Fluorescent Probes in Model Membranes I: Anthroyl Fatty Acid Derivatives in Monolayers and Liposomes of Dipalmitoylphosphatidylcholine†

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ABSTRACT: A study of three fluorescent anthroyl probes has been carried out using pure and mixed monomolecular films with dipalmitoylphosphatidylcholine. In addition, fluorescence depolarization and differential scanning calorimetry data were obtained from dipalmitoylphosphatidylcholine vesicles with incorporated anthroyl probes. The three probes used were 2-(9-anthroyl)palmitic acid, 12-(9-anthroyl)stearic acid, and 16-(9-anthroyl)palmitic acid. The latter probe was synthesized for these studies. In monolayers the probes shifted the onset of the liquid-condensed/liquid-expanded monolayer phase transition with the extent of the shift decreasing in the order: 2-(9-anthroyl)palmitic acid > 12-(9-anthroyl)stearic acid > 16-(9-anthroyl)stearic acid. A corresponding decrease in the gel–liquid crystalline bilayer transition temperature (T_c) showed the same order of perturbation in both the fluorescence depolarization and differential scanning calorimetry data. Locating the anthroyl entity in the center of the bilayer would appear to provide a minimum perturbation.

In a recent article, a comparison was made of the pure monomolecular films of two membrane probes, one a spin-label probe (12-nitroxide stearic acid, 12-NS)[1] and one a fluorescent probe (12-(9-anthroyl)stearic acid, 12-AS), with their "parent" stearic acid (Cadenhead et al., 1975). It was shown that the substitution of either the oxazolidine ring or the anthroyl group at the 12-position led to a significant increase in the area occupied per molecule, but the smaller oxazolidine ring had a much greater effect. These data were interpreted to mean that the shift in behavior from that of the "parent" molecule was dependent, not only on the size, but also on the nature (polar or nonpolar) of the substituted group.

Previously it was shown that the behavior of pure films of fatty acid spin-label probes was very much dependent on the location of the oxazolidine ring on the alkane chain (Cadenhead and Müller-Landau, 1975). It was also demonstrated that a full understanding of a probe's ability to perturb its environment required the study of a series of mixed films of the probe and a suitable host lipid (Cadenhead and Müller-Landau, 1976). Here we will demonstrate the effect of shifting the anthroyl group along the fatty acid alkane chain and evaluate the data from the mixed film systems: 2-(9-anthroyl)palmitic acid (2-AP)/dipalmitoylphosphatidylcholine (DPPC), 12-AS/DPPC; and 16-(9-anthroyl)palmitic acid (16-AP)/DPPC. In addition, the results of fluorescence depolarization and DSC studies on DPPC multilamellar vesicles (liposomes) with incorporated anthroyl probes will be correlated with the monolayer data. Previous fluorescence polarization studies were carried out with 12-AS in oxidized cholesterol oriented spherical bilayers (Yguerabide and Stryer, 1971), with 12-AS in egg-lecithin multibilayers (Badley et al., 1973), with 12-AS in DPPC sonicated liposomes (Vanderkooi et al., 1974) and with 12-AS and 2-AP in sonicated DPPC liposomes (Bashford et al., 1976). The results obtained here demonstrate that all three probes perturb both the monolayer and the bilayer packing, but that the extent of the perturbation decreases as the anthroyl moiety is moved from the 2- to the 16-position.

Experimental Section

Materials

DPPC for the monolayer studies was purchased from Applied Science Laboratories (99+% pure) and was used without further purification after being checked for purity using thin-layer chromatography (TLC) and by measuring the gel–liquid crystalline transition (T_c) using differential scanning calorimetry (DSC). An even better check was the presence and sharpness of the liquid-expanded/liquid-condensed and liquid-condensed/solid-condensed phase changes in the monomolecular film below the T_c (Cadenhead and Kellner, 1974). DPPC for the fluorescence polarization and DSC studies on liposomes was synthesized as previously described (Papahadjopoulos et al., 1973). Both 12-(9-anthroyl)stearic acid (12-AS) and 2-(9-anthroyl)palmitic acid (2-AP) were supplied by Dr. R. A. Badley of Unilever Research, Bedford, England. TLC, DSC, and melting point checks showed the 12-AS to be better than 99% pure. The 2-AP was recrystallized from hexane before use.

The melting points of 12-AS and 2-AP were respectively 78.5 (Waggoner and Stryer, 1970, reported 78–79 °C) and 91–92 °C. The latter showed a small premelt.

The new fluorescent probe, 16-(9-anthroyl)palmitic acid (16-AP), was synthesized from 16-hydroxypalmitic acid (Aldrich Chemical Co., Milwaukee, Wis.), trifluoroacetic anhydride (Matheson Coleman and Bell, Norwood, Ohio), and

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[1] Abbreviations used are: DPPC, dipalmitoylphospatidylcholine; 2-AP, 2-(9-anthroyl)palmitic acid; 12-AS, 12-(9-anthroyl)stearic acid; 16-AP, 16-(9-anthroyl)palmitic acid; T_c, phase transition midpoint temperature for aqueous dispersions and for monomolecular films; 12-NS, 12-nitroxide stearic acid; TLC, thin-layer chromatography; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; CPK, Corey-Pauling-Koltun; π_T, surface pressure at which the liquid-expanded/liquid-condensed phase transition initiates in monolayers; ΔP, the magnitude of the change in polarization.

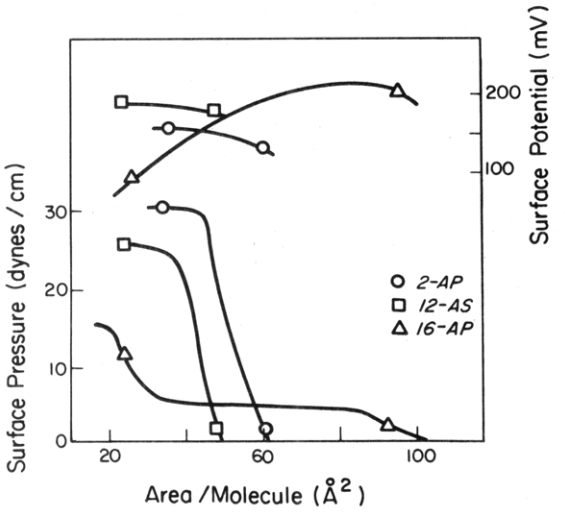

FIGURE 1: Surface pressures (lower curves) and surface potentials (upper curves) of 2-(9-anthroyl)palmitic acid, 12-(9-anthroyl)stearic acid, and 16-(9-anthroyl)palmitic acid, as a function of area/molecule at 23 °C. Data were recorded continuously; symbols are for identification only.

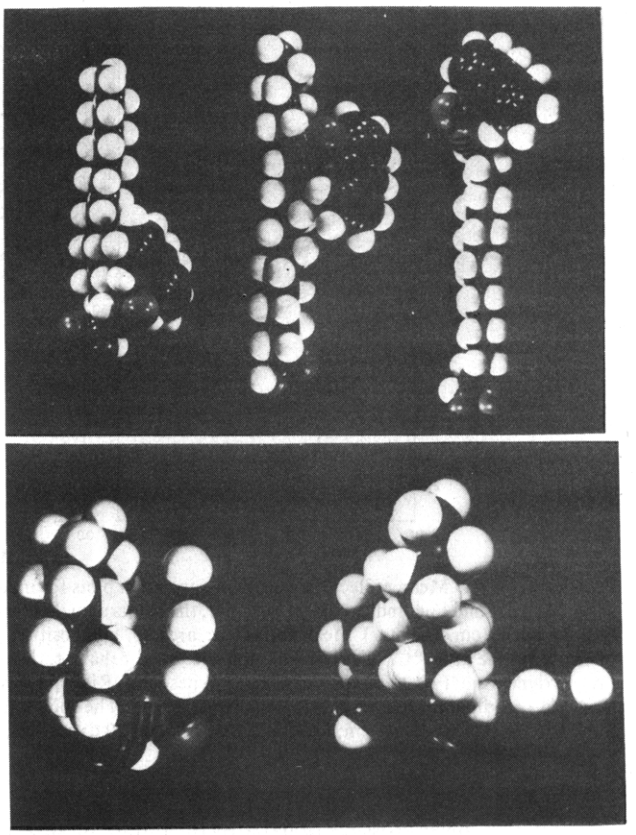

FIGURE 2: (Top) CPK models of (left to right) 2-(9-anthroyl)palmitic acid, 12-(9-anthroyl)stearic acid, and 16-(9-anthroyl)palmitic acid. All three molecules are represented in an erect or close-packed conformation. (Bottom) CPK models of 16-(9-anthroyl)palmitic acid (left) and 12-(9-anthroyl)stearic acid (right), in a bent or bipolar conformation.

anthracene-9-carboxylic acid (Aldrich Chemical Co.) using the method of Parrish and Stock (1965), essentially as modified by Lenard et al. (1974). The product was recrystallized several times from hexane and was identified by means of its NMR spectrum, ultraviolet spectrum, as well as its mass spectrum (Kellner, 1977). 16-AP had a melting point of 57–58 °C, again with a small premelt.

Water for aqueous substrates for the monomolecular films was twice distilled from glass, initially from alkaline permanganate, subsequently from dilute sulfuric acid. It was then twice distilled from quartz before use. The films were spread using either hexane–ethanol (10:1 molar ratio) or Freon-TE (Cadenhead and Kellner, 1974) as the solvents. All spreading solvents were checked for impurities by spreading pure solvent, allowing time for evaporation, and then compressing. No discernable surface pressure changes were observed, indicating an absence of surface active contaminants.

Procedures and Apparatus

(a) *Monolayer Studies.* The automated Wilhelmy film balance which simultaneously and continuously records surface pressure and surface potential as a function of area/film molecule has been adequately described elsewhere (Cadenhead, 1969), as have film handling techniques. Further details may be obtained from B. M. J. Kellner's Ph.D. thesis (Kellner, 1977). At a given area/molecule, surface pressures were determined within ±0.5 dynes/cm, while surface potentials were within ±5.0 mV.

(b) *Phospholipid Vesicle Preparation.* Liposomes were prepared as previously described (Bangham et al., 1965; Papahadjopoulos, 1970). A chloroform solution of the purified DPPC, or the appropriate DPPC–probe mixture, was evaporated under vacuum in a glass tube previously flushed with high purity nitrogen (Linde H. P. Dry 99.996%). The dry lipids were suspended in buffers containing 100 mM NaCl, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), 2 mM L-histidine, and 0.1 mM EDTA by mechanical shaking on a Vortex mixer under nitrogen at 42 °C.

(c) *Fluorescence Measurements.* Vesicles were labeled in the manner previously described (Jacobson and Wobschall, 1974; Papahadjopoulos et al., 1973). Suspensions were equilibrated at temperatures above the T_c for at least an hour and then slowly cooled to room temperature before the upward temperature scan. Fluorescence polarization measurements were made as described previously (Jacobson and Wobschall, 1974; Jacobson and Papahadjopoulos, 1975).

(d) *Differential Scanning Calorimetry.* The transition temperatures (T_c) of the pure and mixed phosphatidylcholine dispersions were determined with a differential scanning calorimeter (Perkin-Elmer DSC-2) calibrated with indium. Suspensions were centrifuged at $10^5 g$ for 30 min at 25 °C and the wet pellets transferred to the calorimeter pans. After cooling to room temperature, the samples were heated with a heating rate of 5 °C/min. Transition enthalpies (ΔH) were evaluated by measuring the area under the excess specific heat curve by paper weighing. Phospholipid was determined directly in the sample by phosphate assay after calorimetry. All experiments were repeated at least twice.

Results and Discussion

(1) *Monolayer Experiments: (a) Pure Films of Fluorescent Probes.* The results of the film studies (surface pressure and surface potential as a function of area/molecule) are shown in Figure 1. The findings may best be interpreted by means of the molecular CPK models shown in Figures 2 (top and bottom). The surface pressure and surface potential data for 12-AS are identical with those previously published (Cadenhead et al., 1975). The areas/molecule around 40 Å^2 are consistent with the conformation of 12-AS shown in Figure 2 (top) with the anthroyl group parallel to the alkane chain. The inflection, which begins at about 20 dynes/cm, indicates the onset of a gradual collapse. In contrast, the collapse of the "parent" stearic acid molecule occurs at higher pressures and is more abrupt. The lower collapse pressure for 12-AS is con-

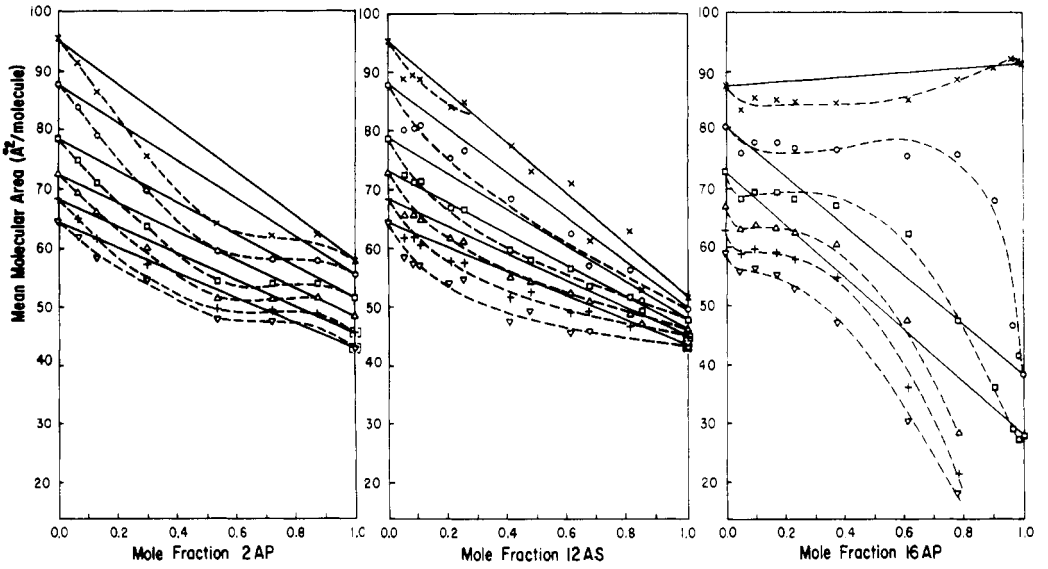

FIGURE 3: (Left) Mean molecular area vs. composition plots for mixed films of 2-(9-anthroyl)palmitic acid and L-α-dipalmitoylphosphatidylcholine at 44 °C. The solid lines indicate ideal behavior, the points actual observed data: (X) 2 dynes/cm; (□) 10 dynes/cm; (△) 15 dynes/cm; (+) 20 dynes/cm; (▽) 25 dynes/cm. (Center) Mean molecular area vs. composition plots for mixed films of 12-(9-anthroyl)stearic acid and L-α-dipalmitoylphosphatidylcholine at 44 °C. The solid lines indicate ideal behavior, the points actual observed data: (X) 2 dynes/cm; (○) 5 dynes/cm; (□) 10 dynes/cm; (△) 15 dynes/cm; (+) 20 dynes/cm; (▽) 25 dynes/cm. (Right) mean molecular area vs. composition plots for mixed films of 16-(9-anthroyl)palmitic acid and L-α-dipalmitoylphosphatidylcholine at 39 °C. The solid lines indicate ideal behavior, the points actual observed data: (X) 2 dynes/cm; (○) 5 dynes/cm; (□) 10 dynes/cm; (△) 15 dynes/cm; (+) 20 dynes/cm; (▽) 25 dynes/cm.

sistent with a lower cross-sectional area of contact of adjacent molecules (that of the anthroyl group). The approach to infinite compressibility as collapse takes over indicates that the collapse phase has a liquid-like nature (Cadenhead, 1969) though less so than that of 12-NS (Cadenhead and Müller-Landau, 1974). The surface potential values are only slightly different from those of stearic acid (Cadenhead et al., 1975), indicating that the ester linkage makes little contribution to the vertical component of the dipole moment.

With 2-AP the isotherm is similar to that of 12-AS, though it is somewhat more expanded and the film does not collapse until about 30 dynes/cm. We had anticipated that the presence of the anthracene group might destabilize the anchoring ability of the carboxyl group. As may be seen from Figure 2 (top), however, it is possible to satisfy the hydrophilic affinities of both the carboxyl and the anthroyl ester group, while packing the anthracene above the water interface. The result is that 2-AP forms a film which is even more stable then 12-AS. However, because of poorer packing of the anthracene and the slightly shorter alkane chain, the film is somewhat more expanded. The slightly lower surface potential of 2-AP reflects the effect of the altered polar head group less the accommodation of the aqueous substrate.

It is interesting to note that, although 12-AS and 2-AP each possess two polar groups, both behave as monopolar compounds; that is the isotherms indicate that only one polar group is immersed in the aqueous substrate. From Figure 2 (top), the explanation for 2-AP is obvious: the carboxyl and ester groups are located so closely together they behave as one. It is not clear, however, why 12-AS behaves as a monopolar compound and why in contrast 16-AP behaves as a bipolar substance. Thus, the 16-AP isotherm (Figure 1) "lifts-off" above 100 Å^2/molecule then undergoes a sharp inflection followed by a short condensed region at about 25 Å^2/molecule and a collapse at 15 dynes/cm. Likewise, the surface potential behavior (Figure 1) of 16-AP differs considerably from that of the other two anthroyl probes. Initially at a higher value, the potential begins to decrease as the film is compressed, dropping to about half the original value, behavior similar to that of β-estradiol diacetate (Cadenhead and Phillips, 1967), another bipolar compound.

The most reasonable explanation for all of this is that, at high areas/molecule, both the carboxyl and the anthroyl ester groups of 16-AP are immersed in the aqueous substrate. As the film is compressed, the ester anthroyl group (the less polar) is forced out of the water, erecting the molecule to the conformation shown in Figure 2 (top). The lack of a linear portion in the condensed region of the isotherm, the low collapse pressure, and the failure of the surface potential to cease decreasing with compression all indicate that the erect conformation has limited stability.

In Figure 2 (bottom) a bent or bipolar conformation (both polar groups in the aqueous substrate) is shown for 16-AP. If we attempt to achieve a similar conformation for 12-AS, we are forced to locate the anthracene moiety in the aqueous interface. As Figure 2 (bottom) shows, this may be avoided with 16-AP. What this means is that, although 12-AS may have both polar groups in the aqueous substrate at very low pressures (<0.1 dyne/cm), such a conformation is unstable over the pressure range displayed in Figure 1 and 12-AS behaves essentially as a monopolar compound.

(b) *Mixed Films with DPPC.* Data from the three mixed systems 2-AP, 12-AS, and 16-AP with DPPC are represented in Figure 3 as plots of mean molecular area as a function of composition. These plots are constructed from the original isotherms by recording, at selected pressures, the mean molecular area [total area/total number of mixed molecules] for each isotherm corresponding to a number of compositions ranging from pure probe to pure DPPC. Should the two components of the system form either an ideal mixture, or be completely immiscible, the mean molecular area plot will be a straight line connecting the data points for the two pure components. Miscibility and nonideal behavior are indicated by deviations from this straight line. The mean molecular area plots were constructed for a number of selected surface pressures (2–25 dynes/cm) at approximately 40 °C. At 40 °C, the

isotherm for DPPC is fully (liquid) expanded over the pressure range considered, and complications due to a DPPC liquid-expanded/liquid-condensed phase change are avoided.

Isotherms were also obtained at 23 °C where a well-pronounced phase change occurs for pure DPPC. The plot shown in Figure 4 illustrates the shift in the onset of the DPPC liquid-condensed/liquid-expanded phase change (π_T) as a function of probe concentration at low probe concentrations for all three probe molecules.

A better understanding of the data in Figures 3 and 4 may be obtained by considering the schematic shown in Figure 5 where the process of mixing of the two components is shown in two separate steps: insertion and accommodation. Stage 1 represents the two pure separate films of the two components. Stage 2 shows an ideal mixture, i.e., an intimate molecular mixture of the two components with both occupying the same area/molecule they occupied in stage 1. Stage 3 shows the final accommodated state in which either or both of the components may occupy a greater or smaller area/molecule than in stage 1.

It is obvious that the phase change shift is a measure of the differences between the pure DPPC in stage 1 and the final mixed film in stage 3. In other words, the phase change shift (see Figure 4) is a measure of the "net perturbation" (insertion plus accommodation) produced by the probe. In contrast, the mean molecular area plots (Figure 3) compare the ideal mixed state (stage 2) with the final state (stage 3) and therefore give a measure of the accommodation only.

Interpretation of Figure 4 is now relatively simple. All three probes eventually shift the π_T to higher surface pressures. Initially the shifts for all three are small, with those for the 2-AP and the 12-AS being positive while that for 16-AP is essentially zero. Eventually (at ~0.2 mol fraction of probe) we find a small positive shift for 16-AP, a much larger one for 12-AS, while the DPPC phase change has been completely wiped out by 2-AP. The order of increasing perturbation is therefore 2-AP > 12-AS > 16-AP. A shift of π_T to higher pressures further stabilizes the expanded film. A similar shift for DPPC may be produced by raising the film temperature (Phillips and Chapman, 1968; Hui et al., 1975). At 0.05 mol fraction probe (1:19, probe:host lipid) the $\Delta\pi$ values were approximately 4, 3, and 1 dynes/cm for 2-AP, 12-AS, and 16-AP, respectively. The temperature shifts required for an equivalent effect would be of the order of 3, 2, and less than 1 °C. At 0.20 mol fraction probe (1:4, probe:host lipid) the equivalent temperature shifts would be much larger: greater than 18 °C (2-AP), approximately 13 °C (12-AS), and approximately 3 °C (16-AP) (Figure 4).

Turning to the mean molecular plots in Figure 3, we find that, for 2-AP and 12-AS, the DPPC mixed systems exhibit negative deviations from ideality at almost all concentrations and surface pressures. In contrast, the 16-AP/DPPC system exhibits negative deviations only below 5 dynes/cm; otherwise near ideal behavior is obtained at low probe concentrations with positive deviations at most higher values. The switch from mainly negative to mainly positive deviations at about 4 dynes/cm may clearly be attributed to the phase change at this pressure for pure 16-AP. Below the phase change, 16-AP is in a bipolar configuration and negative deviations below 4 dynes/cm arise through the erection of the probe by DPPC. Above 4 dynes/cm, 16-AP is already erect in the pure state and the mean molecular area plot with DPPC shows mainly positive deviations.

In Figure 3 ideal behavior for 16-AP is indicated by solid lines only for surface pressures below 15 dynes/cm. The reason for this is that there are no data for pure 16-AP above this

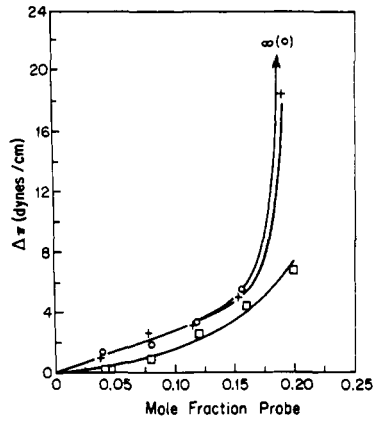

FIGURE 4: The shift in pressure ($\Delta\pi$) of the liquid expanded-liquid condensed phase transition of DPPC at low probe concentrations, as a function of probe concentration at 23 °C: (○) 2-AP; (+) 12-AS; and (□) 16-AP.

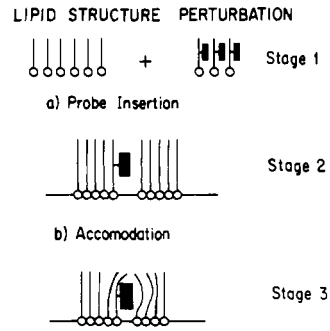

FIGURE 5: Schematic representation of a host lipid structure perturbation by a fluorescent probe molecule. For details, see text.

pressure (Figure 1). One possible solution to obtaining such a value would be to extrapolate the condensed region of the 16-AP isotherm. However, extrapolation of the experimental 15, 20, and 25 dynes/cm mean molecular area plots indicate a zero area/molecule for 16-AP. This suggests that the probe is squeezed out of such high concentration films at high pressures. Using this zero extrapolated value, the behavior at 15–25 dynes/cm and low probe concentrations of 16-AP is essentially ideal. At 25 dynes/cm and low probe concentrations both 2-AP and 12-AS show negative deviations. In other words, the mixed systems of DPPC with these latter two probes show accommodation.

Summarizing the pertinent information from Figures 3 through 4, we see that the net perturbation decreases from 2-AP to 12-AS to 16-AP. For 2-AP and 12-AS, however, the perturbation, due to the physical insertion of the probe in the monolayer, is partially offset by an accommodation of the system. For 16-AP, which produces the least net perturbation, there is also the least accommodation. All of this indicates that locating the anthroyl group at the end of the alkane chain has the least effect on the physical state of the host lipid.

(2) *Bilayer Studies:* (a) *Differential Scanning Calorimetry.* The differential scanning calorimetry data are illustrated in Figure 6 and analyzed in Table I. The obvious trends are that the observed T_c and ΔH show a decrease as the anthroyl group is moved toward the carboxyl group. Simultaneously, the half-width of the peak increases. With the 2-AP/DPPC system, there is an indication that partial separation of two mixtures is occurring (Figure 6). It should be noted, however, that within experimental error the 2-AP/DPPC and 12-AS/DPPC systems have similar ΔH values. This could arise because of

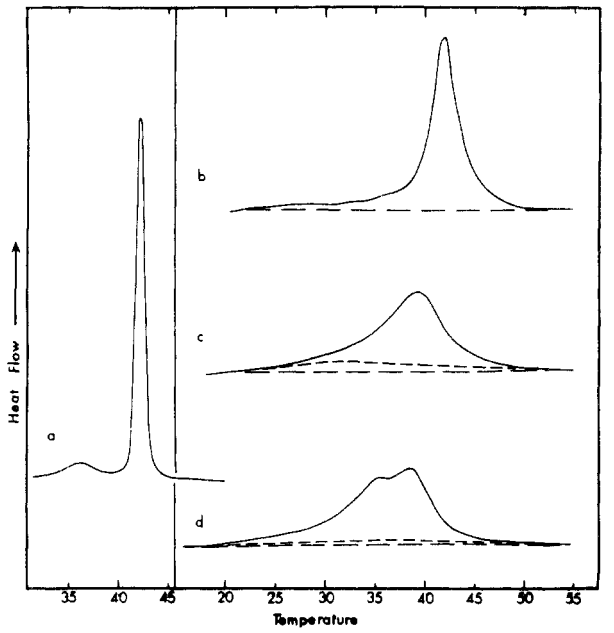

FIGURE 6: Differential scanning calorimetry thermograms for pure DPPC, 20 mol % 16-AP in DPPC, 20 mol % 12-AS in DPPC and 20 mol % 16-AP in DPPC. The ordinate for pure DPPC is reduced by a factor of two. The abscissa markers are in °C.

TABLE I: Differential Scanning Calorimetry Data for Hydrated Dispersions of DPPC and DPPC–Probe Mixtures.

System	T (°C)[a]	Transition width (°C)[b]	ΔH (kcal/mol)[c]
DPPC + 20 mol % 2-AP	37	~25	7.3 (6.7–8.1)
DPPC + 20 mol % 12-AS	39.3	~25	6.5 (5.5–7.4)
DPPC + 20 mol % 16-AP	41.6	~9.5	9.0 (8.9–9.1)
Pure DPPC	42.3	3.6	9.4 ± 0.3

[a] Transition midpoint temperatures reproducible to 0.2 °C on replicate scans. T_c for DPPC + 20 mol % 2-AP is the mean of two peaks located at 35.2 and 38.8 °C. [b] Transition width as defined (Jacobson and Papahadjopoulos, 1975). For DPPC + 20 mol % 2-AP, width is based on entire double peak. [c] ΔH values are means of duplicate scans and are based on the molar amount of DPPC present as determined by phosphate assay. ΔH for pure DPPC includes major and minor transitions. For the DPPC–probe mixtures, ΔH is the average of the two numbers given in parentheses. This range represents the uncertainty in defining the baseline under the peak and estimating the onset and completion temperatures. Our choice of the two extreme cases is shown diagramatically by the broken lines in Figure 6 (curves c and d). It should be noted that the final value of ΔH for the probe–DPPC mixtures could be an overestimate since the probes themselves may participate in the thermotropic transition.

a partial segregation in the 2-AP/DPPC system reducing the probe's perturbing capability. Alternatively, or in addition, the longer alkane chain of 12-AS may increase its perturbing ability in an environment composed of PC with C_{16} chains (Phillips et al., 1970). Note that the minor transition is abolished (or obscured by the broadened main transition) consistent with the effect of other long chain amphipaths on DPPC melting such as myristic acid or lysolecithin (Papahadjopoulos et al., 1976). The latter two compounds, however, also produce an increase in the T_c, a result very different from that reported here for the anthroyl probes.

The smaller values of ΔH found for the mixed (20%) probe/DPPC liposomes probably reflect a more disordered gel state than in pure DPPC, an effect produced by the incorporated probe. Further, if we consider that the breadth of the transition reflects the size of the cooperative melting unit (Hinz and Sturtevant, 1972), the increasing width of transition as the anthroyl group is moved up the acyl chain toward the head group indicates that the probe moiety is more effective at reducing the cooperative unit when placed close to the head group where the acyl chains are most closely packed.

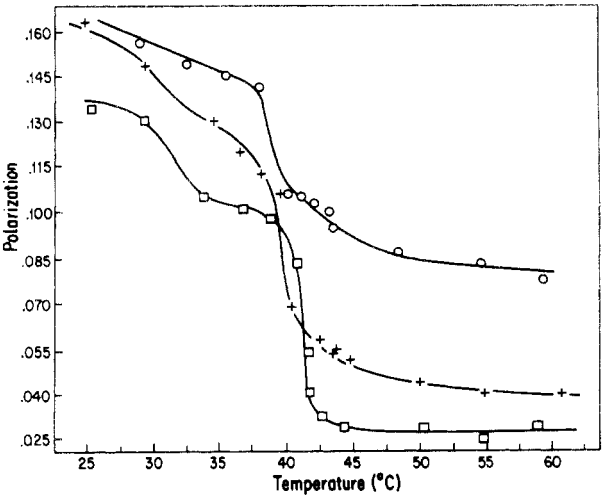

FIGURE 7: Fluorescence polarization vs. temperature for multilamellar vesicles of DPPC with the three incorporated anthroyl probes. Probe: DPPC 1/500; [DPPC] = 0.5 μmol/mL; excitation wavelength was 365 nm (12-nm bandwidth); (○) 2-AP; (X) 12-AS; (□) 16-AP.

We may compare the effects of these probes on both monolayer and bilayer. The temperature shifts for the bilayer from Table I are approximately 5, 3, and less than 1 °C for 2-AP, 12-AS, and 16-AP, respectively. For these temperature shifts from Hui et al. (1975), the corresponding $\Delta\pi$ monolayer shifts should be 12, 5, and 2 dynes/cm. As may be seen from Figure 4 similar shifts may be obtained at about 0.17 to 0.18 mol fraction probe rather than the 0.2 mol fraction used with the bilayer. It is to be noted that there is a reasonable semiquantitative correspondence between the monolayer and bilayer. Unlike the following fluorescence depolarization–monolayer data comparison, *both* the monolayer phase change shift and bilayer DSC T_c shift depend on an average property of the lipid aggregate. The failure to achieve an exact correspondence is hardly surprising considering the fact that the energetics of the monolayer and bilayer are not identical.

(b) *Fluorescence Depolarization.* The results of the fluorescence depolarization studies on unsonicated DPPC vesicles with 0.20% anthroyl probe are shown in Figure 7. All three probes can detect the main DPPC phase transition which shifts from 39 °C (2-AP) to 40 °C (12-AS) to 41.5 °C (16-AP). The corresponding polarization changes during the main phase transition are 0.037 (2-AP), 0.047 (12-AS), and 0.066 (16-AP). The data also suggest that 12-AS and 16-AP report the pretransition at about 32 °C; it is not clear whether 2-AP can report on this transition since the relatively poor precision of the 2-AP data, as determined by duplicate experiments, for $T < T_c$ may have obscured the pretransition. Typical values of the DPPC gel–liquid crystalline transition, as detected using DSC, range from 41.5 to 42.5 °C with the pretransition at about 30–37 °C depending on the physical technique employed (Jacobson and Papahadjopoulos, 1975).

By several criteria these data indicate that an anthroyl group near the bilayer midplane causes less perturbation than one near the polar interface. From the above data we see that, while all three probes detect the main transition, there would appear to be a perturbation, as judged from the change in T_c, which

increases going from 16-AP to 12-AS to 2-AP, the same order found for the mixed monolayer systems.

Polarization, for $T > T_c$ and also for $T < T_c$, is the highest for 2-AP, intermediate for 12-AS, and lowest for 16-AP. From the Perrin equation, the polarization will be inversely related to the rotational mobility of the probe assuming lifetime to be constant (Weber, 1953).[2]

Thus, this result indicates that the rotational freedom of the probe moiety is greatest for 16-AP and least for 2-AP. These mobility results can be correlated with earlier spin label results (Hubbell and McConnell, 1971), yielding a decreasing order parameter as the spin label is moved along the acyl chain toward the bilayer midplane. It should be noted that mobility (time rate of change of position) is not simply related to ordering in the system (Seelig and Seelig, 1974).

Since various nonperturbing magnetic resonance methods indicate that considerable order exists up to near the terminal methyl group (Seelig and Niederberger, 1974), it is reasonable to conclude that anthroyl probes at the 2- and 12-positions perturb their microenvironment to a greater extent than at the 16-position in analogy to the perturbation exerted by spin labels (Seelig and Seelig, 1974).

The magnitude of the change in polarization (ΔP) at the T_c reflects how the microenvironment of the probe is altered on melting of the host lipid. The greatest change is sensed by 16-AP, while the smallest change is detected by 2-AP. Considering the massive structural change during the phase transition, we conclude that the microenvironment of 16-AP is the least perturbed since the probe in the 16 position undergoes the largest change in polarization at the T_c.

All the fluorescence data listed above are for unsonicated vesicles. Exploratory studies with sonicated vesicles showed that sonication produces a further small lowering in the observed T_c and a broadening of the main transition. Sonication also effectively eliminates the pretransition. These observations help explain small differences between our results and those recently published by Bashford et al. (1976) and are not inconsistent with recent studies on sonicated and multilamellar vesicles using the fluorescent probe, 1,6-diphenylhexatriene (Lentz et al., 1976). Our polarization values below the T_c are, however, somewhat low when compared with those obtained with 1,6-diphenylhexatriene. They are nevertheless consistent with those obtained by others using anthracene derivatives (Vanderkooi et al., 1974; Bashford et al., 1976). Thus, it is likely that some rotational motion is retained below the T_c and that anthracene derivatives perturb the bilayer, even in the gel state.

Although the mole fractions of probe used for the fluorescence studies would not be expected to alter the pure DPPC thermotropic behavior as judged from earlier work with perylene (Jacobson and Papahadjopoulos, 1975), several correlations can be made between the calorimetric data and fluorescence polarization data. The former technique provides information on the probe-induced perturbations of the bulk membrane properties while the latter method gives information on the local environment of the probe which is perturbed by its presence. It should be pointed out that the relationship between the probe's perturbation of its microenvironment and the alteration in the bulk thermotropic properties caused by the probe is not necessarily simple. For example, while the probe may tend to "fluidize" their own microenvironment, this effect may not be propagated to all points through the bilayer; in fact, the presence of 12-AS has been reported to inhibit the acyl chain motion of DPPC molecules between the 12- and 16-positions in fluid DPPC bilayers (Podo and Blasie, 1977).

The magnitudes of the perturbations due to the "impurity" effects of the three probes, as judged by the depressions in T_c, are in the same order, whether one studies the perturbation on individual microenvironments by polarization or the cumulative effect of many of these perturbations on the melting of the bulk membranes. Further, the enthalpy of transition (ΔH) gives a thermodynamic measure of the difference in structure between the gel and liquid crystalline phases. The change in polarization at T_c (ΔP) provides a measure of the change in rotational freedom of the probe allowed by its microenvironment when the host lipid undergoes a phase transition. A comparison of the relative magnitudes of ΔH and ΔP reveals a reasonable correlation between the two methods and suggests that the structural differences between the gel and fluid phase are progressively diminished by the presence of the probe, as it is moved toward the polar interface.

It should be noted that probes such as the anthroyl fatty acids obviate one serious difficulty with apolar fluorescence probes such as perylene and diphenylhexatriene: these latter probes probably occupy more than one site in bilayer membranes (Chen et al., 1977; Veatch and Stryer, 1977), whereas the location of the anthroyl moieties with respect to the width of the membrane would appear to be well defined. However, the probe group may exhibit anisotropic rotational motions within the site as suggested by the data of Vanderkooi et al. (1974).

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[2] P_0, the limiting polarization of the probes, measured in glycerol solutions at 0 °C, was about 0.33 for all three anthroyl derivatives. This is consistent with an angle of 30° between the absorption and transition moments of 12-AS as measured by Badley et al. (1973).

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