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PERTURBATIONS TO LIPID BILAYERS BY SPECTROSCOPIC PROBES AS DETERMINED BY DIELECTRIC MEASUREMENTS

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Summary

Dielectric measurements on lecithin/cholesterol bimolecular lipid membranes have indicated that the series of extrinsic fluorescent probe molecules, the *n*-(9-anthroyloxy) fatty acids, cause significant perturbation to the bilayer structure at concentrations equivalent to those used in fluorescence experiments (0.1 mol%). Perturbations were observed in the capacitance and conductance of the electrically distinct substructural regions of the bilayer that were consistent with the putative location of the probe molecules. Inclusion of stearic acid decreased the thickness of the hydrocarbon region of the membrane, presumably by expanding the average surface area per unit membrane mass, and also significantly disrupted the surface regions. The attachment of the anthroyloxy moiety to position 2 of a fatty acid accentuated both these effects. Attachment at position 12 had the reverse effect by increasing the volume of the hydrocarbon region without further disturbance of the surface organisation. The 9-positioned probe had an intermediate effect. The degree of perturbation by the 2-positioned probe was dependent on the probe concentration within the range (probe:lipid) 1:1000 to 1:10 000. The technique therefore detects perturbation of structure at probe levels which are lower than those commonly used in fluorescence-labelling experiments.

Introduction

Spectroscopic probes incorporated into membranes have been widely used to report structural changes occurring during cellular processes (transformation,

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differentiation [1,2], and to determine basic properties of the lipid bilayer such as the polarity and viscosity of micro-environments [3] and the lateral diffusion of lipid components [4]. Of particular interest is the series of *n*-(9-anthroyloxy) fatty acid probes which position a fluorophore at graded depths into the lipid bilayer [5—7]. The anthroyloxy fluorophore is a bulky group, of which the chemical structure is quite foreign to the lipid bilayer, and the question naturally arises as to what extent these probes modify or perturb the membrane system.

Such disturbances have been detected at high probe:lipid ratios. Podo and Blasie [8] employed pulse Fourier-transform-HNMR techniques to show dynamic perturbation, not necessarily corresponding to the location of the fluorophore, of 2-(9-anthroyloxy)palmitic acid and 12-(9-anthroyloxy)stearic acid in the bilayer structure. Cadenhead et al. [9] found that the probes shifted the onset of the lipid condensed-liquid-to-expanded-monolayer phase transition, the extent of the shift being in the order 2-(9-anthroyloxy)palmitic acid > 12-(9-anthroyloxy)stearic acid > 16-(9-anthroyloxy)palmitic acid. The degree to which these probes decreased the phase transition temperature of dipalmitoyl phosphatidylcholine liposomes as determined by differential scanning calorimetry was in the same order.

At the low probe:lipid ratios normally used in fluorescence experiments the extent of perturbation is far less certain. In the range (probe:lipid) of 1:150 to 1:500 no shift was seen in the phase transition temperature of dipalmitoyl phosphatidylcholine liposomes for 2-(9-anthroyloxy)palmitic acid, 6-(9-anthroyloxy)stearic acid, 12-(9-anthroyloxy)stearic acid and 16-(9-anthroyloxy)-palmitic acid [5], nor did these probes alter the permeability of liposomes towards glucose [10]. However, the detection of perturbation depends not only on the probe:lipid ratio but also on the sensitivity of the method employed.

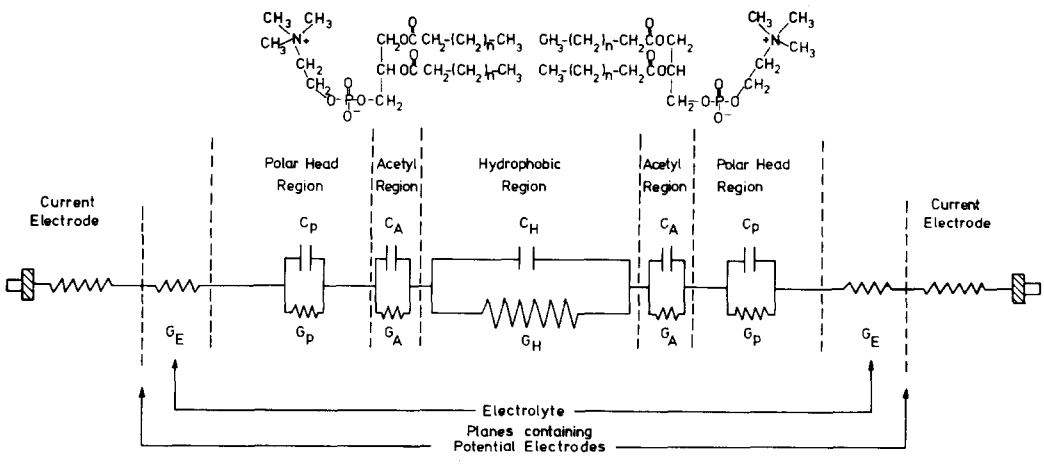

Fig. 1. The equivalent circuit for the bilayer. Each substructural layer is represented by an equivalent parallel capacitance and conductance. The subscripts are as follows: H, hydrocarbon; A, acetyl; P, polar head; E, bulk electrolyte.

In this report, we examine the use of low-frequency dielectric measurements to determine the perturbation of planar bilayer lipid membranes by such spectroscopic probes. This technique measures the frequency dependence of membrane capacitance, C, and conductance, G, over the frequency range 0.01—250 Hz [11]. The presence of regions of different dielectric properties within the membrane leads to a Maxwell-Wagner dispersion in C and G. By fitting the measured dispersions to an equivalent circuit containing a parallel C-G network for each substructural region, as depicted in Fig. 1, the dielectric parameters of these regions can be obtained [12]. The dispersions found for lecithin and lecithin/cholesterol bilayers are consistent with there being (a) a hydrophobic region associated with the phospholipid acyl chains, (b) a polar head region associated with the choline-phosphate dipoles, and (c) an intermediate, acetyl, region associated with the linkage of the fatty acids to the glycerol backbone (Ref. 13 and Ashcroft, R.G., Coster, H.G.L. and Smith, J.R., unpublished data). These regions are denoted by subscripts H, P and A, respectively.

Measurement of these substructural parameters (C and G for each region) in the presence of spectroscopic probes can indicate the extent of disruptions to the basic bilayer structure and the possible location of the perturbing group. To this end, the effects on egg lecithin/cholesterol bilayers of three n-(9-anthroyloxy) fatty acid probes (2-(9-anthroyloxy)palmatic acid, 9-(9-anthroyloxy)-stearic acid, 12-(9-anthroyloxy) stearic acid) and of the simpler molecules, stearic acid and methyl-9-anthroate, were investigated at probe:lipid ratios of 1:10000 to 1:100. Additionally, one ESR probe, 12-nitroxystearic acid, was included in this study for comparison with 12-(9-anthroyloxy)stearic acid.

Materials and Methods

Egg lecithin was obtained from Lipid Products, U.K. Cholesterol (BDH Chemicals) was recrystallized three times from ethanol. Stearic acid (Judex Chemicals) was recrystallized from petroleum ether (60—80°C fraction) and then twice from acetone. The synthesis of the n-(9-anthroyloxy) fatty acids has been described previously [5]. 12-Nitroxystearic acid was from Syva Corp., CA. Other reagents were of the highest grade available.

The bilayers were formed by painting the egg phosphatidylcholine/cholesterol mixture (molar ratio 5:1) dissolved in n-decane or n-tetradecane across a 1.5 mm diameter aperture in a polycarbonate septum separating the two compartments containing the current and voltage electrodes immersed in the aqueous solution, 1 mM KCl. Spectroscopic probes were added to the lecithin/cholesterol mixture before forming the membrane. The thinning of the film was observed optically and by monitoring the capacitance at 1 Hz, over several hours. All bilayers used were at least 4 h old ensuring that, for n-tetradecane, solvent expulsion had produced equilibrium concentrations of the alkane in the bilayer (some solvent would still be present in the membranes). At this stage, the rates of change in capacitance and conductance, measured at 1 Hz, were less than 1%/h.

Details of the four terminal digital impedance measuring apparatus have been given previously [11,14]. All measurements were made at 18—22°C. Mea-

surements of equivalent circuits of resistors and capacitors have demonstrated that this technique has the requisite accuracy to resolve the properties of the three distinct substructural regions (Ref. 13 and Ashcroft, R.G., Coster, H.G.L. and Smith, J.R., unpublished data). Once the impedance of the bilayer had settled, a series of frequency scans, each involving 25—30 measurements of C and G in the frequency range 0.03—220 Hz, were made until the bilayer broke. Data from the final three or four scans were averaged and the standard errors calculated. The data were fitted to the equivalent circuit (Fig. 1) by a nonlinear least-squares computer procedure. The best fit was that set of model parameters which (a) gave values less than unity for the reduced χ^2 statistics for the measured impedance and phase, and (b) gave the minimum value of the variance of the impedance fit. This data-fitting routine returned the substructural values of C and G for each region which were then used to generate the theoretical plots shown as smooth curves in Figs. 2—4. The capacitance, C, of each substructural region is related to its thickness, δ, by :

$$C = \frac{\epsilon_r \epsilon_0 A}{\delta} \tag{1}$$

where A is the area of the membrance, ϵ_r is the dielectric constant of the substructural region and ϵ_0 is the permittivity of free space.

Results

In all cases, the addition of spectroscopic probes appeared to improve the mechanical stability of the bilayers. The average values of substructural parameters for lecithin/cholesterol membranes are shown in Table I, together with the standard errors for seven membranes. These errors serve to indicate the significance of the changes induced by probes described in the following experi-

TABLE I

THE DIELECTRIC PROPERTIES OF EGG LECITHIN/CHOLESTEROL BILAYERS (MOLE RATIO 5:1) FOR THE FIVE-LAYER MODEL DEPICTED IN FIG. 1

Membranes were formed from lipids dissolved in *n*-tetradecane and data were obtained from fitting 30 frequency scans (0.01—220 Hz) on seven membranes. The C and G values in the table are the average values of each fit parameter ± S.D. KCl concentration was 1 mM.

Property	Region		
	Hydrocarbon	Acetyl	Polar-head
Capacitance (mF/m^2)	5.66 ± 0.09 C_H	195 ± 15 C_A	600 ± 20 C_p
Conductance (mS/m^2)	0.20 ± 0.09 G_H	210 ± 15 G_A	29 000 ± 3000 G_p
Dielectric constant *	2.13	10—40	10—40
Calculated thickness (nm)	3.3	~0.5—2	~0.15—0.6

* These values of the dielectric constant are arrived at on the assumption that each region of the membrane has a dielectric constant similar to those of comparable non-aqueous liquid phases. The value for ϵ_H has been widely used (e.g., see Refs. 12 and 15).

TABLE II

PROPERTIES OF MEMBRANES FORMED FROM LIPIDS IN *n*-DECANE AND *n*-TETRADECANE (EGG LECITHIN/CHOLESTEROL MEMBRANES; MOLE RATIO 5:1)

12-AS, 12-(9-anthroyloxy)stearic acid.

Membrane	Time (h)	Scans	Substructural parameters					
			Capacitance (mF/m^2)			Conductance (mS/m^2)		
			C_H	C_P	C_A	G_H	G_P	G_A
Lecithin/cholesterol from *n*-tetradecane	4	30	5.66	600	195	0.2	29 000	210
Lecithin/cholesterol from *n*-decane	4	4	4.0	360	84	0.8	22 000	96
	24	4	4.6	940	110	1.0	44 000	140
Lecithin/cholesterol from *n*-decane 1 mol% 12-AS	4	3	3.0	540	840	0.01	62 000	7800
	16	3	3.2	620	300	0.01	73 000	140

ments. An example of the C and G dispersion for a probe-free membrane is included in Fig. 2.

The effect of the alkane solvent

A comparison of the substructural parameters for membranes formed in *n*-decane and *n*-tetradecane is shown in Table II. The effect of *n*-decane was chiefly to decrease C_H and therefore to increase the thickness of the hydropho-

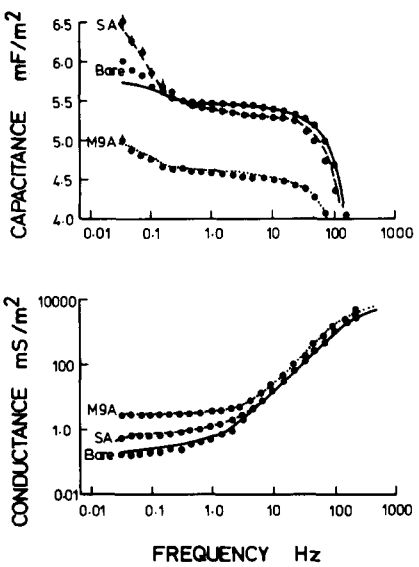

Fig. 2. Capacitance and conductance data and theoretical plots for lecithin/cholesterol membranes (mole ratio 5:1) prepared using *n*-tetradecane as the solvent. SA, stearic acid (two scans averaged); Bare, normal membrane (four scans averaged); M-9A, methyl-9-anthroate (four scans averaged). The probes were at 1 mol%. The parameters used to generate the theoretical curves are presented in Table III. The standard error in each C and G measurement is shown as a vertical bar, though, in most cases, it is obscured by the larger datum symbol.

bic region. Not only was *n*-decane retained in the bilayer to a far greater extent but it also drained more slowly and therefore extended the thinning period. Such retention of *n*-decane has also been documented by Fettiplace et al. [15] and White [16]. *n*-Decane also had some effect on the substructural parameters for the polar and acetyl regions of the membrane. The inclusion of 12-(9-anthroyloxy)stearic acid at 1 mol% further reduced the extent and rate of drainage of *n*-decane (Table II). Similar effects were seen for stearic acid, methyl-9-anthroate and 2-(9-anthroyloxy)palmitic acid at the same concentration. These results indicate that at 1% concentrations, these probe molecules certainly affect the bilayer in at least the formative stages. Because of the problems of retained solvent and a slow drainage rate, the remaining experiments were carried out using *n*-tetradecane as the solvent, with a thinning period of at least 4 h.

The effects of probe molecules

Stearic acid and methyl-9-anthroate were used to determine the effects of the substituent parts of the anthroyloxy fatty acid probes on the substructural parameters. Fig. 2 shows the dispersions in C and G for both these additives and Table III lists the derived substructural parameters. Stearic acid caused a particularly large dispersion between 0.03 and 0.3 Hz. In addition, methyl-9-anthroate caused a 25% decrease in the mid-range value of the capacitance found for probe-free bilayers.

The inclusion of any of the *n*-(9-anthroyloxy) fatty acids at 0.1 mol% caused substantial changes in membrane capacitance, and in the case of 2-(9-anthroyloxy)palmitic acid, in the form of the dispersion at low frequencies (Fig. 3). The forms of the dispersion of C and G for 9-(9-anthroyloxy)stearic acid and 12-(9-anthroyloxy)stearic acid were similar but the overall capacitance of the bilayer containing 12-(9-anthroyloxy)stearic acid was about 15% smaller than that of a normal membrane. On the other hand, the bilayer con-

TABLE III

EFFECTS OF VARIOUS PROBES ON THE SUBSTRUCTURAL PARAMETERS OF LECITHIN/CHOLESTEROL MEMBRANES (MOLE RATIO 5:1) MADE WITH *n*-TETRADECANE AS THE SOLVENT

Measurements were commenced at least 4 h after membrane formation. SA, stearic acid; M-9A, methyl-9-anthroate; 2-AP, 2-(9-anthroyloxy)palmitic acid; 9-AS, 9-(9-anthroyloxy)stearic acid; 12-AS, 12-(9-anthroyloxy)stearic acid; 12-NS, 12-nitroxystearic acid.

Probe (mol%)	Number of scans averaged	Substructural parameters					
		Capacitance (mF/m^2)			Conductance (mS/m^2)		
		C_H	C_P	C_A	G_H	G_P	G_A
Control	30	5.66	600	195	0.2	29 000	210
SA (1.0)	2	6.9	300	55	0.5	7 100	32
M-9A (1.0)	4	8.0	280	220	2.7	28 000	16
2-AP (0.1)	3	7.5	280	90	0.05	5 600	60
9-AS (0.1)	2	7.0	580	120	5.4	36 000	190
12-AS (0.1)	3	5.2	840	225	12.0	20 000	270
12-NS (0.1)	4	5.2	340	280	0.1	22 000	780

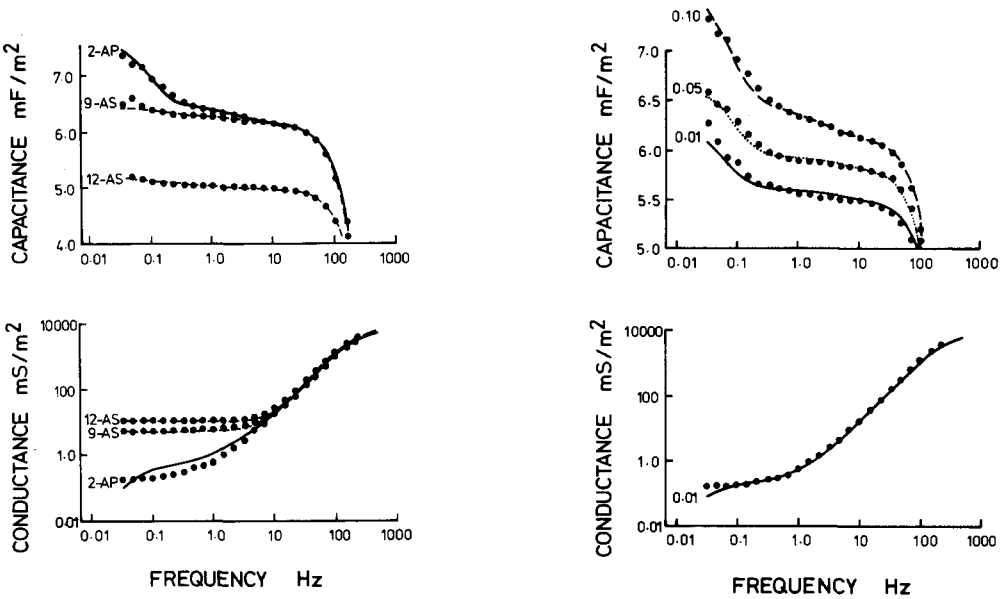

Fig. 3. The effects of inclusion of probe molecules at 0.1 mol% on the capacitance and conductance of lecithin/cholesterol membranes (mole ratio 5:1) prepared using *n*-tetradecane as the solvent. 2-AP, 2-(9-anthroyloxy)palmitic acid (three scans averaged); 9-AS, 9-(9-anthroyloxy)stearic acid (two scans averaged); 12-AS, 12-(9-anthroyloxy)stearic acid (three scans averaged). The parameters used to generate the theoretical curves are listed in Table III.

Fig. 4. Effects of the concentration of the probe, 2-(9-anthroyloxy)palmitic acid, on the capacitance and conductance of lecithin/cholesterol membranes (mole ratio 5:1). Probe concentrations (mol%) were 0.1, 0.05, 0.01. Three scans were averaged for each membrane. The parameters used in generating the theoretical curves are listed in Table IV. For clarity, only the conductance data and theoretical curve for a bilayer with 0.01% probe present are shown, as both the data and theoretical conductances at the three concentrations studied are very similar.

taining 9-(9-anthroyloxy)stearic acid had an overall capacitance about 20% higher than that of the control membrane. The relevant dielectric parameters are listed in Table III. The capacitance of the hydrophobic region (C_H) progressively decreased as the anthroyloxy group was moved further along the acyl chain and hence deeper into the membrane. The capacitance of the polar (C_P) and acetyl (C_A) regions progressively increased during this process.

Comparison of fluorescent and spin-labelled fatty acids

The capacitance dispersions for membranes containing 12-(9-anthroyloxy)-stearic acid and the ESR probe, 12-nitroxystearic acid, were similar as indicated by the similarity of the substructural capacitance values listed in Table III. The main difference between these two additives occurred in the conductance at low frequencies (less than 10 Hz).

Effects of probe concentration

2-(9-Anthroyloxy)palmitic acid was incorporated into bilayers at three different probe concentrations, viz., 0.01, 0.05 and 0.1 mol%. The dispersions in C and G are depicted in Fig. 4 and the derived substructural parameters are listed in Table IV. The progressive disturbance of membrane structure can be seen

TABLE IV

EFFECTS OF VARIOUS CONCENTRATIONS OF 2-(9-ANTHROYLOXY)PALMITIC ACID (2-AP) ON THE SUBSTRUCTURAL PARAMETERS OF LECITHIN/CHOLESTEROL MEMBRANES (MOLE RATIO 5:1) MADE WITH *n*-TETRADECANE AS THE SOVLENT

Concentration of 2-AP probe (mol%)	Number of scans averaged	Substructural parameters					
		Capacitance (mF/m^2)			Conductance (mS/m^2)		
		C_H	C_P	C_A	G_H	G_P	G_A
0	30	5.66	600	195	0.2	29 000	210
0.01	3	6.2	600	110	0.04	16 000	50
0.05	3	6.6	560	110	0.01	24 000	65
0.10	3	7.5	280	90	0.05	5 600	60

by the shifting of the low-frequency capacitance to higher levels. Similarly, progressive changes in the C and G parameters are seen at all substructural levels. However, the conductance of the hydrophobic region (G_H) is little affected.

Discussion

We direct attention first to the major changes seen in the capacitance of the hydrophobic region of the membrane (C_H). Stearic acid, 2-(9-anthroyloxy)-palmitic acid and 9-(9-anthroyloxy)stearic acid each cause C_H to increase by 20—30%. It is very unlikely that this is due to increase in the dielectric constant (ϵ_H) of this region. Measurements of polarity-sensitive fluorescence parameters of the anthroyloxy fatty acids (lifetime, quantum yield) show that the dielectric constant of this region is little different from that of a pure hydrocarbon [3]. Thus, the increase in C_H is caused by a decrease in the thickness (δ_H) of the hydrocarbon region according to Eq. 1. This could result from an increase in the surface area per unit membrane mass and an associated decrease in membrane width. The occurrence of such perturbations at the surface is reflected in the changes in the capacitance for the polar (C_p) and acetyl (C_A) regions of the membrane (Table III) although the precise forms of these changes are difficult to interpret on a molecular basis. Clearly, stearic acid itself, by virtue of its carboxyl group located at the membrane surface, is capable of decreasing δ_H via a change in surface organization of the bilayer. Thus, the probe molecules can be expected to cause a similar reduction in δ_H due to interactions at the carboxyl group alone. The additional effect of the anthroyloxy group would be expected to depend on its location along the acyl chain. If located near the bilayer surface it should cause an additional increase in the average area per unit membrane mass and thereby reduce δ_H still further. Its position here should also disturb the polar and acetyl regions. Indeed, the results for 2-(9-anthroyloxy)palmitic acid show a large decrease in δ_H and substantial changes in the dielectric parameters of the polar and acetyl regions. The results for 9-(9-anthroyloxy)stearic acid show only a slight disturbance of the polar and acetyl region parameters and a value of δ_H equal to that found for stearic acid alone, but at 1 mol%.

When the reporter group is located deeper in the bilayer as for 12-(9-anthroyloxy)stearic acid and 12-nitroxystearic acid, bulk is added to the region, the thickness should increase, and C_H should decrease as is indeed observed (Table III). The similarity of the decrease in C_H for 12-(9-anthroyloxy)-stearic acid and 12-nitroxystearic acid (approx. 8%) indicates that the nitroxyl and anthroyloxy groups can be accommodated equally well in this region, presumably due to the fluidity of the phospholipid environment. It is also possible that these 12-nitroxystearic acid and 12-(9-anthroyloxy)stearic acid results, to some degree, simply reflect an enhanced equilibrium concentration of solvent in the bilayer. Table III indicates that 12-(9-anthroyloxy)stearic acid disturbed the polar and acetyl region parameters to a lesser degree than 12-nitroxystearic acid.

Methyl-9-anthroate partitions into the centre of the lipid bilayer [5] and the small size of the esterified component ($-CH_3$) may permit water molecules to be associated with the ester bond and to be carried into the bilayer and thus increase the effective dielectric constant of the hydrophobic region, ϵ_H. The C_H and G_H changes are not simply due to an increase in ϵ_H, since the increase in G_H, although 10-fold, is very much smaller than expected for a 40% increase in ϵ_H. The dependence of perturbation on probe concentration is demonstrated clearly by the data for 2-(9-anthroyloxy)palmitic acid in Fig. 4 and Table IV. Perturbation is detectable down to probe:lipid ratios of 1:10 000. The large changes in C_A, G_P and G_A indicate that the fluorophore of 2-(9-anthroyloxy)-palmitic acid dominates the dielectric and conductive properties in these regions. The changes in C_A suggest that the acetyl region is thicker or that there is a decrease in the dielectric constant of this region (or both). The change in C_P at 0.1 mol% but not at the lower levels shows that a certain threshold level must be reached before this parameter is detectably altered.

This study shows that perturbation can be detected down to very low probe: lipid ratios. At these levels, perturbation by 12-(9-anthroyloxy)stearic acid is probably no more severe than that seen for stearic acid and 12-nitroxystearic acid. The probe:lipid ratios referred to in the above studies are those pertaining to the solution used to form the membrane, and the actual ratios in the thinned membranes may be different. Thus, extrapolation of these data to studies involving liposomes and natural membranes must be approached with caution. It is clear, however, that the perturbation becomes more severe as the anthroyl-oxy group is moved closer to the surface. It is significant that 2-(9-anthroyl-oxy)palmitic acid causes lysis of human erythrocytes and lymphocytes in a concentration-dependent manner whereas the 6, 9, 12 and 16 derivatives have no effect [10, 17] indicating that maintenance of lipid-lipid and lipid-protein interactions at the membrane surface is extremely important in maintaining the integrity of the cell. The effects on lecithin/cholesterol membranes of some added reagents, e.g., benzyl alcohol, are strongly dependent on KCl concentration [13,18]. It may be that the effects of these fluorescent probes are different, at physiological salt concentrations, from the effects at 1 mM KCl described here. Some membrane phenomena are unaffected by low concentrations of spectroscopic probes (e.g., phase transition temperature), and a judicious choice of probe and probe concentration may frequently be sufficient to ensure that perturbation is minimized.

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