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ELECTROSTATIC INTERACTIONS IN THE BINDING OF FLUORESCENT PROBES TO LIPID MEMBRANES

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SUMMARY

1. The electrostatic interactions involved in the binding of 1-anilinonaphthalene-8-sulphonate (ANS) to several phosphatide liposomes have been examined by varying the surface charge, *i.e.* by lowering the pH, by adding divalent cations, or by including charged components or cholesterol in the liposome. In all cases a more negative surface charge inhibited binding.

2. The binding of the neutral dye, *N*-phenyl-1-naphthylamine, was not affected by the divalent cations or the lowered pH but was inhibited by the addition of cholesterol suggesting that direct competition between cholesterol and these probes may occur.

3. The order of effectiveness of the divalent cations in facilitating ANS binding suggests that they chelate to the lipid phosphate groups. This order is shown to be the same as that in which they facilitate the action of phospholipase C.

4. The action of phospholipase C on sphingomyelin was shown to be strongly dependent on temperature and this dependence as well as that of the binding of ANS to sphingomyelin are consistent with a phase transition in this lipid beginning at 30 °C.

INTRODUCTION

The quantum yield and emission maximum of 1-anilinonaphthalene-8-sulphonate (ANS) are increased and blue shifted respectively when this dye is transferred from a polar to non-polar environment[1]. A similar blue shift and fluorescence enhancement is observed when ANS is added to solutions of several proteins[1,2] and to suspensions of lipids and membrane extracts[3–7]. It was initially thought that the change in the fluorescence characteristics of ANS on binding to a biological macromolecule could be related to the non-polar character of the binding site[2] but more recent work has suggested that the fluorescence spectra of ANS are too complex for this to be so[8,9]. Even though ANS may not be a good hydrophobic probe, however, its binding to membranes appears to be strongly dependent on the surface charge of the membrane and consequently it may prove to be a useful probe for indicating changes in surface potential.

Rubalcava *et al.*[7] have shown that Ca^{2+} enhances the binding of ANS to ery-

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonate.

throcyte ghosts. Vanderkooi and Martonosi[10], Gomperts *et al.*[4] and Träuble[11] have all shown that the enhancement of ANS fluorescence in the presence of simple liposomes or microsomal suspensions is increased by the addition of mono-, di- or trivalent cations. In all cases the fluorescence enhancement reaches a plateau as the cation concentration is increased and the concentration of cation needed to reach this plateau falls as the ionic charge increases. The results of Vanderkooi and Martonosi, using egg lecithin and dipalmitoyllecithin liposomal suspensions, imply that the maximum enhancement achieved with increasing cation concentration is identical for all ions of a given valency, whereas those of Träuble, using dipalmitoyllecithin, indicate that for constant charge the maximum enhancement increases as the ionic radius falls.

The increased fluorescence has been interpreted as an increased binding facilitated by the suppression of electrostatic repulsion between the anionic ANS and the negative phosphate or carboxylate groups of the lipid as these are neutralised by the chelation of the cation. The importance of such an electrostatic repulsion is further emphasized by the observations that the fluorescence intensity of ANS increases as the pH of the suspension is lowered to a value below the pK of the lipid head group[3,10] and also when cationic anaesthetics are added to the lipid or membrane preparation[3,5]. In this paper we have examined the interaction of ANS and its non-sulphonated analogue *N*-phenyl-1-naphthylamine with liposomal suspensions of four phosphatides, namely egg lecithin, dipalmitoyllecithin, lysolecithin and sphingomyelin, under conditions varied so as to alter the effective surface charge.

MATERIALS

N-Phenyl-1-naphthylamine (British Drug Houses laboratory reagent) was recrystallised twice from ethanol and once from petroleum ether. ANS (Eastman Organic Chemicals) was purified by the method of Weber and Young[12].

Lysolecithin, egg lecithin and synthetic, dipalmitoyl-L-3-lecithin were obtained from Koch-Light Laboratories, Buckinghamshire, England; sphingomyelin was extracted from pigs kidneys. Each phospholipid gave a single spot using the thin-layer chromatography technique of Payne[13]. Phospholipase C (from *Clostridium perfringens*) was obtained from Sigma, London, and dissolved in 2 mM Tris–HCl buffer, pH 7.8 (up to a maximum concentration of 12 mg/ml).

METHODS

Phospholipids, with dicetyl phosphate, stearylamine or cholesterol as required, were dissolved in chloroform–methanol (2:1, v/v) and the solvent evaporated under nitrogen to give a thin film of lipid to which glass-distilled water was normally added to give a final concentration of phospholipid in the aqueous phase of 0.5 mg/ml. For the phospholipase C experiments the aqueous phase was 2 mM Tris–HCl (pH 7.8) and the phospholipid concentration in the stock solution 5 mg/ml. The egg lecithin and lysolecithin were dispersed by Vortex mixing and the sphingomyelin and dipalmitoyllecithin by 2 min sonication in a Kerry ultrasonic bath. The solutions were then allowed to stand for at least 1 h before use.

Fluorescence emissions were measured at 90° with respect to the exciting beam

using an Aminco-Bowman spectrofluorimeter. Measurements were performed at 25 °C, except where otherwise stated, in a temperature-controlled cell holder. ANS and *N*-phenyl-1-naphthylamine were excited at 350 and 340 nm, respectively and their emissions observed at 450 and 420 nm, respectively. The final concentrations of ANS and *N*-phenyl-1-naphthylamine were 15 and 3 μM, respectively, and of phospholipid 0.025 mg/ml except in the pH titrations when the lipid concentration was 0.05 mg/ml.

The degree of hydrolysis of phospholipid by phospholipase C was measured by titration of protons released with 10 mM KOH, using a Radiometer TTTl titrator and a Radiometer SBR2c recorder. The reaction was carried out at 25 °C for egg lecithin, at 37 °C for sphingomyelin and at 45 °C for dipalmitoyllecithin in a final volume of 2.5 ml 2 mM Tris–HCl (pH 7.8) for 30 min. Each reaction mixture received 3.0 mg of phospholipid and 25 μmole metal chloride (10 mM) and alkali consumption in each reaction has been rationalised to be equivalent to the addition of 2.5 μg of phospholipase C in each case.

RESULTS

Titration curves

Fig. 1 shows the fluorescence intensity of ANS in the presence of lysolecithin, egg lecithin, dipalmitoyllecithin and sphingomyelin, and of *N*-phenyl-1-naphthylamine in the presence of egg lecithin and dipalmitoyllecithin as a function of pH. As the pH is lowered from 5.0 to 1.5 the fluorescence intensity of ANS increases sharply in the presence of each lipid. When the pH is lowered a further pH unit the intensity remains constant with lysolecithin and egg lecithin but falls with sphingomyelin and dipalmitoyllecithin. The fluorescence intensity of *N*-phenyl-1-naphthylamine in the egg and dipalmitoyllecithin systems remains constant between pH 5.0 and 2.0 and then declines as the pH is lowered below 2.0. These fluorescence changes are reversible and therefore the fall in fluorescence is not due to lipid hydrolysis.

On lowering the pH through the above range the amino group of both these dyes will be protonated. Protonation blue-shifts the last absorption band of the aryl amines[14] thus providing a method for measuring the degree of protonation. The absorption of ANS at 350 nm and of *N*-phenyl-1-naphthylamine at 335 nm, the wavelengths of maximum absorption in the unprotonated state, are plotted in Fig. 2 as a function of pH. The pK of the amino group lies in the region 0.0 to 0.5 for both ANS and for *N*-phenyl-1-naphthylamine.

Effect of net lipid charge

Net negative or positive charge was conferred on egg lecithin liposomes by the addition of dicetyl phosphate or sterylamine respectively, up to a molar ratio of 1 : 1. In Fig. 3b the fluorescence intensity of ANS is plotted as a function of the mole fraction of charge component in the liposomes. The intensity falls as the dicetyl phosphate concentration increases and increases with increasing sterylamine concentration.

A partition coefficient, K, for the distribution of ANS between the aqueous and lipid phases of these charged liposomal suspensions was calculated from

$$K = \frac{\text{bound ANS}}{\text{free ANS}} = \frac{F - F_0}{F_m - F}$$

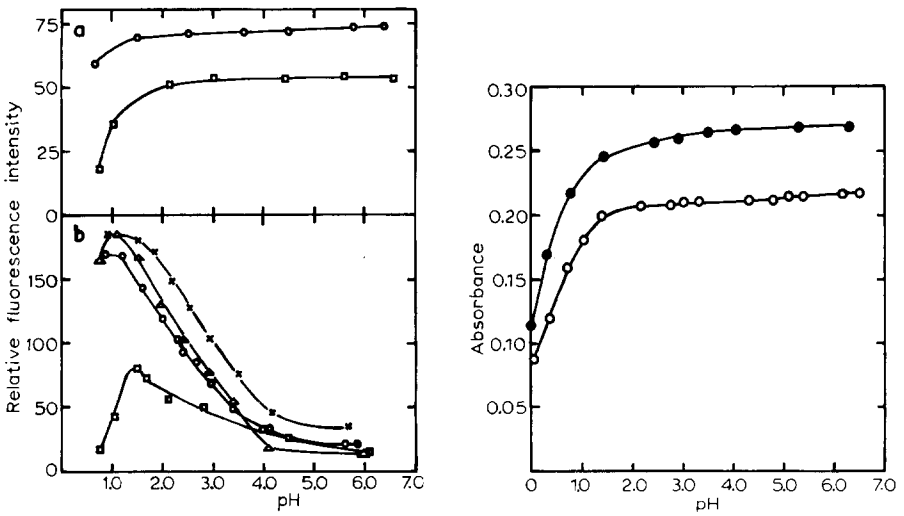

Fig. 1. The relative fluorescence intensity of (a) *N*-phenyl-1-naphthylamine in the presence of egg lecithin (○—○) and dipalmitoyllecithin (□—□), and of (b) ANS in the presence of lysolecithin (×—×), egg lecithin (○—○), sphingomyelin (△—△), and dipalmitoyllecithin (□—□), as a function of pH. The fluorescence intensity units are arbitrary.

Fig. 2. The absorbance of $2.2 \cdot 10^{-5}$ M ANS in water at 350 nm (○—○) and of $3.1 \cdot 10^{-5}$ M *N*-phenyl-1-naphthylamine in 10% ethanol–90% water at 335 nm (●—●) as a function of pH. The *N*-phenyl-1-naphthylamine is not soluble in water alone to this concentration.

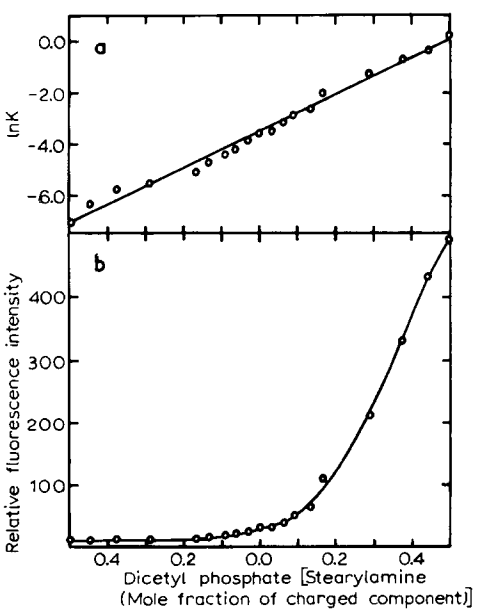

Fig. 3. Plots of (a) the logarithm of the partition coefficient of ANS between lecithin dispersions and water, and of (b) the fluorescence intensity of the ANS in the presence of these dispersions as a function of the mole fraction of dicetyl phosphate or stearylamine added to the egg lecithin. The fluorescence intensity units are arbitrary.

where F is the fluorescence intensity at a given net charge, F_0 is the intensity in the absence of lipid and F_m is the maximum intensity obtained by increasing the lipid concentration. It can be seen from Fig. 3a that log K is almost linear with the mole fraction of charged component in the liposome.

The effect of divalent cations

As there appears to be some doubt over the maximum enhancement of the fluorescence intensity of ANS that can be achieved by increasing the concentration of different cations in a lipid suspension we have measured the intensity of ANS fluorescence in the presence of three phosphatides on adding the group IIA cations Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} in concentrations at which the fluorescence intensity is maximal. The fluorescence intensity increased as the cation concentration was increased and, in the cases of all four ions, reached a plateau at 0.1 M. Fluorescence measurements referred to as plateau levels were made at both 0.1 and 0.5 M. The results are shown in Fig. 4 where the maximum fluorescence is plotted against the ionic radii[15] of the cations. In each case it increases with decreasing radius for Ba^{2+}, Sr^{2+} and Ca^{2+} but then falls for the smallest ion, Mg^{2+}. Addition of these four ions to egg lecithin in the presence of *N*-phenyl-1-naphthylamine had no effect on the fluorescence intensity of this dye (Fig. 4).

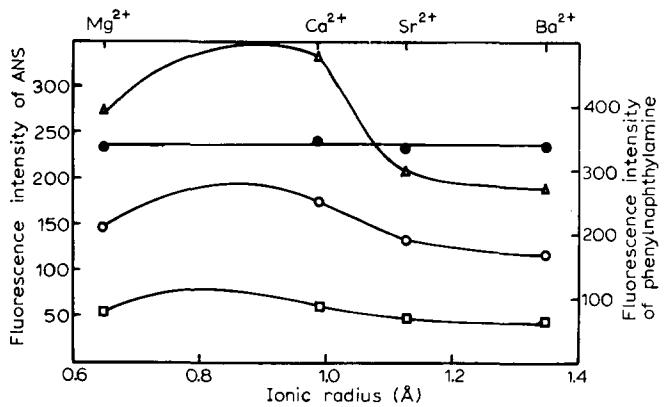

Fig. 4. The maximum fluorescence intensity of ANS achieved in the presence of sphingomyelin (△—△), egg lecithin (○—○), and dipalmitoyllecithin (□—□) and of *N*-phenyl-1-naphthylamine in the presence of egg lecithin (●—●), on adding divalent cations as a function of the ionic radius of the cation. The fluorescence intensity units are arbitrary.

Bangham and Dawson[16] have suggested that Ca^{2+} activates the phospholipase C hydrolysis of phospholipids by masking the negative charges of the phosphate moiety of the lipid head groups, thus reducing electrostatic repulsive forces between the lipid surface and the net negatively charged enzyme. As this mechanism is directly analogous to that suggested by several workers[3,4,10,11] to explain the enhancement of ANS fluorescence when divalent cations are added to the liposomal suspension, we have measured the rate of hydrolysis by phospholipase C of the three phosphatides in the presence of Mg^{2+}, Ca^{2+}, Sr^{2+} or Ba^{2+}. The cation concentration was 10 mM in each case, greater concentrations having been shown to be inhibitory[16].

The degree of hydrolysis of egg lecithin after 30 min incubation at 25 °C exhibits a cation dependence similar to that found for ANS fluorescence, *i.e.* the order of efficiency of activation is $Ba^{2+} < Sr^{2+} < Mg^{2+} < Ca^{2+}$ (Fig. 5). The same order was found for a sphingomyelin substrate incubated at 37 °C, but for dipalmitoyllecithin at 45 °C the order was $Mg^{2+} < Sr^{2+} < Ba^{2+} < Ca^{2+}$. In this latter case, however, the degree of hydrolysis was three orders of magnitude less than for egg lecithin and the comparison between the four cations is of little significance.

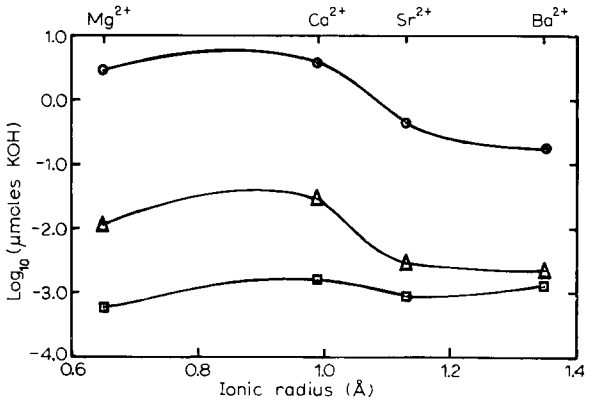

Fig. 5. The degree of hydrolysis of egg lecithin (○—○), sphingomyelin (△—△), and dipalmitoyllecithin (□—□), catalysed by phospholipase C, in 30 min in the presence of 10 mM divalent cation as a function of the radius of the cation. See Methods for the assay procedure and conditions.

No detectable hydrolysis of dipalmitoyllecithin occurred below its transition temperature of 41 °C and no hydrolysis of sphingomyelin was found at temperatures below 30 °C. This may be due to the physical state of the phospholipid at these temperatures. Oldfield and Chapman[17] have used differential scanning calorimetry to show that sphingomyelin isolated from ox brain enters a phase transition at 32 °C and that this transition is characterised by a maximum rate of melting at 40 °C. Vanderkooi and Martonosi[5] and Träuble[11] have demonstrated that the phase transition of dipalmitoyllecithin may be determined from the change in ANS fluorescence with temperature. They found that, on increasing the temperature, the fluorescence intensity increases as the lipid enters the transition region and then falls once the lipid is in the liquid–crystalline state. We therefore measured the fluorescence intensity of ANS in the presence of sphingomyelin as a function of temperature. The intensity exhibits a gradual increase as the temperature rises from approximately 30 to 42 °C and declines with further temperature increase (Fig. 6). This behaviour suggests that sphingomyelin undergoes a phase transition in this temperature range.

Effect of adding cholesterol

Cholesterol is known to change the surface potential of lipid monolayers[18]. Therefore we have examined the effect of the addition of cholesterol to the phosphatide suspensions on the enhancement of ANS fluorescence. In the presence of egg lecithin, dipalmitoyllecithin and sphingomyelin the fluorescence intensity falls as the cholesterol content is increased, irrespective of whether cations are present or not (Fig. 7). In the case of the latter two lipids the fluorescence enhancement is virtually

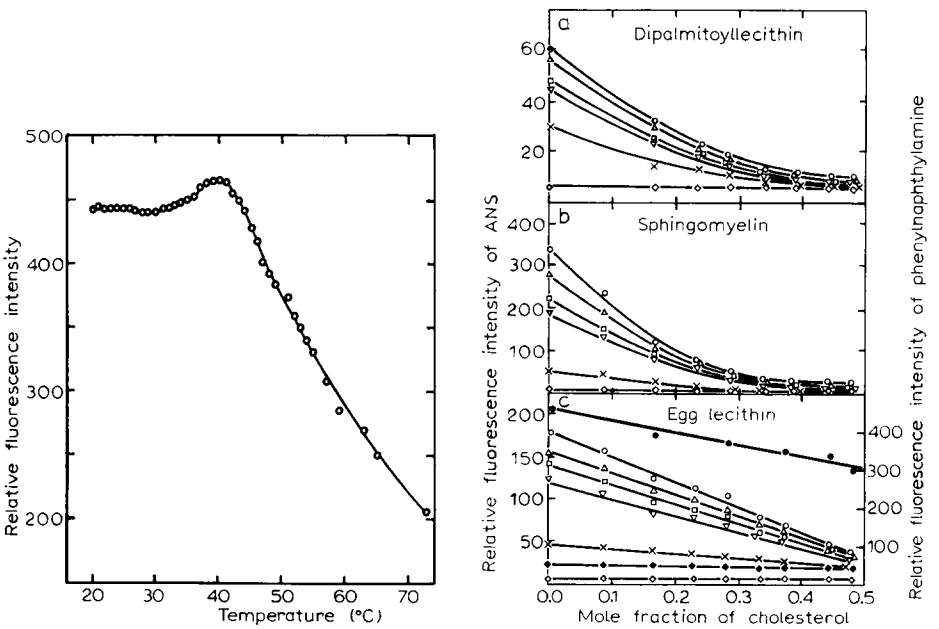

Fig. 6. The relative fluorescence intensity of $1.25 \cdot 10^{-4}$ M ANS in the presence of sphingomyelin (0.025 mg/ml), 10 mM $CaCl_2$, and 2.0 mM Tris at pH 7.8 as a function of temperature.

Fig. 7. The relative fluorescence intensity of ANS in the presence of (a) dipalmitoyllecithin, (b) sphingomyelin and (c) egg lecithin and of *N*-phenyl-1-naphthylamine in the presence of (c) egg lecithin as a function of the mole fraction of cholesterol added to the phospholipid. The fluorescence intensity units are arbitrary. ○—○, ANS+lipid+$CaCl_2$; △—△, ANS+lipid+$MgCl_2$; □—□, ANS+lipid+$SrCl_2$; ▽—▽, ANS+lipid+$BaCl_2$; × — ×, ANS+lipid, no divalent cation; ◇—◇, ANS alone; ●—●, *N*-phenyl-1-naphthylamine+lipid; ◆—◆, *N*-phenyl-1-naphthylamine alone. The intensity of the *N*-phenyl-1-naphthylamine fluorescence in the presence of egg lecithin was independent of the presence of any of the four cation species. The divalent cations were 0.1 M; all other concentrations were as stated in Methods.

eliminated at cholesterol mole fractions greater than 0.3, but with egg lecithin there is still a significant enhancement at 0.5 mole fraction of cholesterol. The experiment with egg lecithin was repeated using *N*-phenyl-1-naphthylamine instead of ANS. In this case the fluorescence intensity falls less markedly with increasing cholesterol concentration (Fig. 7).

DISCUSSION

The results presented in this paper all support the hypothesis that electrostatic forces are important in determining the extent of binding of ANS to phospholipids. Davies[19] has derived the equation which relates the amount of a charged solute that is absorbed at a hydrophobic interface to the surface potential at the interface. For low solute concentrations this takes the form of a Boltzmann equation and the logarithm of the partition coefficient of the solute between the hydrophobic interface and the aqueous phase is, to a first approximation, linearly dependent on the surface potential. An adequate expression describing the dependence of the surface potential

of phospholipid dispersions on surface charge density has not been derived, but attempts to do so suggest that, for low surface potentials, it is related linearly to the surface charge density[20,21]. The linear dependence of log K on the mole fraction of dicetyl phosphate or stearylamine present in the egg lecithin liposomes demonstrates the importance of electrostatic interactions and is consistent with the theory described above. The zeta potential (ζ), which, for low surface charge densities, is identical to the surface potential[22] in the above described equations, has been measured by Bangham and Dawson for egg lecithin–dicetyl phosphate[23] and egg lecithin–stearylamine[16] dispersions. They found that ζ became more negative with increasing dicetyl phosphate content but more positive with increasing stearylamine.

The enhancement of ANS fluorescence as the pH is lowered through the region of the lipid phosphate pK has been confirmed for egg lecithin and sphingomyelin and has been shown to occur also with dipalmitoyllecithin and lysolecithin. The constant fluorescence intensity of N-phenyl-1-naphthylamine in the presence of egg and dipalmitoyllecithin when the pH is lowered through the same region confirms that the ANS fluorescence enhancement is due to facilitated binding as a result of reduced electrostatic repulsion as the phosphate groups are neutralised. If the enhancement were due to a change in the lipid environment one would expect the neutral dye to respond in a similar manner to ANS as their chromophoric groups are identical; the neutral dye does not possess a negatively charged sulphonate group, however, and its binding will therefore not be affected by the degree of ionization of the phosphate groups.

The tendency for the fluorescence intensity of both ANS and N-phenyl-1-naphthylamine to diminish at pH values below 1.5 may be due to the protonation of the dye amino group, the pK of this group having been shown to be in this region (Fig. 2). On protonation the N-phenyl-1-naphthylamine becomes a cation and the ANS a zwitterion, but as the pK of the aryl sulphonate group has been reported to be approximately 0.7 (ref. 22) the ANS will rapidly pass from the zwitterionic to the cationic form. Consequently the dyes will be repelled by the quaternary amino groups of the lipid, the lipid phosphate groups having been neutralised at this pH. Vanderkooi and Martonosi[10] observed a similar diminution of intensity when titrating ANS in the presence of sarcoplasmic reticulum microsomes. Nevertheless, the possibility that the diminution of fluorescence at low pH may be due to the protonation of the bound dyes and a concomitant decrease in the quantum efficiency cannot be excluded.

The hypothesis that the enhancement of ANS fluorescence observed when cations are added to a phospholipid suspension is caused by facilitated binding due to the masking of the lipid phosphate moieties by the cation is borne out by the order of effectiveness of the group IIA cations. The stability of chelate complexes involving cations of the first two groups of the periodic table generally increases, for a given subgroup, with decreasing ionic radius[25], with one exception. The second element of group IIA, Mg^{2+}, often behaves anomalously and the stability constants increase in the order $Ba^{2+} < Sr^{2+} < Mg^{2+} < Ca^{2+}$. Williams[25] has suggested that Mg^{2+} is less readily dehydrated than the other cations but Care and Staveley[26] and Deamer *et al.*[27], who have shown that the stability constants of Mg-EDTA^{2-} and magnesium stearate respectively are anomalously low, have suggested that the ligands possess a relatively rigid geometry and that the Ca^{2+} ion is of the optimum size to fill the chelation cavity. To our knowledge the stability constant of the complexes of these four ions with egg

lecithin, dipalmitoyllecithin and sphingomyelin have not been measured directly, but Shah and Schulman[28] have measured the change of surface potential across monolayers of both egg and dipalmitoyllecithin when these ions are added to the subphase. They found, particularly at high monolayer compressions, that the potential increased in the order $Ba^{2+} < Sr^{2+} < Mg^{2+} < Ca^{2+}$. They attributed this increase to the binding of the cations to the phosphate moiety of the lipid head groups. We observed the same order when measuring the degree of enhancement of ANS fluorescence induced by these ions in the presence of egg lecithin, dipalmitoyllecithin and sphingomyelin. The insensitivity of the fluorescence enhancement of *N*-phenyl-1-naphthylamine to the presence of the cations further confirms that no major changes in the lipid environment are responsible for the cation-induced enhancement.

The similarity in the effectiveness of the group IIA cations with egg lecithin and sphingomyelin in activating phospholipase C and in enhancing ANS fluorescence suggests that, in both cases, they may be acting by masking the negative phosphate moieties of the phospholipid, this mechanism having been shown to occur in the lipase reaction[16]. Dipalmitoyllecithin is such a poor substrate for the phospholipase C reaction that the differences we observed in the degree of hydrolysis in the presence of the different cations were of the same order as the experimental error involved in the assay procedure.

Shah and Schulman[18] and Chapman *et al.*[29] have shown that the surface area and surface potential per molecule of both egg and dipalmitoyllecithin decrease as the cholesterol content of the monolayer is increased. Chapman and Penkett[30] have observed a broadening of the fatty acid chain proton NMR signal when the cholesterol content of egg lecithin suspensions is increased and have associated this with a loss of rotational freedom of these chains due to complex formation with the cholesterol. They offer this as an explanation of the condensation effect in monolayers and therefore one might expect the surface potential of liposomal suspensions also to be lowered on the addition of cholesterol. If this is in fact the case, the effect on ANS binding would be similar to that obtained on adding a negatively charged component to the suspension, *e.g.* dicetyl phosphate, and the level of ANS binding should be lowered. This hypothesis is supported by the decline in fluorescence intensity of ANS in the presence of egg lecithin, dipalmitoyllecithin and sphingomyelin as the cholesterol content is increased. The decrease in ANS fluorescence intensity as the cholesterol content increases also occurs in the presence of group IIA cations and the point at which fluorescence enhancement becomes negligible is independent of the presence of the cations. Unfortunately, the mechanism of direct competition between ANS and cholesterol, both of similar size and shape, for binding sites in the phospholipid layer cannot be discounted as the fluorescence intensity of *N*-phenyl-1-naphthylamine also decreases to some extent with increasing cholesterol in egg lecithin liposomes (Fig. 7).

The experiments described in this paper suggest that the sulphonate group of ANS, when bound to a phospholipid, is in close proximity to the lipid phosphate groups. From earlier work on the binding of ANS to detergent micelles[31] this not only seems probable but obligatory. It was demonstrated that ANS is insoluble in hydrocarbon solvents and therefore cannot penetrate into the interior of a micelle but must partition at the surface with the aromatic group in the hydrocarbon phase and the sulphonate group either solvated in the aqueous phase or salt linked to a cationic charge on the micelle surface. It was also shown that substituted naphthylamine sul-

phonates will bind to positively charged micelles, will not bind to negatively charged micelles and will only bind to neutral micelles if the aromatic residue is above a certain size. These results emphasize the partitioning nature of ANS binding and the importance of electrostatic interactions in its binding. As phospholipid and detergent micelles do not differ in the orientation of the polar and non-polar residues of their constituent molecules one would also expect the binding of ANS to phospholipids to be a partitioning at the lipid surface, thereby requiring the sulphonate group to be close to the phosphate group. This form of binding has recently received confirmation from X-ray crystallographic work. Lesslauer *et al.* have examined the diffraction patterns of dipalmitoyllecithin[32] and lecithin–cardiolipin[33] vesicles containing ANS and have concluded that the sulphonate group of the ANS lies in the plane of the polar head groups with the aromatic residue protruding into the fatty acid side chain layer.

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