Calorimetric Investigation of the Phase Partitioning of the Fluorescent Carbocyanine Probes in Phosphatidylcholine Bilayers†

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ABSTRACT: The partition behavior of fluorescent 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine (C_NDiI) probes in bilayers of various synthetic phosphatidylcholines was studied by differential scanning calorimetry. These studies were carried out at different probe concentrations on a series of different chain length C_NDiI's (even N = 10–22). All of the C_NDiI's lowered the phase transition onset temperatures of dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine bilayers. The melting point depressions and heats of transition varied systematically as a function of both probe length and lipid chain length. Since these probes alter the heats of transition, their partition between fluid and gel regions of the bilayer is not simply defined by the elevation or depression of bilayer melting point (ideal mixing). The range over which dilute solution theory can be applied to these systems is narrow. The behavior of the probes is best treated in terms of binary mixtures, considering both the onset and completion temperatures of the transition.

While lateral segregation in synthetic lipid membranes resulting in domains of different composition and physical properties has been demonstrated directly by a number of techniques [Shimshick & McConnell, 1973; Wu & McConnell, 1975; for review, see Melchior & Steim (1979)], evidence for the existence of domains in cell membranes has, until recently, been indirect (Melchior, 1982; Pessin & Glaser, 1980; Setlow et al., 1979; Bearer & Friend, 1982; Buhr et al., 1979; Wolf et al., 1981). Application of biophysical techniques to elucidate domain organization in biological membranes has been hampered by the need for methodologies of probing, not the whole membrane, but specific domains within it.

A number of spectroscopic techniques have been developed, which make use of the selective partition of amphiphiles either between the water phase and membrane or between domains within the membrane. Shimshick & McConnell (1973) and Wu & McConnell (1975) have used the partition of the spin probe Tempo[1] to demonstrate that many lipid mixtures in addition to exhibiting two coexistent solid phases, and a coexistent solid and fluid phase, can also exhibit two coexistent fluid phases. Sklar et al. (1979) have shown that the trans isomer of the fluorescent probe paranaric acid prefers solid phase, while the cis isomer prefers fluid phase. Klausner et al. (1980) demonstrated a similar preference for the cis and trans isomers of common fatty acids, and similar results were obtained by Pringle & Miller (1979) for 9-tetradecen-10-ol. Klausner & Wolf (1980) have shown that the partition of the 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanines (C_NDiI's)! (Figure 1) between the gel and fluid phases of disaturated lecithins is highly dependent upon the probe's alkyl chain length.

Some of these studies have utilized simple thermodynamic properties such as perturbations of phase transition temperature (T_m) to indicate phase partition preference. Klausner & Wolf (1980) on the basis of both changes in T_m and quenching due to self-association investigated the phase partitioning of the C_NDiI's in disaturated lecithin bilayers. They found that probes with acyl chains shorter than and much longer than those of the lipid preferentially partition into the fluid phase, while probes with chain lengths slightly longer than those of the lipid preferentially partition in the gel phase. This study and others involving amphiphile partition (Klausner & Wolf, 1980; Klausner et al., 1980; Pringle & Miller, 1979) did not take into account changes in phase transition enthalpy (ΔH) resulting from addition of the probe.

In the present study we have examined by differential scanning calorimetry (DSC) the effect of adding C_NDiI probes into dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) bilayers. These results have enabled us to examine the applicability of ideal solution theory to questions of probe partition within bilayers and have shed light on the interactions between C_NDiI's and phosphatidylcholines.

Materials and Methods

DMPC, DPPC, and DSPC were purchased from Sigma (St. Louis, MO) and stored in crystalline form at −20 °C. Lipid purity was verified to be 99+% by thin-layer chromatography and quantification with an Iatroscan TH-10 analyzer (Ackman, 1981). The C_NDiI's were prepared by Molecular Probes (Plano, TX) as perchlorates. Their structure is shown in Figure 1. Purity of the dyes was confirmed by thin-layer chromatography.

For sample preparation, the dyes were dissolved in absolute ethanol, and their concentration was determined spectroscopically (Sims et al., 1974). For preparation of liposomes, 50–500 μg of the appropriate lipid was placed in a tared calorimeter pan and the sample mass determined with a Cahn electrobalance to within 1 μg. The lipid was then dissolved in absolute ethanol containing the appropriate molar ratio of

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[1] Abbreviations: C_NDiI, 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine, previously referred to as 3,3′-diacylindocarbocyanines [see Klausner & Wolf (1980)]; T_m, transition onset temperature; T_m', transition completion temperature; ΔH, transition enthalpy; ΔQ, heat of transition; DSC, differential scanning calorimetry; PC, phosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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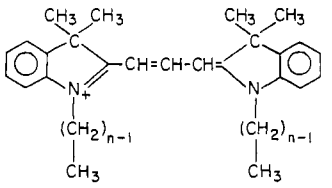

FIGURE 1: Structure of 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine.

C_NDiI and dried to a thin film. Samples containing no dye were dissolved in equivalent volumes of pure ethanol. The remaining solvent was removed from the samples under vacuum (100 μmHg) at room temperature overnight. So that the samples would be fully hydrated, 5 μL of H_2O was added to 15-μL sample pans, and the pans were hermetically sealed. The samples were repeatedly cycled above and below the lipid transition temperature at least 3 times to ensure full hydration. Full hydration of the bilayers was confirmed by reproducible, characteristic thermograms.

DSC measurements were made with a Du Pont 1090 thermal analyzer. The general methodology is similar to that described previously (Melchior et al., 1977). Peak areas, onset temperatures, and completion temperatures were calculated by using Du Pont's Advanced DSC VI.0 and General Utility Programs. Completion temperatures were corrected for finite scan rates by subtracting the transition width of the pure material and adding 0.25 °C (Mabrey & Sturtevant, 1976). Sample sizes ranged from 50 to 500 μg dry weight. The majority of the calorimetry was performed as upscans at 10 °C/min. All samples were prepared in duplicate, and at least two quantitative scans were performed on each sample. Additional scans performed at slower rates (5 °C/min and 1 °C/min) gave identical T_m's and ΔQ's. The calorimeter heats were calibrated with indium, and temperature was calibrated with benzene and naphthalene.

Results

C_{10}DiI and C_{18}DiI were incorporated at molar ratios of 0.005, 0.01, 0.03, 0.05, and 0.10 into lipid bilayers of approximately 50 μg of DMPC, DPPC, or DSPC. Figure 2 presents a series of thermograms showing changes in the phase transition of DPPC liposomes induced by incorporation of increasing mole percent C_{18}DiI. Increasing the dye in DPPC bilayers to 10 mol % lowers the onset temperature (T_m) from 40.7 to 39.8 °C, raises the completion temperature (T_m') from 44.3 to 44.5 °C, and increases the heat of transition from 36.7 to 43.5 kJ/mol. When the concentration of C_{18}DiI in DPPC bilayers was increased to an amount greater than 20 mol %, a shoulder appeared on the main transition, and a small peak was evident at approximately 54 °C. This most likely derives from C_{18}DiI saturation of the DPPC phase, leading to the formation of a separate C_{18}DiI-rich phase in the bilayer.

As seen in Figure 2, addition of C_{18}DiI to DPPC liposomes abolishes the pretransition at approximately 3 mol %. Loss of the pretransition was consistently observed for all C_NDiI's in all lipids studied. In contrast, the effect of C_NDiI incorporation on the T_m, T_m', and heat of transition was markedly dependent upon both the lipid and C_NDiI chain lengths.

Figure 3 shows changes in T_m and T_m' of DMPC, DPPC, and DSPC as a function of C_{10}DiI and C_{18}DiI concentration. It effectively represents the low concentration ends of PC–C_NDiI phase diagrams with T_m being the solidus line and T_m' the liquidus line. C_{10}DiI depresses T_m for all three disaturated phospholipids. The order of depression is DMPC > DPPC > DSPC. C_{18}DiI shows only a slight concentration effect on T_m for all three lipids.

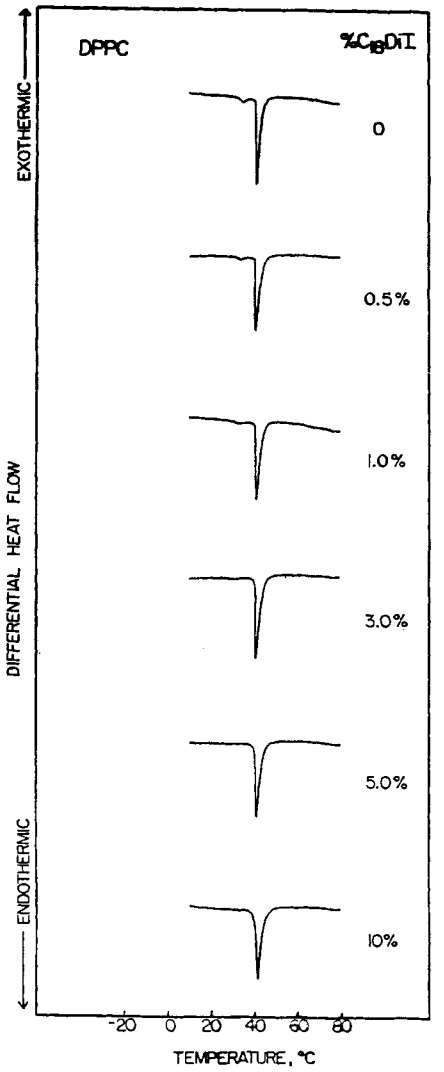

FIGURE 2: Thermograms of DPPC bilayers containing increasing amounts of C_{18}DiI. Thermograms have been normalized to sample mass.

Figure 4 shows the heats of the endothermic lipid phase transition (ΔQ), for each lipid as a function of C_{10}DiI and C_{18}DiI concentration. Heats for DMPC liposomes increase to a maximum at 1 mol % C_{10}DiI and then decrease. There is a slight increase in the heat of transition with increasing C_{10}DiI concentrations for DPPC, and a stronger increase for DSPC. In contrast, with increasing C_{18}DiI concentration, ΔQ increases in a similar fashion for all the phosphatidylcholines.

These concentration curves suggest that the behavior of C_NDiI's in bilayers is dependent upon their carbon chain length as well as the phospholipid into which they are incorporated. So that this behavior could be better defined, DSC measurements were made on samples containing each of the even chain length C_NDiI's (N = 10–22) incorporated into disaturated PC bilayers at a concentration of 4.76 mol %.

Figure 5 shows the change in T_m and T_m' in each of the three lipid bilayers brought about by the incorporations of 4.76 mol % of the even chain length C_NDiI's. All of the dyes lower T_m in each of the three lipids. The most severe depression in T_m occurs at C_NDiI chain lengths of two or more carbons less than the chain length of the lipid into which it is incorporated. T_m depression becomes minimal and levels off at C_NDiI chain lengths equal to or greater than those of the phospholipid. All of the dyes depress T_m' in DSPC and DPPC with the exception of C_{10}DiI which elevates T_m' in DPPC. In DMPC, all of the

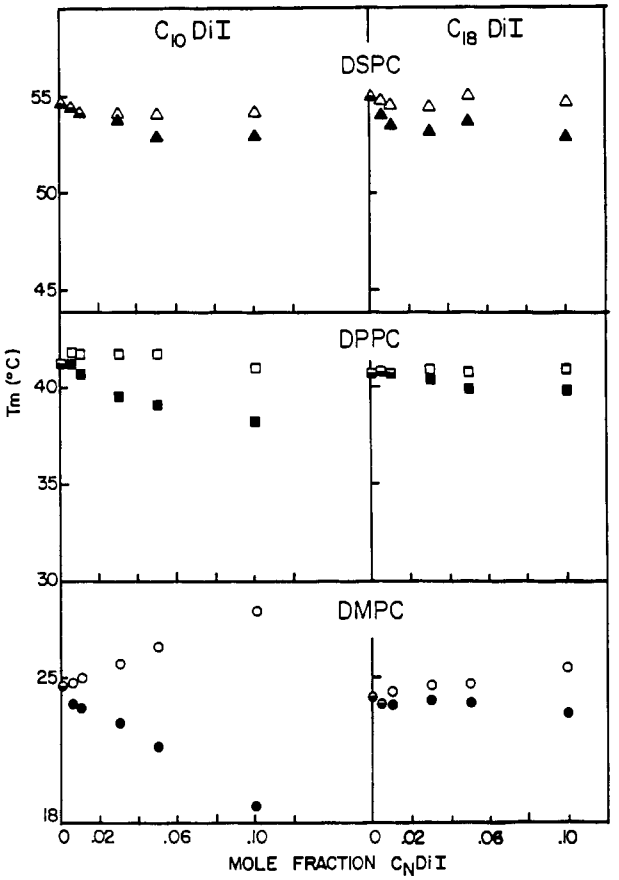

FIGURE 3: Effect of C_{10}DiI and C_{18}DiI on the phase transition onset (closed symbols) and completion (open symbols) temperatures of DMPC (○), DPPC (□), and DSPC (△) as a function of probe concentration. Uncertainties (SEM) are contained within the symbols.

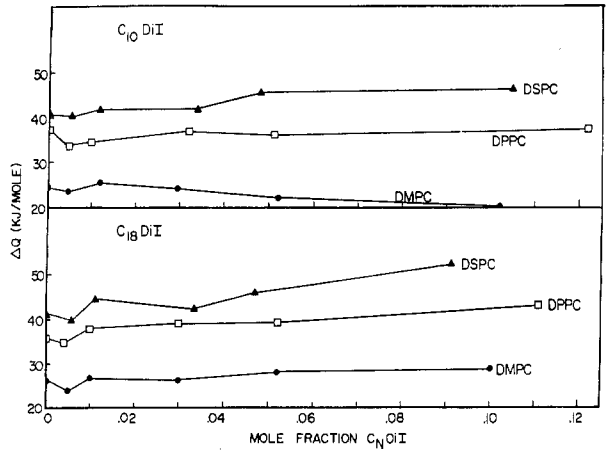

FIGURE 4: Effect of C_{10}DiI and C_{18}DiI on the heat of melt (ΔQ) of DMPC (●), DPPC (□), and DSPC (▲) as a function of probe concentration. Plotted is ΔQ vs. mole fraction of C_NDiI. Uncertainties (SEM) are contained within the symbols.

dyes elevate T_m' with the exception of C_{12}DiI which has no effect. The transition is narrowest for C_{16}DiI in DMPC, C_{18}DiI in DPPC, and for C_{10}-, C_{18}-, C_{20}-, and C_{22}DiI in DSPC.

Changes in heats of transition induced by 4.76 mol % of the dye are shown in Figure 6. For C_NDiI's with carbon chain lengths equal to or less than those of the phospholipid bilayer, ΔQ is independent of chain length. With further increase in chain length, ΔQ increases and then decreases to the previous level.

The major qualitative features of both the ΔQ and T_m vs. N plots are the same for the three phospholipids. With increasing phospholipid chain length, the general pattern shifts to longer C_NDiI chain length.

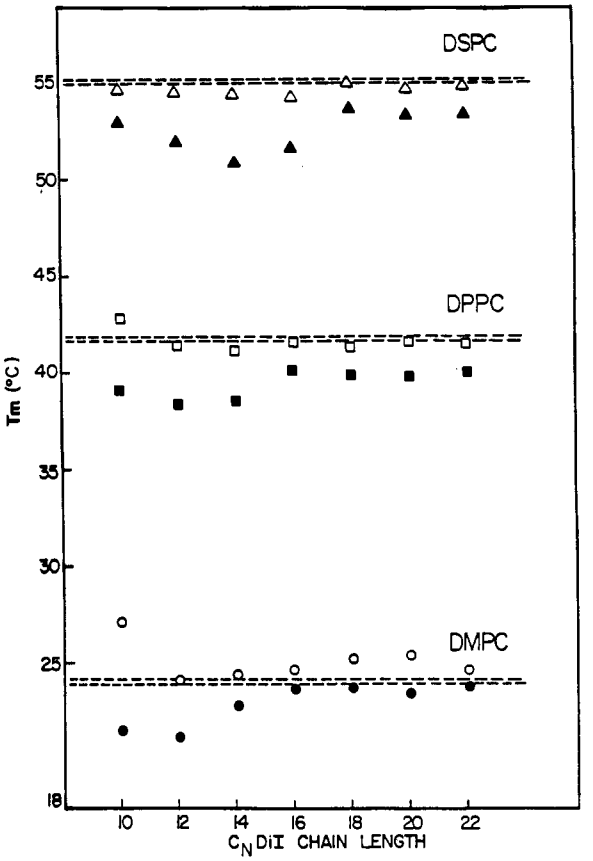

FIGURE 5: Melting onset temperature (T_m) (closed symbols) and completion temperature (T_m') (open symbols) as a function of C_NDiI chain length (N) in DMPC (○), DPPC (□), and DSPC (△) at a C_NDiI concentration of 4.76 mol %. Dashed lines represent T_m and T_m' for the main transition of the pure lipid. Uncertainties (SEM) are contained within the symbols.

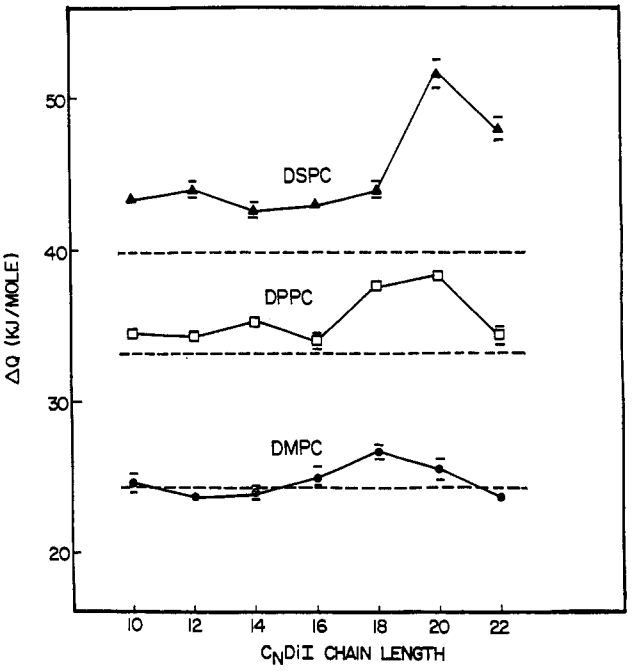

FIGURE 6: Heat of melt (ΔQ) as a function of C_NDiI chain length (N) in DMPC (●), DPPC (□), and DSPC (▲) at a C_NDiI concentration of 4.76 mol %. Dash lines represent ΔQ for the main transition of the pure lipid. Error bars represent ±1 SEM; where not shown, they are contained within the symbol.

Discussion

A number of groups have studied selective partition of spectroscopic amphiphiles between coexisting phases in lipid bilayers (Sklar et al., 1979; Klausner et al., 1980; Klausner & Wolf, 1980; Foster & Yguerabide, 1981; Pringle & Miller, 1979). Some of these studies have used depression or elevation of phase transition temperature as an indication of solubility preference. Klausner et al. (1980) have derived the following formula based on ideal solution theory to describe this process:

$$\Delta H^\circ(1/T - 1/T^\circ) = R \ln (X^g/X^f) \quad (1)$$

where ΔH° is the enthalpy of the pure lipid phase transition, T is the measured phase transition temperature, T° is the transition temperature without solute, and X^g and X^f are the mole fractions of lipid in the gel and fluid states, respectively. Investigators using this formula have assumed there is no change in ΔH in the presence of probe but have not used a thermodynamic technique such as calorimetry to verify this.

In the present study we have applied DSC to a system previously studied strictly by fluorescence. Clearly, our measurements show the assumption that C_NDiI does not alter enthalpy is an oversimplification. Mathematically, a simple treatment that allows for changes in enthalpy is to assume "regular", rather than ideal, solution behavior (Hildebrand et al., 1970). That is, we assume that the enthalpies of interaction are altered by addition of solute. The entropic effect of solute addition is assumed to be the same as in an ideal solution. Thus if S^g, S^f, $S^{g\circ}$, and $S^{f\circ}$ are the partial molar entropies of the gel and fluid states with and without solute, we may express the entropy difference for the two states due to the addition of solute as

$$S^g - S^{g\circ} = -R \ln X^g \quad (2)$$

for the gel and

$$S^f - S^{f\circ} = -R \ln X^f \quad (3)$$

for the fluid. Thus

$$(S_L{}^f - S_L{}^g) - (S_L{}^{f\circ} - S_L{}^{g\circ}) = R \ln (X^g/X^f) \quad (4)$$

This may be rewritten as

$$\Delta S - \Delta S^\circ = R \ln (X^g/X^f) \quad (5)$$

where ΔS and ΔS° are the change in entropy for the transition with and without solute. Therefore instead of (1) we obtain

$$\Delta H/T - \Delta H^\circ/T^\circ = R \ln X^g/X^f \quad (6)$$

We see that the enthalpic effects are likely to dominate partition behavior since T is expressed on an absolute temperature scale.

This second-order approximation attempts to take into account solvation effects. An alternative approach would be to introduce activity coefficients into the ln term. Our data indicate that the concentration range over which eq 6 is valid may be extremely narrow. For example, in Figure 3 with respect to transition onset we see linear relationships in C_{10}DiI temperature depression but not in C_{18}DiI. A practical problem in obtaining absolute partition coefficients from eq 6 is that fractional errors in the difference between $\Delta H/T$ and $\Delta H^\circ/T^\circ$ tend to be large.

The question of how to treat the pretransition introduces a further ambiguity into the absolute determination of $\Delta H/T - \Delta H^\circ/T^\circ$. Two endotherms occur in pure lecithin bilayers: the pretransition, in which the lipids are converted from an $L_{\beta'}$ to a $P_{\beta'}$ configuration, and the main transition, from $P_{\beta'}$ to an L_α configuration (Ranck et al., 1974). The $L_{\beta'}$ state is evidently disrupted by addition of the probe since the pretransition is abolished at probe concentrations of 3 mol % or greater. The question exists whether one should consider the pretransition to be part of a single bilayer transition from gel to fluid state. If one includes the enthalpy of the pretransition, then ΔH is less than ΔH° for all points but one in Figure 6. If the pretransition enthalpy is not included, then ΔH is greater than ΔH° for all points but two. One can readily see how inclusion or exclusion of pretransition enthalpy can completely reverse the effect as predicted by eq 6. In most calorimetric studies the pretransition is not considered part of the main transition. Inclusion of the pretransition would also necessitate determination of a weighted average transition temperature. It should be emphasized that inclusion or exclusion of the pretransition does not alter the shape of the curves, or the relative changes seen in Figures 3–6.

Each of the enthalpy curves in Figure 6 display three major regions. As an example consider the curve for DMPC. ΔQ is essentially constant in the first region N = 10–14, rises in the second region N = 16–18, and returns to the previous value in the third region N = 22. Relative to DMPC, the curve shifts to larger N by approximately two carbons for DPPC and four carbons for DSPC. We know from absorbance measurements (Klausner & Wolf, 1980) that unless the C_NDiI chain length is approximately the same as that of the lipid, the dye aggregates in the gel state of the lipid. Dispersion of these aggregated C_NDiI's would require an energy of solvation. Since C_NDiI's are positively charged, individual molecules might be expected to repel one another with the result that their heats of solvation would be exothermic. The observed endothermic heat of the lipid transition would thus be diminished in phospholipid bilayers containing aggregated probes (regions I and III) as compared to bilayers in which the probes do not aggregate in either state (region II). To further examine this hypothesis, it would be useful to study the effect of pH upon ΔQ. One would expect higher pH to decrease C_NDiI aggregation.

As discussed above, even regular solution theory (eq 6) does not adequately describe the thermodynamic behavior of C_NDiI PC mixtures. Such a treatment does not allow for the finite width of transition ($T_m' - T_m$). Rather than considering only the onset transition temperature, it is useful to also consider the completion temperature of the transition and to plot the data as phase diagrams for pseudo-binary mixtures of the C_NDiI's and PC's. Figure 3 shows the low C_NDiI concentration end of such phase diagrams for C_{10}- and C_{18}DiI in DMPC, DPPC, and DSPC. Certain of these phase diagrams suggest the existence of either eutectic points (see for instance C_{10}DiI in DSPC) or peritectic points (see for instance C_{10}DiI in DPPC) at low concentration (<1 mol %). This can only be considered suggestive since this is beyond the resolution of these measurements.

Information about the preference of the various C_NDiI's for the gel vs. fluid phase of a given PC can be obtained from Figure 5 by comparing the effect of 4.76 mol % of the C_NDiI's on the two ends of the transition. Probes which prefer the fluid phase can be expected to melt primarily with the first lipids to melt, thus lowering the onset of the transition. Probes which prefer the gel can be expected to melt primarily with the last lipids to melt, thus affecting the end of the transition (in either direction). Thus an understanding of preference can be obtained by comparing the effects of a given probe on T_m and T_m'. By these criteria all of the probes show systematic variation in gel to fluid preference in the three lipids. A large preference for gel over fluid, however, is only observed for C_{18}- and C_{20}DiI in DMPC. We suspect that the behavior of C_{10}DiI

is somewhat anomalous and are presently exploring this point.

In general terms these results agree with the fluorescence results of Klausner & Wolf (1981). As discussed above, the application of simple solution theories to the determination of phase partition in this system is limited. Partition is not sufficiently defined by the effect of solute upon transition temperature. Furthermore, use of fluorescence as a measure of the transition is complicated by the aggregation state and relative fluorescence quantium efficiency of the probe in the two phases as well as by the algorithm used to determine transition temperature. Calorimetric studies typically report T_m as defined here, while fluorescence studies define T_m as the temperature of half-effect (Klausner & Wolf, 1980). Figure 5 suggests that such a definition would considerably alter the dependence of of T_m upon N. Application of fluorescent amphiphiles as probes of domain organization requires a knowledge of both their absolute partition and the partition of fluorescence.

Registry No. DMPC, 18194-24-6; DPPC, 63-89-8; DSPC, 816-94-4; C_{10}DiI, 84109-06-8; C_{12}DiI, 84109-08-0; C_{14}DiI, 84109-10-4; C_{16}DiI, 84109-11-5; C_{18}DiI, 41085-99-8; C_{20}DiI, 84109-13-7; C_{22}DiI, 84109-15-9.

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Mapping of Hydrophobic Sites on the Surface of Myosin and Its Fragments†

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ABSTRACT: The interaction of the hydrophobic probe conjugated polyene fatty acid *cis*-parinaric acid (PA) with myosin and its fragments was studied by measuring the enhancement of PA fluorescence following binding. The measurements point to the presence of about 1.34 hydrophobic sites per mol of myosin or per mol of double-headed fragment heavy meromyosin and 0.65 site per mol of myosin subfragment 1 (S-1). The binding constants were all in the range of 10^7 M^{-1}. The S-1 isoenzyme containing alkali light chain 1 [S-1(A1)] bound strongly close to 1 mol of PA per mol of S-1(A1) while the isoenzyme containing alkali light chain 2 [S-1(A2)] bound no PA. Isolated alkali light chains A1 and A2 bound PA very weakly. Mg–nucleotide binding to the S-1 active site had little effect on the stoichiometry or affinity of PA binding; binding of actin to myosin carrying the probe either in solution or in an organized system of myofibrils had no effect on the magnitude of probe fluorescence but dramatically increased the rotational relaxation time of bound PA. These data indicated that parinaric acid bound to the hydrophobic pocket formed between the 41-residue region at the N-terminal end of A1 and the heavy chain of S-1 and that the hydrophobic pocket is distant from both actin and the nucleotide binding sites.

The question of the effective (surface) hydrophobicity of the contractile proteins has not received much attention in the past. Although many hydrophobic residues are buried in the interior of native proteins, some hydrophobic groups may remain exposed at the molecular surface or in crevices; in fact, the nucleotide binding site of myosin may be such a crevice. It is conceivable that some hydrophobic groups are involved in the hydrolysis of ATP and in the process of force development in muscle. For example, hydrophobic groups may have their accessibility to the solvent changed as a result of orientational alteration of the cross-bridge attitude believed to occur during muscle contraction (Huxley, 1969; Huxley & Simmons, 1971). Further, binding of substrate or change in the area of the

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