Table 1				
Cations	*See* Table 2				
Chaotrops					
Guanidine	B↑	500	500	>0.95	1.0
$KClO_4$	D↑	45	45	1.0	1.0

Table 4 (continued)

Compound	Type of profile	HHW'_{100} (mM)	HHW_{100} (mM)	α	β
NaSCN	B↑	340	340	>0.95	1.0
NaI	D↓	250	330	0.85	0.756
Detergents					
Dodecyl sulfate	E	AH_{50}=4.5 mM			
Dodecyl pridinium chloride	B↓	0.458	0.590	0.833	0.778
Dodecyl acid phosphate	B↓	3.82	3.82	>0.95	1.0
Octadecylamine	E	AH_{50}=2.2 mM			
Octadecyltrimethylammonium	D↑	0.58	0.89	0.72	0.65
Digitonin	D↓	0.422	0.422	1.0	1.0
Cholic acid	D↓	1.64	2.2	0.78	0.75
Doxycholic acid	B↓	0.188	0.3	0.6	0.627
Brig 36f	A↓	9.7 [a]	18.2 [a]	0.76	0.53
Emulphogene BC-720	A↓	4.2 [a]	7.4 [a]	0.8	0.57
Triton X-100	A↓	4.48 [a]	8.96 [a]	0.8	0.5
Triton CF-32	A↓	2.52 [a]	2.82 [a]	0.85	0.89
Lubrol PX	A↓	10.6 [a]	14.6 [a]	0.81	0.73
Ionophores					
Gramicidin	No significant effect up to 5 mM				
Valinomycin	A↓	2.0	2.1	>0.90	0.95
Monensin [b]	B↓	>2	>2	>0.95	1.0
Nigericin	B↓	1.5	1.5	>0.95	1.0
Dianemycin [b]	B↓	>2.5	>2.5	>0.95	1.0
A23187	B↓	3.1	3.1	>0.95	1.0
Tetraphenylboron	A↓	4.0	4.8	0.85	0.83
Dicyclohexyl-18-crown-6	A↓	4.3	13.0	0.60	0.30
Di-*t*-butyl-18-crown-6	A↓	0.4	1.0	0.66	0.40
Probes					
TEMPO	No significant effect up to 15 mM				
TEMPONE	No significant effect up to 15 mM				
TEMPOL	C↓	12	–	0.5	<0.5
3-Nitroso-Androstan	A	1.4	1.4	>0.95	1.0
4-Nitroso-stearate	No significant effect up to 10 mM				
12-Nitroso-stearate	A	5.5	5.5	0.95	1.0
PCA	A↓	8.38	8.95	0.76	0.936
9-vinyl anthracene 9,10-Dimethylanthracene 9,10-Diphenylanthracene Diphenylhexatriene Perylene	No noticeable effect at least up to 10 mM				
N-Phenyl-1-naphthylamine	A↓	0.119	0.33	0.642	0.361
8-Anilino-1-naphthalene-sulfonic acid	B↓	0.456	0.57	0.82	0.766

[a] These values are in mg/ml.

[b] These compounds do not show any significant concentration dependence, therefore, the values of HHW'_{100} correspond to the concentration for half maximal effect.

if one were to arrange different classes of compounds in the order of increasing polarity, the order would be Type C < Type A < Type B < Type D solutes. That is type C solute would be localized farthest away from the interface and the type D solute would be localized closest to or on the interface.

This order of localization of the various solutes is qualitatively consistent with the type of profiles they induce. As indicated earlier the phase transition profile arises from the change in the organization of acyl chains. Since the methyl end region of the bilayer is in a relative state of disorder, a molecule localized in this region would have little or no effect on the transition profile. Thus, as a function of increasing solute concentration, one would expect a change in the packing but only a slight (if any) change in the size of the cooperative unit; that is, a continuous lowering of the transition temperature and no change in the area or the shape of the transition profile. Type C molecules fall into this category. Because of their weak dipolar character they may show some tendency to have their average position of localization slightly towards the polar region as opposed to the completely nonpolar hydrocarbons which would be localized in the center of the bilayer without significantly perturbing the packing within the cooperative unit. Indeed, most small hydrocarbons do not show any effect on phase transition profile unless present in high concentration, when they may be pushed towards the closely packed region. The weakly dipolar molecules would tend to localize more towards the interface thereby weakening the intermolecular packing. However, this is not expected to be adequate to disrupt the cooperative unit. Thus, a solute localized in the region of the 9th or higher carbon of the acyl chain would not modify the cooperativity of transition (sharpness of the peak) although it may modify (weaken) the packing within the cooperative unit. Indeed, the behavior of type C solutes is consistent with such an expectation: they lower the transition temperature without any significant effect on the shape and the area of the transition profile.

As noted earlier some of the organic solvents induce type C profile only at lower additive concentration, whereas they induce type A profile at higher concentrations. This would imply that at lower additive concentrations the additive is localized in the terminal methyl group regions. Only at higher concentrations is the additive localized in the C_1–C_8 region inducing type A profile. This new region of additive localization could be due to a modification of the partitioning properties of the C_1–C_8 region after the additive is localized in the terminal methyl region.

Another possibility is that these two regions have intrinsically different partitioning characteristics for small solutes. Thus, at low concentrations an additive would preferentially partition in the border domain between gel and liquid crystalline region. However, at higher additive concentrations when the gel border domains are "saturated", the additive would modify the gel domains. Such an effect is indeed observed with some solvents and with some fluorescent and spin-labelled probes which induce a change in transition profiles abruptly at high additive concentration. The possibility of selective localization of probes only in certain regions in the plane of the bilayer has been expressed in literature (Keith *et al.*, 1973; Bieri, Wallach & Lin, 1974). Similarly, a lack of additive-induced change in the transition profile does not necessarily rule out their perturbing effect. It only indicates that the additive does not modify the gel phase. However, these probes may be localized either in the terminal methyl group region or in the gel border domains where they will not affect thermotropic transition.

Type A profiles appear to involve a significant change both in the packing and the size of the cooperative unit. This would be expected if these solutes disrupt the interactions that stabilize the cooperative unit. Such a situation would arise if type A solutes were to be localized in the vicinity of the first eight carbons of the acyl chain. Such a localization requires that the solute be relatively large and moderately amphipathic. Indeed, most of the type A solutes (*n*-alkanols, alkanoic acids, detergents) fit into this general category. Amphipaths with strong polar groups may not satisfy this requirement. The polar groups would repel each other and thus modify or disrupt the cooperative unit completely. Such effects could account for a qualitatively different behavior of dodecanoic acid (type A), dodecylamine, N-dodecylpyridinium chloride, dodecylphosphate (all type B) and dodecylsulfate (type E).

The type D profiles result from a change in the packing within the cooperative unit, whereas the size of the cooperative unit remains essentially unchanged. If one assumes that the type D solutes interact stoichiometrically with lipid in the polar group region, one would expect the appearance of a new phase that coexists with the unmodified phase. The cooperative units of these two phases have different packing characteristics and, therefore, different transition temperatures. If the packing in the new phase is tighter, its transition temperature will be higher. Indeed, large organic cations induce a new peak at lower transition temperatures, and the small inorganic cations induce a new peak at higher transition temperature. One would expect a limiting value for

the size of a hypothetical cation which may bind to the polar group region without altering the packing characteristics and, therefore, the transition characteristics of the modified bilayer.

If the interaction between a type D solute and lipid in the bilayer is assumed to be stoichiometric, the appearance of new phase would be concentration-dependent. Thus, as a function of type D additive concentration the area of the new peak would change. Moreover, the appearance of a new phase by a type D additive may not necessarily accompany the disappearance or reduction in the area of the original peak. A decrease in the area of the main peak would occur only if the solute interacts with the lipid in the gel phase responsible for the parent peak. In contrast, if the solute interacts with the lipid in the liquid crystalline phase then the appearance of new peak would not accompany the disappearance of the original unmodified phase. Indeed, both of these possibilities have been observed. Appearance of the new peak accompanies the disappearance of unmodified phase with Ca^{2+} ions. On the other hand, the area of the original peaks remains unchanged when at low concentrations Eu^{3+} ions induce a new peak having transition at higher temperature.

It is not certain whether the formation of the new phase by type D solutes occurs by the formation of individual stoichiometric complex or if the whole cooperative unit of the new phase is formed at once. The latter possibility would lead to large microscopic statistical fluctuations in membrane properties in response to the type D additives.

Type B profiles would arise from a combination of the mechanisms proposed for type D and type A profiles. The phase modified by type B solutes does co-exist with the unmodified phase. However, the modified phase has small cooperative units and/or has distinctly different packing characteristics. Such a perturbation would be expected if the solute was localized in the glycerol backbone region. The structure of uncouplers appears to be well suited for such a localization. Evidence based on ESR studies also leads to similar conclusions. For example, both forms of the uncoupler 2,4-dinitrophenol, are predominantly localized at the polar head-group regions of the membrane (Chen & Hsia, 1974).

Thus, our hypothesis appears not only to be capable of qualitatively accounting for the data presented in this paper, but it is also consistent with current concepts of bilayer structure and organization. So far, we have tried to explain only the broad aspects of solute-induced phase change and phase separation. It appears that the results and the consequent hypothesis presented in this paper also provide a rationale for

certain subtle aspects of solute-induced changes in membranes. Some of these are elaborated below:

(1) As a function of increasing chain length *n*-alkanols show not only a quantitative change (in the HHW'_{100} values) but also a qualitative change in the type of profiles they induce. Lower alcohols up to C_4 induce type C profiles since they may not be able to perturb the interactions in the C_1–C_8 region. Higher alcohols (C_5–C_{10}) are capable of localizing themselves in this region and thus they induce type A profile. Interestingly enough, however, the potency of C_9 and higher alcohols drops such that C_{11} and higher alcohols induce little change in the phase transition profile. This is consistent with the hypothesis which we have elaborated earlier. Although undecanol and higher alcohols would go in between the lipid acyl chain in the C_1–C_8 region, they are long enough so that they can substitute for the lipid acyl chains and thus stabilize the cooperative unit. Indeed, such a reversal and loss of activity with increasing chain length has been observed for some other properties of the bilayer (Jain & Cordes, 1973; Jain, Toussaint & Cordes, 1973) and biomembrane (Sullivan, Jain & Koch, 1974). An abrupt change in induced profiles from *n*-butanol to *n*-pentanol is also quite intriguing. It may be pointed out that a difference in their behavior has been noticed in the studies on solubilization of membrane proteins and lipid by these alcohols (Nachbar, Winkler & Salton, 1972, and references therein). Our results may have some bearing on these observations.

(2) Behavior similar to the one just described is also observed for *n*-alkanoic acids. C_5 and C_6 acids do not show any effect. C_7–C_{10} acids induce type A profile. For alcohols such transition from type C to A occurs between C_4 and C_5, and the reversal from the maximum occurs after C_8. Thus, there appears to be a shift of two carbon lengths for the induction of the same phenomenon by alkanols and by alkanoic acids. These observations could be rationalized if one assumes that *n*-alkanols localize two methylene deeper than the corresponding alkanoic acids. This could be accomplished if the localization of alkanols is regulated by hydrogen bonding of hydroxyl group with the carbonyl oxygens of the acyl residues of lecithin. In contrast, the carboxyl group of alkanoic acids may interact electrostatically with, say, the quaternary ammonium group of choline. Some support for such speculation can be derived from the studies that invoke hydrogen bonding between β-OH of sterols and the acyl carbonyl of phospholipids (Jain, 1975; Huang, 1976). The suggestion of an interaction between carboxyl and quaternary ammonium group implies a specific and probably stoichiometric interaction.

(3) Fig. 13 and 14 show a qualitative difference in the behavior of the various surfactants in modifying the bilayer. This could have a functional significance in explaining their ability to solubilize membrane components. For erythrocyte membrane, for example, it has been observed that sodium dodecyl sulfate extracts individual components separately. In contrast, bile salts and Triton X-100 solubilizes different components simultaneously (Kirkpatrick, Gordesky & Marinetti, 1974). In terms of the observations presented in this paper, it would mean that SDS, which reduces the total number of cooperative units without affecting the size of the residual cooperative units, would successively solubilize the most susceptible of the membrane domains. In contrast, the bile salts and nonionic detergents would decrease the size of the cooperative units presumably without affecting their number. Thus, different membrane domains will be solubilized simultaneously.

(4) Mellitin, a lytic peptide from bee venom, is known to interact with biomembrane and lipid bilayer (Sessa, Freer, Colacicco & Weissmann, 1969). It induces a type E profile indicating that the modified phase is liquid crystalline. A large molecule inserted between phospholipid molecules in the gel phase would break up the cooperative units. Such a view is consistent with the conclusions based on the spectroscopic studies (Verma, Wallach & Smith, 1974).

(5) The even-odd paraffin chain length difference in the effect of *n*-alkanols (Fig. 4) can be understood in terms of the conformation of polymethylene chain. A difference in the packing properties of odd and even chains for paraffins of chain length less than 25 is well documented (Malkin, 1931; Broadhurst, 1963; Mnyukh, 1963; Yemni & McCullough, 1973). Such differences are, for example, manifested in the transition temperature and crystal structure of homologous *n*-alkanes. The even-odd alterations are a consequence of the difference in the symmetry of the alkyl chain, which leads to a difference in the packing of end group. Maximum interaction between all *trans* alkyl chains is possible only when they assume a staggered arrangement. As shown in Fig. 19, such an arrangement of chains would lead to an apparent tilting of the chain axis at an angle from the plane perpendicular to the bilayer. Incorporation of *n*-alkan-1-ols in between the lecithin chains would also require a staggered arrangement. The terminal groups of alkanol molecule are somewhat larger and asymmetric than the methylene residues. As schematized in Fig. 19, due to a greater symmetry, the even chain staggers itself in between the acyl chains. If an odd-numbered chain is staggered only one end can assume an advantageous low energy position. The

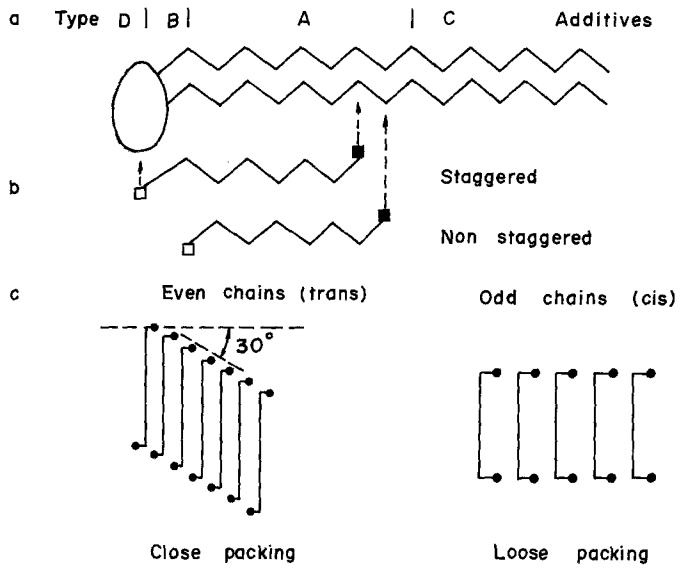

Fig. 19. A schematic diagram (not drawn to scale) that illustrates the various features of intermolecular packing within a bilayer phase. (*a*) The possible regions of localization of the various additives that induce qualitatively distinct phase transition profiles when incorporated in dipalmitoyl lecithin bilayer. Solutes of type D may be localized in the phosphorylcholine region; type A in C_1–C_9 region; type C in C_{10}–C_{16} region. (*b*) Staggered and nonstaggered arrangement of two all-transpolymethylene chains. If the end group regions are larger than the methylene groups (which they usually are), and alternation in the properties of odd and even chain lengths would results. (*c*) The even chains can stagger with a slight shift along the long axis. This would result in a close packed arrangement in which the plane of the film is tilted by about 30° from the long axis of the chain. The odd chains are less symmetrical, therefore, the bulkier end groups will interfere with a staggered closed pack arrangement. Thus, the packing of odd chains would be loose and energetically less favorable

other end is necessarily forced into some apparently high energy position. This is because both the ends are on the same side ("cis"), a feature that would interfere with the staggered arrangement of the chains. Thus, if the hydroxyl group of the odd chain alkanol is hydrogen bonded to the carbonyl oxygen, the methyl end would also be *cis* to the hydroxyl group. Thus, the terminal methyl group of an odd chain length alcohol would interfere with the staggered arrangement. In even chain length alcohols, the terminal methyl group would be *trans,* and thus it would be better accommodated with staggered chains. Since the difference in the sizes of methylene and methyl groups is rather small, the free energy

change associated with even-odd chain lengths would also be small. The value of 200 cal, which we have observed, is not unreasonable.

(6) We have consistently observed that most hydrocarbons and highly symmetrical nonpolar molecules (e.g. data in Figs. 16 and 17) do not modify transition profiles. Since these molecules do get incorporated into the bilayer, it is tempting to suggest that they are localized near the terminal methyl group in between two monolayer halves of the bilayer. Such a localization would neither modify packing and/or size of the cooperative unit. Such a behavior of TEMPO and TEMPONE is consistent with that of di-tert-butyl-nitroxide observed by ESR spectroscopy (Dix, Diamond & Kevelson, 1974; Tomkiewicz & Corker, 1975).

In this paper we have made several provocative statements. The supporting evidence for some of which is not as strong as one would like it to be. However, the overall hypothesis and the evidence appears to be internally consistent. The phenomenon of localization of solutes in different regions along the thickness of the bilayer, and the phenomenon of solute-induced changes in the phase properties of the bilayer offers a rational explanation for a varity of biochemical processes induced in biomembranes by additives such as drugs, probes, lytic agents, ionophores, detergents and fusogenic agents. A meaningful interpretation and comparison of the concentration dependence of the effects reported in this paper can be made to biological dose response relationships. However, one has to keep two factors in mind. First, the effects on transition profile as reported here correspond to a modification of large membrane area. Biological effects may, in contrast, be manifested when only a small fraction of the membrane is modified. In most cases, the effects may be intrapolated linearly to the lower concentration range. The second factor arises due to the experimental conditions used. All the experiments were done by mixing small volumes of concentrated lipid and additive solutions, and in all cases the lipid-to-additive concentration ratio was greater than 5. Typically, it is 15 or more. These factors make a correlation between the effects on phase transition and partition coefficients somewhat indirect but still meaningful. It may, however, be noted that quite a few compounds modify the phase transition profile at as small as 1:300 additive to phospholipid ratio. One of the simplest interpretations of this would be that only a few additive molecules in a cooperative unit (about one mole percent) can modify its packing characteristics.

In the context of biomembrane phenomena, the additive-induced changes in the phase properties would involve domain boundary effects

in the bilayer, and the aggregation, segregation, and conformational change of membrane components (*see* Jain & White, 1976 for a review). We are also aware that our observations have implications for possible modes of action of "lipid-soluble" drugs. The phenomenon which we have described here not only illustrates the limitations of the bulk organic solvent/water partition coefficients, but it also provides an explanation as to how the different classes of lipid-soluble drugs could induce qualitatively different responses in biomembranes.

We appreciate several discussions with Drs. Roy McCullough and Roger Murray. We would also like to thank Dr. A. Keith (Pennsylvania State University), Dr. R. Hamill (Eli Lilly), and Dr. P. Heytler (Dupont) for making several compounds available for this study. This work was supported by the Delaware Institute of Medical Research and Education, and by the University of Delaware Research Foundation.

References

Ahkong, Q.F., Fisher, D., Tampion, W., Lucy, J.A., 1973. The fusion of erythrocytes by fatty acids, esters, retinol and α-tocopherol. *Biochem. J.* **136**:147

Bieri, V.G., Wallach, D.F.H., Lin, P.S. 1974. Focal erythrocyte membrane perturbations caused by nitroxide lipid analogues. *Proc. Nat. Acad. Sci. USA* **71**:4797

Brink, F., Posternak, J.M. 1948. Thermodynamic analysis of the relative effectiveness of narcotics. *J. Cell. Comp. Physiol.* **32**:211

Broadhurst, M.G. 1963. An analysis of the solid phase behavior of the normal paraffins. *J. Res. NBS* **66A**:241

Butler, J.A.V., Ramchandrani, C.N., Thomson, D.W. 1935. The solubility of non-electrolytes. Part I. The free energy of hydration of some aliphatic alcohols. *J. Chem. Soc.* p. 280

Cadenhead, D.A., Muller-Landau, F. 1973. Pure and mixed monomolecular films of 12-nitroxide stearate. *Biochim. Biophys. Acta* **307**:279

Chen, W.L., Hsia, J.C. 1974. Studies on motional characteristics and distribution of protonated and anionic forms of spin-labeled 2,4-dinitrophenol in phospholipid bilayer membranes. *Biochemistry* **13**:4948

Cunarro, J., Weiner, M.W. 1975. Mechanism of action of agents which uncouple oxidative phosphorylation: Direct correlation between proton carrying and respiratory-releasing properties using rat liver mitochondria. *Biochim. Biophys. Acta* **387**:234

Dix, J.A., Diamond, J.M., Kevelson, D. 1974. Translational diffusion coefficient and partition coefficient of a spin-labelled solute in lecithin bilayer membranes. *Proc. Nat. Acad. Sci. USA* **71**:474

Fourcans, B., Jain, M.K. 1975. Role of phospholipids in transport and enzymic reactions. *Adv. Lipid Res.* **12**:147

Freese, E., Sheu, C.W., Galliers, E. 1973. Function of lipophilic acids as microbial food additives. *Nature (London)* **241**:321

Galla, H.J., Sackmann, E. 1975. Chemically induced phase separation in mixed vesicles containing phosphatidic acid. An optical study. *J. Am. Chem. Soc.* **97**:4114

Ginsburg, E., Salomon, D., Sreevalsan, T., Freese, E. 1973. Growth inhibition and morphological changes caused by lipophilic acids in mammalian cells. *Proc. Nat. Acad. Sci. USA* **70**:2457

Goddard, E.D., Kung, H.C. 1966. Molecular association in pairs of long chain compounds. *Soap Chem. Spec.* **42**:60

Hansch, C., Dunn, W.J. 1972. Linear relationships between lipophilic character and biological activity of drugs. *J. Pharm. Sci.* **61**:1

Hatefi, Y., Hanstein, W.G. 1969. Solubilization of particulate proteins and nonelectrolytes by chaotropic agents. *Proc. Nat. Acad. Sci. USA* **62**:1129

Haydon, D.A., Taylor, F.H. 1960. Adsorption at the oil-water interface and the calculation of electrical potentials in the aqueous surface. Phase I. Neutral molecules and a simplified treatment for ions. *Phil. Trans.* **252**:225

Helenius, A., Simons, K. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta* **415**:29

Hersh, L.S. 1971. Cellular narcosis and hydrophobic bonding. *In*: The Chemistry of Biosurfaces. M.L. Hair, editor. Vol. 1, p. 349. Mercel Dekker Inc., New York

Herzfeld, S.H., Corrin, M.L., Harkin, W.D. 1950. The effect of alcohols and of alcohols and salts on the critical micelle concentration of dodecylammonium chloride. *J. Phys. Chem.* **54**:271

Hill, M.W. 1974. The effect of anesthetic-like molecules on the phase transition in smectic mesophases of dipalmitoyl lecithin. I. The normal alcohol up to C=9 and three inhalation anesthetics. *Biochim. Biophys. Acta* **356**:117

Hill, M.W. 1975. Partition coefficients of some anesthetic-like molecules between water and smectic mesophases of dipalmitoyl phosphatidylcholine. *Chem. Soc. Trans.* **3**:149

Hinz, H.J., Sturtevant, J.M. 1972. Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins. *J. Biol. Chem.* **247**:6071

Huang, C.-H. 1976. Roles of carbonyl oxygens at the bilayer interface in phospholipid-sterol interaction. *Nature (London)* **259**:242

Hubbell, W.L., McConnell, H.M. 1971. The molecular motion in spin-labeled phospholipids and membranes. *J. Am. Chem. Soc.* **93**:314

Jacobson, K., Papahadjopoulos, D. 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentration of bivalent cations. *Biochemistry* **14**:152

Jain, M.K. 1972. The Bimolecular Lipid Membrane: A system. Van Nostrand Reinhold Co., New York

Jain, M.K. 1975. Role of cholesterol in biomembranes and related systems. *Curr. Top. Membr. Trans.* **6**:1

Jain, M.K., Cordes, E.H. 1973. Phospholipase K. Effect of *n*-alkanols on the rate of enzymatic hydrolysis of egg phosphatidylcholine. *J. Membrane Biol.* **14**:101

Jain, M.K., Toussaint, D.G., Cordes, E.H. 1973. Kinetics of water penetration into unsonicated liposomes: Effects of *n*-alkanols and cholesterol. *J. Membrane Biol.* **14**:1

Jain, M.K., White, H.B. 1977. Long-range order in biomembrane. *Advanc. Lipid Res.* **15**:1

Jain, M.K., Wu, N., Wray, L.V. 1975. Drug induced phase change in bilayer as possible mode of action of lipid soluble drugs. *Nature (London)* **255**:494

Jain, M.K., Wu, N., Morgan, T.K., Briggs, M.S., Murray, R.K. 1976. Phase transition in lipid bilayer. II. Influence of adamantane derivatives. *Chem. Phys. Lipids* **17**:71

Kantor, H.L., Prestegard, J.H. 1975. Fusion of fatty acid containing lecithin vesicles. *Biochemistry* **14**:1790

Keith, A.D., Sharnoff, M., Cohn, G.E. 1973. A summary and evaluation of spin labels as probes for biological membrane structure. *Biochim. Biophys. Acta* **300**:379

Kinoshita, K., Ishikawa, H., Shinoda, K. 1958. Solubility of alcohols in water determined by the surface tension measurement. *Bull. Chem. Soc. (Jap)* **31**:1081

Kirkpatrick, F.H., Gordesky, S.E., Marinetti, G.V. 1974. Differential solubilization of proteins, phospholipids and cholesterol of erythrocyte membranes by detergents. *Biochim. Biophys. Acta* **345**:154

Kosower, E.M., Kosower, N.S., Faltin, Z., Diver, A., Saltoun, G., Frensdorf, A. 1974. Membrane mobility agents. A new class of biologically active molecules. *Biochim. Biophys. Acta* **363**:261

Kosower, N.S., Kosower, E.M., Wegman, P. 1975. Membrane mobility agents. II. Active promoters of cell fusion. *Biochim. Biophys. Acta* **404**:530

Krishnan, K.S., Balaram, P. 1975. Perturbation of lipid structures by fluorescent probes. *FEBS Lett.* **60**:419

Ladbrooke, B.D., Chapman, D. 1969. Thermal analysis of lipids, proteins and biological membranes. A review and summary of some recent studies. *Chem. Phys. Lipids* **3**:304

Lee, A.G. 1975. Functional properties of biological membranes: A physical-chemical approach. *Progr. Biophys. Mol. Biol.* **29**:3

Leo, A., Hansch, C., Elkins, D. 1971. Partition coefficients and their uses. *Chem. Rev.* **71**:525

Levine, Y.K. 1973. X-ray diffraction studies of membranes. *Progr. Surface Sci.* **3**:279

Lis, L.J., Kauffman, J.W., Shriver, D.F. 1975. Effect of ions on phospholipid layer structure as indicated by Raman spectroscopy. *Biochim. Biophys. Acta* **406**:453

Maggio, B., Lucy, J.A. 1975. Studies on mixed monolayers of phospholipids and fusogenic lipids. *Biochem. J.* **149**:597

Malkin, T. 1931. Alternation in properties of long-carbon-chain compounds. *Nature (London)* **127**:126

Mnyukh, Yu. V. 1963. Laws of phase transformation in a series of normal paraffins. *J. Phys. Chem. Solids* **24**:631

Molyneux, P., Rhodes, C.T., Swarbrick, J. 1965. Thermodynamics of micellization of N-alkylbetaines. *Trans. Faraday Soc.* **61**:1043

Nachbar, M.S., Winkler, W.J., Salton, M.R.J. 1972. The effect of aliphatic alcohols upon the dissociation of *Micrococcus lysodeikticus* membrane lipids and proteins. *Biochim. Biophys. Acta.* **274**:83

Ohnishi, S., Ito, T. 1974. Calcium-induced phase separations in phosphatidyl-serine-phosphatidyl-choline membranes. *Biochemistry* **13**:881

Paterson, S.J., Butler, K.W., Huang, P., Labelle, J., Smith, I.C.P., Schneider, H. 1972. The effects of alcohols on lipid bilayers: A spin label study. *Biochim. Biophys. Acta* **266**:597

Phillips, M.C. 1972. The physical state of phospholipids and cholesterol in monolayers, bilayers, and membranes. *Prog. Surface Membr. Sci.* **5**:139

Phillips, M.C., Williams, R.M., Chapman, D. 1969. On the nature of hydrocarbon chain motions in lipid liquid crystals. *Chem Phys. Lipids* **3**:234

Posner, A.M., Anderson, J.R., Alexander, A.E. 1952. The surface tension and surface potential of aqueous solutions of normal aliphatic alcohols. *J. Colloid Sci.* **7**:625

Reynolds, J.A. 1972. Are inorganic cations essential for the stability of biological membranes? *Ann. N.Y. Acad. Sci.* **195**:75

Rothman, J.E. 1973. The molecular basis of mesomorphic phase transitions in phospholipid systems. *J. Theoret. Biol.* **38**:1

Seelig, A., Seelig, J. 1974. The dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. *Biochemistry* **13**:4839

Seeman, P. 1972. The membrane actions of anesthetics and tranquillizers. *Pharmacol. Rev.* **24**:583

Sessa, G., Freer, J.H., Colacicco, G., Weissmann, G. 1969. Interaction of a lytic polypeptide, mellitin, with lipid membrane systems. *J. Biol. Chem.* **244**:3575

Shah, D.O., Schulman, J.H. 1965. Binding of metal ions to monolayers of lecithins, plasmalogen, cardiolipin, and dicetylphosphate. *J. Lipid Res.* **6**:341

Shah, D.O., Schulman, J.H. 1967. The ionic structure of lecithin monolayers. *J. Lipid Res.* **8**:227

Sheetz, M.P., Chan, S.I. 1972. Effect of sonication on the structure of lecithin bilayers. *Biochemistry* **11**:4573

Shimshick, E.J., McConnell, H.M. 1973. Lateral phase separation in phospholipid membranes. *Biochemistry* **12**:2351

Simon, S.A., Lis, L.J., Kauffman, J.W., MacDonald, R.C. 1975. A calorimetric and monolayer investigation of the influence of ions on the thermodynamic properties of phosphatidylcholine. *Biochim. Biophys. Acta* **375**:317

Smith, R., Tanford, C. 1973. Hydrophobicity of long chain *n*-alkyl carboxylic acids, as measured by their distribution between heptane and aqueous solution. *Proc. Nat. Acad. Sci. USA* **70**:289

Stowe, B.B., Dotts, M.A. 1971. The molecular length of effective lipids. *Plant Physiol.* **48**:559

Sullivan, K.H., Jain, M.K., Koch, A.L. 1974. Activation of the β-galactoside transport system in *Escherichia coli* ML-308 by *n*-alkanols: Modification of lipid-protein interaction by a change in bilayer fluidity. *Biochim. Biophys. Acta* **352**:288

Sundaralingam, M., Jensen, L.H. 1965. Crystal and molecular structure of a phospholipid component: L-α-glycerophosphorylcholine cadmium chloride trihydrate. *Science* **150**:1035

Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L., Thompson, T.E. 1976. A calorimetric and fluorescent probe study of the gel-liquid crystalline phase transition in small, single-lamellar dipalmitoylphosphatidylcholine vesicles. *Biochemistry* **15**:1393

Tanford, C. 1972. Hydrophobic free energy, micelle formation and the association of proteins with amphiphiles. *J. Mol. Biol.* **67**:59

Tinoco, J.D., Ghosh, A.D., Keith, A.D. 1972. Interaction of spin-labelled lipid molecules with natural lipids in monolayers at the air-water interface. *Biochim. Biophys. Acta* **274**:279

Tomkiewicz, M., Corker, G.A. 1975. Rotational correlation times and partition coefficients of a spin label solute in lecithin vesicles. *Biochim. Biophys. Acta* **406**:197

Trauble, H. 1971. Phase numwandlungen im lipiden magliche schaltprozesse im biologischen membranes. *Naturwissenschaften* **58**:277

Trauble, H., Eibl, H. 1974. Electrostatic effects on lipid phase transitions: Membrane structure and ionic environment. *Proc. Nat. Acad. Sci. USA* **71**:214

Verma, S.P., Wallach, D.F.H., Smith, I.C.P. 1974. The action of mellitin on phosphatide multibilayers as studied by infrared dichroism and spin labeling. A model approach to lipid-protein interactions. *Biochim. Biophys. Acta* **345**:129

Yemni, T., McCullough, R.L. 1973. Energetics of phase transformations in polyethylene. *J. Polymer Sci.* **11**:1385