J. Membrane Biol. 17, 313–340 (1974)

1-Anilino-8-Naphthalenesulfonate: A Fluorescent Probe of Membrane Surface Structure, Composition and Mobility

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Received 19 November 1973; revised 21 February 1974

Summary. The binding of the hydrophobic fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS^-) on phospholipid vesicle membranes was studied to gain information about the structure and mobility in the polar head group region, and to determine the degree of mixing of lipids on the microscopic scale. The maximal degree of binding of ANS^- on dimyristoyl and dipalmitoyl-L-lecithin membranes is one ANS^- per four lecithin molecules, indicating a binding site composed of four polar head groups. ANS^- bound in this site has a long fluorescent lifetime (5 to 9 nsec) and high quantum yield (0.2 to 0.3), indicating that it is relatively inaccessible to the solvent water. The lack of paramagnetic quenching by added Mn^{2+} indicates that ANS^- bound to those four-membered sites is also well shielded from added cations. Similarities in the temperature dependence of the binding constant and the reciprocal fluorescent lifetime indicate that the latter is determined by the propensity for polar head group motion and for water and ANS^- reorientation during the excited state of the molecule.

Membranes composed of lipids which lack a semi-polar head group (phosphatidic acid) or which have unfavorable polar head group conformations or strong interactions between the polar head groups (dimyristoyl ethanolamine) do not support the binding of ANS^- with a high quantum yield and long fluorescent lifetime. Incorporation of these lipids in a lecithin membrane decreases the maximal binding of ANS^- to a greater extent than can be explained on the basis of dilution of the lecithin with these lipids. A statistical model is presented, in which incorporation of one or more molecules of the second type into a four-membered lecithin binding site destroys the ability of this site to bind ANS^- with a long fluorescence lifetime. Agreement between this model and the results obtained with lipid mixtures indicate that egg phosphatidic acid and dimyristoyl ethanolamine mix well with dimyristoyl lecithin on the microscopic scale. The above criterion was also used to show that cholesterol mixes randomly with lecithin. In contrast to the behavior of the phospholipid mixtures, there was evidence for short-lived ANS^- species for cholesterol/lecithin mixtures with mole ratios between 0.6 and 1.0. This species is associated with binding sites in which the lecithin polar head groups

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are spaced out by the inclusion of cholesterol between the hydrocarbon chains of the lipid molecules. The effects of the ion carrier valinomycin and local anesthetics on the lecithin binding sites are discussed.

The fluorescent probe technique [24, 29, 31; *cf.* 3] has seen increasing application in the study of the physical properties of membranes. The technique has been used to make deductions about membrane "polarity", membrane "viscosity" and freedom of lateral and rotational diffusion, and has yielded information about proximity relationships within membranes (*cf.* [22] for references). The most widely used fluorescent probe is 1-anilino-8-naphthalenesulfonate (ANS^-) [24, 29]. This fluorophore has an inverse relationship between its fluorescent quantum yield and solvent polarity, and is thus a very sensitive probe for hydrophobic binding sites.

The most important information delivered by ANS^- can be summarized as follows: (a) The probe can be used to measure membrane "polarity" (*cf.* [22]). (b) The binding of the probe responds to the cation composition on the medium. (c) The fluorescence of the probe can respond to the degree of energization in mitochondria [1], can follow the action potential in nerve [7] and has been correlated with Ca^{2+} uptake in isolated sarcoplasmic reticula [30]. However, the usefulness of these observations is limited by a lack of understanding of exactly what effects are being reported by ANS^- on the microscopic scale.

The present study is an attempt to determine what information can be obtained on the molecular level by a study of the behavior of ANS^- in simple phospholipid systems. This work was initiated in the hope that a systematic study of the binding and fluorescent behavior of ANS^- could provide important information about the structure and reactions in the polar head group region of phospholipid membranes [13]. The investigation was sustained in the prejudice that the functionally correlated ANS^- fluorescence changes in mitochondria, sarcoplasmic reticulum and nerve, might be endowed with greater predictive value if the behavior of ANS in simple phospholipid membranes were better understood.

The present paper, the first in a series of four, deals with the effect of lipid composition, temperature, local anesthetics and ionophores on membrane surface structure, as reported by ANS^-. The second (companion) paper deals with ANS^- as an indicator of membrane surface potential and cation binding. The third paper will deal with the ionophore complexation reactions in the membranes as reported by fluorescent methods. The fourth paper will report the application of rapid kinetic techniques to the problems treated in the first three papers.

The experiments to be reported here were carried out on synthetic lipids with saturated hydrocarbon chains of uniform length. Membranes composed of these lipids in the pure form demonstrate thermal phase transition behavior [19]. Below a critical temperature, T_t, the hydrocarbon chains of the lipid molecules are in an extended conformation [19, 27]. Heating the preparation above T_t produces a lateral expansion of the membrane, a decrease in membrane thickness, and an expansion of the membrane associated with the formation of "kinks" in the hydrocarbon chains (*cf.* [27]). Thus, the choice of lipids exhibiting the thermal phase transition allows a membrane of one lipid composition to be studied in two different physical states, and facilitates the study of membrane properties in a biophysical context.

Materials and Methods

1,2-Dimyristoyl-L-lecithin (PC), dimyristoyl-L-ethanolamine (PE) and phosphatidic acid (PA) derived from egg lecithin were obtained from Koch Light Laboratories. Dipalmitoyl-L-PC was purchased from Fluka. All other chemicals were reagent grade, purchased commercially. 1,8-ANS$^-$ was obtained as the ammonium salt from Pierce Chemical Co. *n*-Dibutyl ether-$CHCl_3$ monolayer vesicle preparations containing 6.8×10^{-4} M lipid (determined weight) were made as described by Träuble and Grell [26]. The monolayer vesicles have been characterized by electron-microscopy as about 500-Å-diameter spheres of organic solvent, covered with a monolayer of phospholipid molecules [25]. Mixed lipid vesicles were obtained by cosonication of the components. It is important that the sonication be conducted above the phase transition temperatures of all components of the system. The duration of sonication was 15 min or longer, when necessary to achieve optical clarity. The preparations were stored above their phase transition temperature, and were used within two days of preparation. It was our experience that vesicle aggregation and separation of components of mixed systems were promoted by aging below T_t. Unless otherwise specified, all of the results of the present study are for monolayer vesicles. In some cases dispersions of phospholipids in water ("bilayer vesicles" here) were studied for comparison.

Routine fluorescent measurements were made on an Aminco-Bowman SPF Ratio Fluorimeter. Corrected fluorescent spectra were taken on a FICA 55 Spectrofluorimeter, which gives quantum-corrected excitation (220 to 550 nm) and emission (200 to 800 nm) spectra. The instrument is fitted with an additional rhodamine B quantum counter behind the sample cuvette, enabling sample transmission to be recorded simultaneously with fluorescence on a Bryans 2600 A 3 y_1, y_2, x recorder. Inner filter effects at the excitation wavelength were in most cases negligible. When necessary, corrections were made using the fluorescence/transmission characteristics of the cuvette (5 mm) and slit (1 mm) combination, as determined by use of standard fluorescent compounds. Additional absorption spectra were taken on a Cary 14 spectrophotometer.

In the steady-state fluorescent measurements, the fluorescent signal observed (in absence of inner filter effects or quantum transfer, *cf.* [3]) is given by:

$$Fl_t = k \sum_i^n \varepsilon_i * Q_i * c_i \qquad (1)$$

where Fl_t is the emission signal, integrated over all emission wavelengths and where Q_i, c_i and ε_i are the quantum yields, the concentrations and the extinction coefficients at the exciting wavelength of the ith species. The value of k, an instrumental proportionality constant, is independent of wavelength for the FICA instrument. Since the excitation and emission wavelength maxima (368 and 480 nm, respectively) and half-widths of the emission spectra were essentially invariant in the membrane preparations and reference solvents used here, Fl_t was measured routinely at fixed wavelengths.

Fluorescent lifetimes and fluorescent decay functions were obtained by the single photon time correlation method. The samples were excited with 1.5 to 2.0 nsec (FWHM) pulses at a repetition rate of 50 kHz from a free-running air lamp at 1 atm. A Jarell Ash 250 cm monochromator was used to select the excitation wavelength (337.1 nm). A GG 400 Schott glass filter was placed in front of the single photon detector (Quantacon type 8850, RCA) to eliminate stray light. The ORTEC time-to-amplitude converter and the associated circuitry including the multichannel pulse height analyzer (Geoscience 8040/7010) were repeatedly checked for linearity with a random noise generator (hp 8057 A).

In the fluorescence decay measurement, the system is excited with a pulse of light, and the probability of fluorescence (the decay of fluorescence) is measured as a function of time. For a system containing more than one fluorescent species, the fluorescence as a function of time is expressed as:

$$Fl(t)=\sum_{i}^{n} a_i * \exp(-t/tau_i) \tag{2}$$

where a_i is a constant which depends upon the concentration and the lifetime, tau_i of the ith species. The total fluorescence of the sample, Fl_t, is given as the time integral of Eq. (2).

Determination of the quantum yield of the bound species was determined by either (a) the method of vesicle titration, or (b) by calculation from the fluorescent lifetime. In method (a) the fluorescent signal and extinction coefficient of the bound form were obtained by extrapolation of a double reciprocal plot based on lipid concentration. Comparison between the extrapolated fluorescence signal and the signal from a known concentration of ANS^- in methanol ($Q=0.22$, [24]) allows the calculation of Q for membrane-bound ANS^-. Values for the extinction coefficients within the membrane and in methanol were equal within an experimental uncertainty of 10%. In method (b), the quantum yield of the ANS^- is determined as:

$$Q=tau/tau_0 \tag{3}$$

where tau_0 is the natural lifetime (for a quantum yield of 1.0). Since the value of tau_0 is known (=24 nsec in methanol [12]) and is relatively insensitive to the environment, Q can be calculated directly from tau. The tau values were evaluated from plots of $\log(Fl(t))$ against t, evaluated after the exciting pulse had fallen to 5% of its maximal value. No time-dependent polarization effects were observed. Method (b) was more accurate, and was thus always used in determining Q unless otherwise noted.

Only one membrane-bound ANS species was observed, unless otherwise noted. The steady-state fluorescence signal and the fluorescence decay curves were composed of a large contribution from the ANS^- in the membrane ($Q\simeq 0.3$; $tau\simeq 7.5$ nsec) and a small contribution from the ANS^- in the aqueous phase ($Q=0.004$ [22]; $tau<1$ nsec). The signals arising from ANS^- in the aqueous phase were subtracted off when necessary. The concentration of ANS^- bound $[ANS]_b$, was calculated from Eq. (1) using the Q value from the fluorescent lifetime experiments and the Fl_t value from the steady-state fluorescence measurements. The Q value was invariant (within an experimental uncertainty of *ca.* 7%) with the degree of ANS^- binding. All measurements were made within

15 min of the ANS$^-$ addition, and 90° light-scattering measurements indicate that the ANS$^-$ addition does not cause vesicle aggregation in this time range.

All experiments involving a high degree of ANS$^-$ binding were carried out at high ionic strength in order that the binding of the probe not be influenced by electrostatic interactions on the membrane surface. Analyses of the ANS$^-$ binding curves to determine the binding constants and the numbers of binding sites were made using a Fortran computer program for least-squares fitting. The experimental and calculated data were plotted by the computer (Univac 1108).

Results and Discussion

ANS$^-$ binds to phospholipid membranes with a fluorescent enhancement. X-ray diffraction [17] and nuclear magnetic resonance (NMR) studies [5], as well as considerations of the quantum yield of the bound form [13, 25] indicate that the probe is located in the polar head group region of the membrane. The binding of ANS$^-$ to these membranes increases with increasing ANS$^-$ concentration, saturating at high concentrations, suggesting that the ANS$^-$ is reacting with "binding sites" in the membrane [13, 25]. The discussion below gives the interpretation of this saturation behavior according to a binding site model.

Binding Site vs. Partition

Two possible modes of probe binding have been considered: (a) the probe can bind to a pre-existing site or (b) the binding of the probe could require displacement or rearrangement of the lipid constituents of the membrane. In case (a), the binding would not necessarily result in an appreciable change in the membrane, since the packing of the lipid constituents would not have to be altered by the binding reaction. The binding reaction to the pre-existing sites B would thus be described as:

$$\mathrm{ANS}^-_{\mathrm{H_2O}} + B_{\mathrm{membrane}} \overset{K_1}{\leftrightarrow} \mathrm{ANS}^- - B_{\mathrm{membrane}} \tag{4}$$

where K_1 is the equilibrium constant for the reaction. In case (b), the binding would be described as a simple partition process according to:

$$\mathrm{ANS}^-_{\mathrm{H_2O}} \overset{P}{\leftrightarrow} \mathrm{ANS}^-_{\mathrm{membrane}} \tag{5}$$

in which the partition coefficient P can be assumed to be independent of the degree of advancement of the reaction when the *solubility* of the molecule in one or the other phase is not exceeded. The corresponding equilibrium

expressions are:

$$K_1 = \frac{[\mathrm{ANS}-B]_{\mathrm{membrane}}}{[\mathrm{ANS}]_{\mathrm{H_2O}} * [B]_{\mathrm{membrane}}} \tag{6}$$

$$P = \frac{[\mathrm{ANS}]_{\mathrm{membrane}}}{[\mathrm{ANS}]_{\mathrm{H_2O}}}. \tag{7}$$

Case (a) predicts that in an experiment in which a constant concentration of membranes is titrated with ANS^-, the degree of binding will increase linearly at low concentrations, saturating at high concentrations. For case (b), no saturation behavior would be observed and the amount of ANS^- bound will be proportional to the concentration in the aqueous phase.

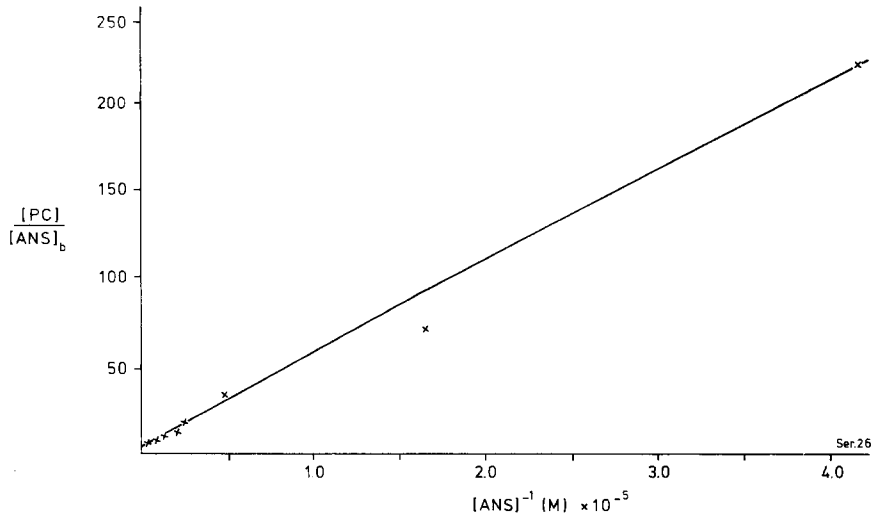
Fig. 1 is a double reciprocal plot of ANS binding to dimyristoyl lecithin monolayer vesicles. The experiment was carried out at high ionic strength to ensure that the binding constant K_1 was not influenced by electrostatic interactions on the membrane [6, 13][1]. The experiment shows that ANS binding demonstrates saturation behavior for degrees of saturation between 2 and 80%, in agreement with the expectations of case (a). The binding of ANS^- can thus be considered as the process of binding into a pre-existing site. The value of K_{10} is 7.54×10^3 and the number of binding sites $[B]_t$ was determined as 0.25 per lecithin molecule. The value of K_{10} is a function of temperature, but the number of binding sites $[B]_t$ is independent of temperature and the phase transition, and has always been determined as 0.25 for monolayer or bilayer membranes composed of pure dimyristoyl or dipalmitoyl PC[2]. The value of $[B]_t$ would thus appear to be determined solely by the geometrical relationship of the polar head groups. The discussion below shows that a systematic study of K_{10} and Q can give information about the degree of mixing of two types of lipids within the membrane.

Binding Site Behavior

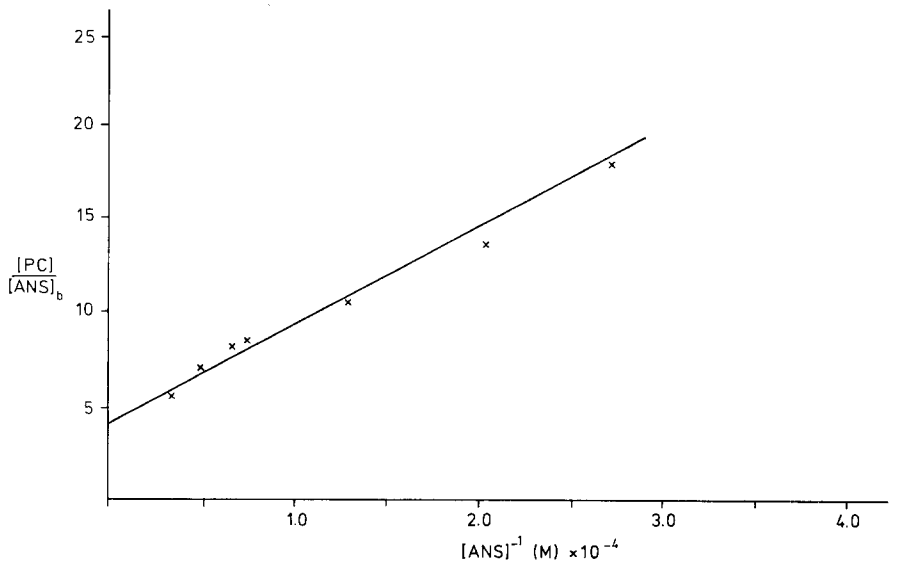
X-ray diffraction studies have shown that the hydrocarbon chains of the lipid molecules are packed in a hexagonal or disordered hexagonal arrays [11, 18]. Possible resulting orientation of the lipid molecules from

1 In a previous paper [13], it was shown that K_1 is a function of the surface potential of the membrane ψ_0 according to $K_1 = K_{10} * \exp(e\psi_0/kT)$, where K_{10} is the binding constant for negligible surface potential. The experiments reported here were conducted at high ionic strength, such that $K_1 = K_{10}$.

2 Values of $[B]_t$ between 0.025 and 0.067 have been reported for dipalmitoyl PC [25]. These values are underestimates since the experiments were performed at low ionic strength, and the value of K_1 (according to the formalism used here) became progressively smaller than K_{10} as the degree of ANS^- binding increased (*cf.* first footnote).



a



b

Fig. 1. (*a*) Double reciprocal plot of ANS^- binding. Dimyristoyl lecithin monolayer vesicles (6.8×10^{-4} M lipid) in a medium containing 1.6 M KCl, and 4.5 mM imidazole buffer, pH 7.3, at 30 °C were titrated with ANS^-. The number of ANS molecules bound per lipid molecule ($[ANS]_b/[PC]$) was calculated by use of Eq. (1) as described in Materials and Methods. The quantum yield of the bound form was 0.31 as determined by the fluorescent lifetime method [*cf.* Eq. (3)]. The values of K_{10} and $[B]_t$ and the solid line were calculated and plotted by a computer program designed for least-squares fitting of the binding according to Eq. (6). The standard deviation of the fitted and experimental data was 0.10. The solid line was calculated using the fitted K_{10} and $[B]_t$ values. (*b*) Same as (*a*) with 10-fold scale expansion

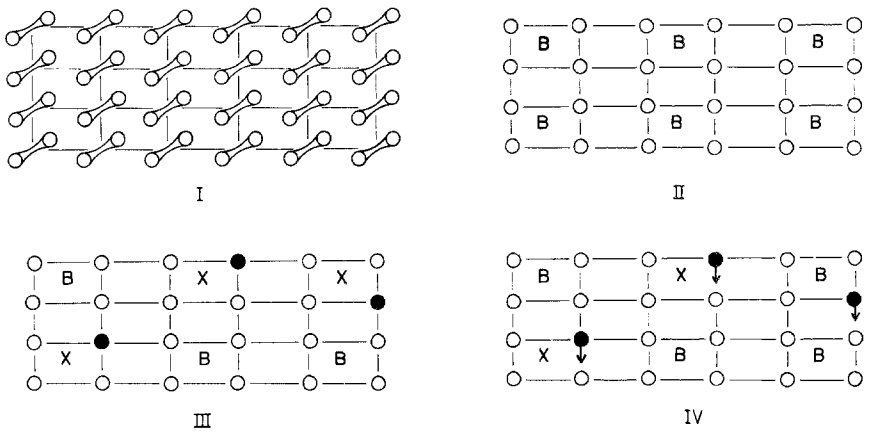


Fig. 2. Model for the origin of binding site behavior in phospholipid membranes: (I) The hexagonal packing of the fatty acid chains (circles) of the phospholipid molecules (dumbbells) gives rise to a "quadratic" array of polar head groups. (II) Binding within one quadratic array precludes binding in the four neighboring arrays. The binding site is denoted with *B*. (III) Insertion of a certain type of foreign lipid molecule with incompatible polar head group precludes binding within this site. (IV) Insertion of a certain type of foreign lipid molecule with polar head group with an unfavorable conformation, or with tendency to form intramolecular hydrogen bonds hinders binding

hexagonal packing are given in Fig. 2-I. When the polar head groups are aligned with the axes of the two hydrocarbon chains of the lipid molecules and if the lipid molecules are packed optimally, then the hexagonal array of hydrocarbon chains will result in a quadratic array of polar head groups. The ANS^- molecule can be inserted into this polar head group array (Fig. 2-I). If it is assumed that the binding in one such site denoted with *B* will preclude binding in the nearest neighboring sites, then the value of 0.25 is derived for $[B]_t$. The blockage of binding at the neighboring sites could result from projection of a section of ANS into this site. Alternatively, the ANS^- binding could affect the polar head group orientation or packing.

This model for ANS^- binding would predict that the ANS^- binding would be sensitive to the binding of other molecules at the membrane surface [13], and that it would be sensitive to the type and conformation of the polar head groups within the membrane. Fig. 3 shows molecular models of dimyristoyl PC and ANS^- and illustrates the expected mode of binding. The ANS^- binding reaction is thus seen as a process of insertion of ANS^- between the polar head groups and displacement of water from this region. The maximal degree of binding is given by geometrical considerations, and we have found no evidence that the ANS^- binding alters any membrane properties. (The single exception to this is that ANS^- as

well as other foreign molecules reduces the half-time of the slow irreversible vesicle aggregation reaction from about 4 days to several hours.) The ANS$^-$ can thus be considered as a *probe* for membrane interactions.

Properties of Lecithin Binding Sites

Table 1 compares dimyristoyl and dipalmitoyl PC as hosts for ANS$^-$. It is seen that the binding constant, number of binding sites, quantum yield and fluorescent lifetime of the bound form are relatively insensitive to the length of the hydrocarbon chains of the lipids. The values of these parameters for dimyristoyl PC monolayer and bilayer vesicles are similar, corroborating our observation that the surface properties of these two types of preparation are essentially the same [13, 14][3]. The quantum yield

Table 1. Comparison of ANS binding parameters for dimyristoyl and dipalmitoyl lecithin vesicles

Lipid	T (°C)	K_{10} ($\times 10^{-4}$ M^{-1})	$[B]_t$	*tau* (nsec)	Q [a]
Dimyristoyl	15	0.95	0.25	9.0	0.37
Monolayer	30	0.75	0.25	7.5 (7.5) [c]	0.31
$T_t=23$ °C [b]	50	0.65	0.25	5.7	0.24
Dimyristoyl Bilayer $T_t=23$ °C [b]	30	0.59	0.25	7.0	0.29
Dipalmitoyl [d]	15	0.10 ± 0.03	0.25	6.8	0.28
Monolayer	30	0.50	0.25	5.8	0.24
$T_t=41$ °C [b]	50	0.41	0.25	5.7	0.24

Experimental conditions: 6.8×10^{-4} M lipid, 10 mM imidazole, pH 7.3, 1.5 M KCl and 5.3×10^{-1} M ANS. *Tau* values were measured in 0.1 M KCl. The experimental uncertainty was 10% unless otherwise indicated.

[a] Determined from Eq. (3), using the experimental values of *tau* and $tau_0=24$ nsec.
[b] Taken from reference [21].
[c] In presence of 0.3 M $MnCl_2$.
[d] There is also evidence for a small portion of a "tight" binding site at 15 °C.

3 The partition coefficient for M^+ANS^- between *n*-dibutyl ether and water is too small for the organic phase of the monolayer vesicles to make any contribution to binding. The identical values of $[B]_t$ for monolayer and bilayer vesicles both above and below the phase transition temperature can thus be taken as evidence that all of the lipid molecules in the monolayer preparation contribute to the membrane surface and that ANS$^-$ has access to both sides of the bilayer vesicle membrane. In both preparations the degree of ANS$^-$ binding is essentially independent of whether the ANS$^-$ is added before or after the sonication.

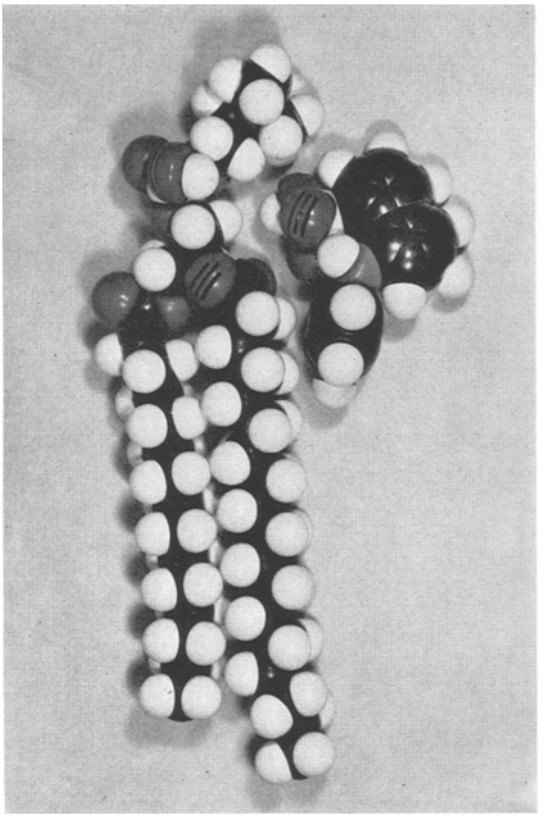


Fig. 3a

Fig. 3. Steric relationships between ANS^- and PC shown with space-filling models. In (*a*) the ANS^- molecule is shown beside the PC. In (*b*) one ANS^- molecule is packed between two PC molecules

of bound ANS^- is high, indicating an environment of rather low "polarity" similar to that of methanol.

The total number of binding sites is independent of temperature and hydrocarbon chain length, in agreement with the binding site model discussed above. However, the binding constants and quantum yields for dipalmitoyl PC are slightly lower than the corresponding values for dimyristoyl PC, indicating that the length of the hydrocarbon chains can exert a minor influence on the polar head groups. It will be shown below that the binding constants and spectral properties are very sensitive to the nature of the polar head group.

The values of Q given in Table 1 are substantially greater than those determined previously [14, 25]. The source of the discrepancy lies in the

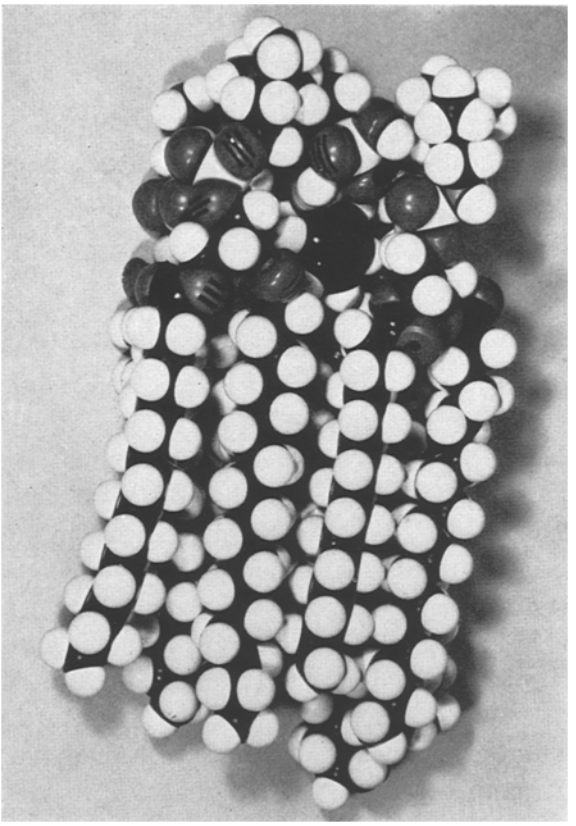


Fig. 3b

fact that the older values were determined by the method of extrapolation of binding data for variable lipid titration. This method is inherently less accurate since less than 1/3 of the ANS$^-$ was bound at the highest usable lipid concentrations, and the determination appeared to be influenced by a reversible vesicle aggregation reaction for lipid concentrations above 1×10^{-3} M.

The quantum yield of the bound form of ANS$^-$ is determined as tau/tau_0. The rate of fluorescence emission $1/tau$ is given as the sum of all quenching processes:

$$1/tau = 1/tau_0 + k_b + \sum_i^n k_i * c_i \qquad (8)$$

where the quenching contributions have been divided formally into contributions from the environment of the bound ANS$^-$ (k_b) and dynamic contributions ($k_i * c_i$) arising from the ability of the binding place to transform

during the lifetime of the excited state. The latter contribution could be determined by the mobility of the polar head groups and the accessibility of H_2O to the ANS^-.

The quantum yield of ANS^- bound to PC membranes was found to be insensitive to pH at values between 3 and 10. This indicates that proton transfer in the excited state makes little contribution to the quenching process. The quantum yield is also insensitive to the concentration of the salts and buffers in the vesicle suspension. Thus, the inequality between $1/tau$ and $1/tau_0$ must result from quenching processes involving either the polar head groups or the solvent water. Brand and Gohlke [2] have proposed that the quantum yield of aryl naphthylene sulfonates gives a measure of the propensity of the environment for quenching via the mechanism of dipole relaxation, during the excited state of the molecule. The dynamic quenching contribution would thus depend upon the strength of these dipole interactions with the excited state, and upon whether these dipoles can reorient in times equal to or shorter than the natural lifetime of the excited state, $tau_0 = 24$ nsec. Daycock *et al.* [9] have given evidence for motion within the choline head groups in dipalmitoyl lecithin membranes with a correlation time of less than 10^{-8} sec. Although this motion may make some contribution to the quenching process, it is more likely that the largest contribution to the quenching reaction is made by reorientation of water in the environment of the ANS^-. Changes in the packing of ANS^- between the polar head groups, and changes in the polar head group mobility would thus express themselves through changes in the accessibility of water to the ANS and the reduction of Q. We would thus adhere to the dipole relaxation interpretation of Brand and Gohlke [2], and consider the difference between $1/tau$ and $1/tau_0$ as a measure of the "looseness" and accessibility of water to ANS^- in the hydrophobic binding site. With this interpretation at hand, we looked at the effect of the crystalline-liquid crystalline phase transition on the quantum yield of bound ANS^-.

Fig. 4 shows the temperature dependence of $1/tau$ for ANS^- bound to dimyristoyl PC monolayer vesicles. For low temperatures, the value of $1/tau$ remains constant, and the rate of the quenching processes is approximately 3 times as great as the "natural" rate of flourescent decay ($1/tau_0$). Above 16 °C, the rate of the quenching processes begins to increase with increasing temperature, such that it is doubled at 50 °C. We interpret these effects as follows: Below 16 °C the mobility of the polar head groups is low. Above 16 °C the mobility of the polar head groups increases, allowing an increase in the accessibility of solvent water to the ANS and allowing the contribution of water reorientation to the relaxation of the excited state to increase

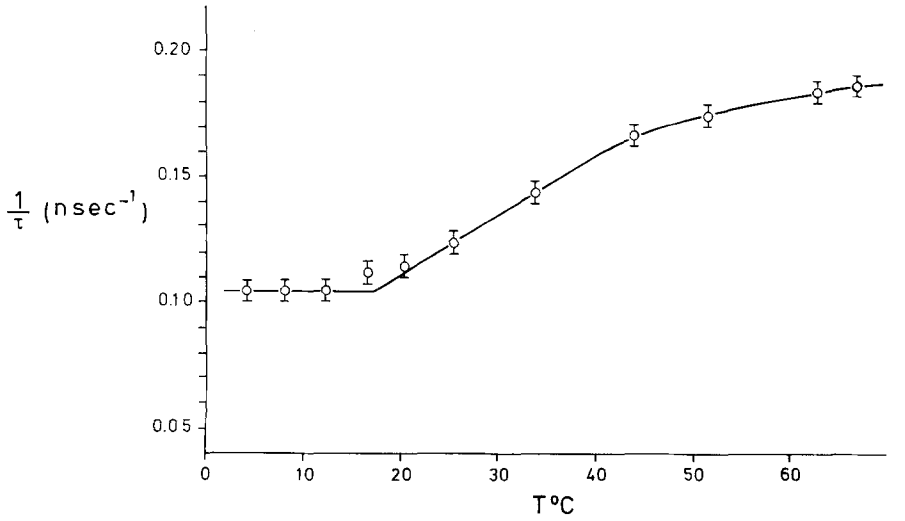


Fig. 4. The temperature dependence of $1/tau$ of ANS^- in dimyristoyl lecithin monolayer vesicles. Experimental conditions: 6.8×10^{-4} M lipid, 5.0 mM imidazole buffer, pH 7.3, 0.3 M KCl, 2.6×10^{-6} M ANS

with increasing temperature. NMR studies indicate that the polar head group mobility of dipalmitoyl PC, as indicated by the width of the N-$(CH_3)_3$ proton resonance, increases with temperature above the phase transition temperature [4].

The polarization of the fluorescence of ANS^- is constant (0.068 ± 0.012) over this temperature range. The rotational correlation times, which would be proportional to the $(tau*P)$, decrease with increasing temperature, indicating that the increase in the quenching rate of ANS^- is accompanied by an increase in the freedom of motion of the bound ANS^-. The concomitant effects of increasing ANS^- mobility and increasing water accessibility could be the result of a general loosening up of binding sites.

This conclusion is supported by temperature-jump relaxation experiments [13], which compared the bimolecular rate constants for the binding reaction of ANS in dimyristoyl PC monolayer vesicles above and below the phase transition temperature. The rate constant for the overall binding reaction at 15 °C is two orders of magnitude slower than at 30 °C, indicating that at the lower temperature the rate of binding was limited by the lower mobility of the polar head groups.

Experiments to measure the temperature-dependence of *tau* for ANS^- in dipalmitoyl PC monolayer and dimyristoyl PC bilayer preparations gave results which were similar to those of Fig. 4. An increase in $1/tau$ is observed

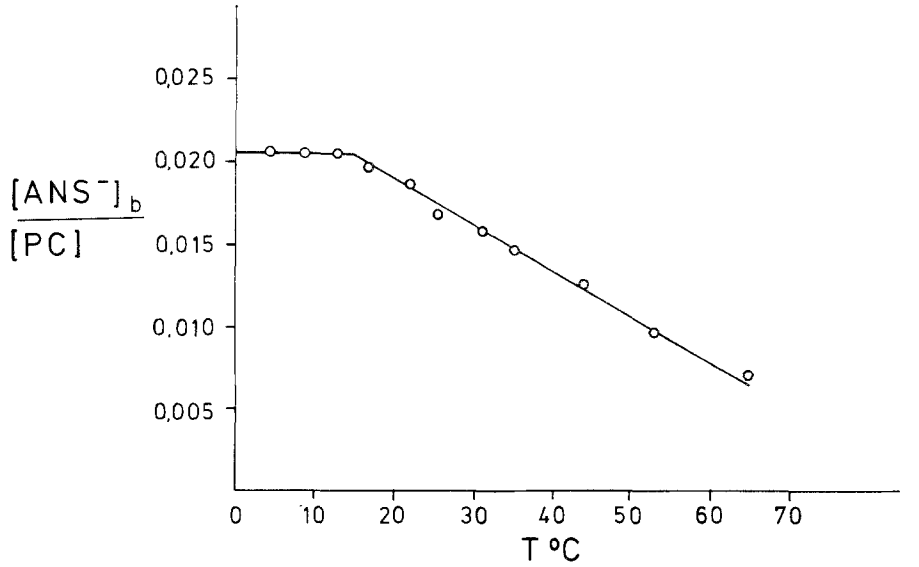


Fig. 5. The temperature dependence of the degree of ANS^- binding in dimyristoyl lecithin monolayer vesicles. Experimental conditions: 6.8×10^{-4} M lipid, 5.0 mM imidazole buffer, pH ca. 7.3, 0.3 M KCl, 8.6×10^{-6} M ANS. Degrees of binding were calculated from the fluorescence amplitude, using the quantum yields calculated from the data of Fig. 4

about 7 degrees below T_t, and a linear increase is observed for this parameter with increasing temperature for temperatures substantially above T_t. The slope of this portion of the curves was 0.0019 ± 0.0001 $nsec^{-1}$ deg^{-1} for all three preparations, suggesting similarity in the surface properties of the membranes above the phase transition temperature. Subtle differences in the temperature-dependence of $1/tau$ were observed in the region of the phase transition, and deserve further study.

Fig. 5 shows that the effect of temperature on $1/tau$ is paralleled by an effect on the binding constant. The increased mobility of the polar head groups is correlated with a decrease in the hydrophobic energy of binding, indicating binding in a less ordered structure at higher temperatures.

The onset of temperature dependence of both $1/tau$ and K_{10} which begins 7 degrees below the phase transition temperature of the membrane (23 °C; *cf.* [25]) correlates with endothermic transitions which have been observed 5 to 10 °C below T_t (*cf.* [15]). It is concluded that the loosening of the polar head groups thus precedes the disordering of the hydrocarbon chains and the lateral expansion of the membrane (*cf.* [27]).

The ANS^- fluorescent signal has been used to report the phase transition of dipalmitoyl PC membranes [25]. In our experiments, this has been

observed only under conditions of low ionic strength and high degrees of ANS^- binding where the membrane surface potential is large.[1] The lateral expansion of the membrane decreases the surface potential which is produced under these experimental conditions allowing a higher degree of ANS^- binding. However, the phase transition, *per se*, does not produce substantial changes in K_{10} or Q, and $[B]_t$ is left entirely unaffected.

Influence of Phosphatidic Acid

ANS^- demonstrates practically no binding to vesicles composed solely of phosphatidic acid (PA), even at high ionic strengths (1.5 M KCl or 50 mM $CaCl_2$) where electrostatic effects are negligible. The absence of the choline group to provide a hydrophobic binding site and the high energy necessary for insertion of the probe between the hydrocarbon chains are probably responsible for the lack of effect. Faced with this negative result, we turned our attention to PC – PA mixtures. Table 2 shows the effect of PA incorporation on K_{10}, $[B]_t$, *tau* and Q. Increasing the PA content leaves the quantum yield of the bound form essentially unaltered, and produces, at most, a 50% decrease in the binding constant. The most dramatic effect is the reduction of $[B]_t$ to as little as 6% of its initial value, and the following analysis will be directed at explaining this finding.

Cosonication of two lipids *a* and *b* can result in three types of mixing behavior: (a) The two types of lipid may be ideally mixed on the microscopic scale. (b) The two types may be only partially mixed on the microscopic scale. (c) The membranes may exist in a mosaic of microdomains of each type of lipid in its pure form. In the latter case, the ANS^- binding would

Table 2. Effect of phosphatidic acid content of dimyristoyl lecithin vesicles on ANS binding parameters at 30 °C

Mole ratio PC:PA	K_{10} ($\times 10^{-4}$ M^{-1})	$[B]_t$ experimental	$[B]_t$ no mixing	$[B]_t$ ideal mixing	*tau* (nsec)	Q
10:0	0.75 ± 0.10	0.25 ± 0.05	–	–	9.0	0.31
9:1	1.08 ± 0.20	0.13 ± 0.03	0.225	0.163	6.2	0.22
8:2	0.66 ± 0.06	0.07 ± 0.02	0.200	0.102	6.4	0.26
7:3	0.66 ± 0.06	0.07 ± 0.02	0.175	0.060	6.1	0.27
5:5	0.40 ± 0.10	0.016 ± 0.002	0.125	0.0154	5.6	0.23

Experimental conditions were identical to those of Fig. (1). Theoretical values of $[B]_t$ for the cases of no mixing and ideal mixing were calculated from Eqs. (9) and (10), respectively. Q values were calculated from *tau* values, using $tau_0 = 24$ nsec. The ± quantities are the standard deviation of four separate experiments.

Table 3. Effect of PA on *tau* in dimyristoyl lecithin phosphatidic acid mixtures

Mole fraction PA	[KCl] (M)	[$CaCl_2$] (M)	$tau_{15°}$ (nsec)	$tau_{30°}$ (nsec)	$tau_{50°}$ (nsec)
0.0	(no significant salt effect)		9.0	7.5	5.7
0.1	0.0	0.0	7.5	6.0	5.4
0.1	0.3	0.0	8.4	6.2	5.7
0.1	0.3	0.008	7.3	6.1	5.7 (5.7)[b]
0.2	0.0	0.0	6.8	6.0	5.8
0.2	0.3	0.0	7.3	6.4	5.6
0.2	0.3	0.008	7.8	6.3	5.7
0.3	0.0	0.0	–	5.4	–
0.3	0.3	0.0	–	6.1	–
0.3	0.3	0.008	–	6.6	–
0.5	0.0	0.0	7.0	5.5	5.5
0.5	0.3	0.0	6.1	5.6	5.6
0.5	0.3	0.008	5.7	5.7 (5.8)[a]	5.8 (5.3)[b]

The measurements were made in 6.8×10^{-4} M monolayer vesicle suspensions, containing 10 mM imidazole buffer, pH 7.3, and 5.3×10^{-5} M ANS. The experimental uncertainty in the *tau* values was about 15%.

[a] $MnCl_2$ was added to a final concentration of 0.3 M.

[b] $LaCl_3$ was added to a final concentration of 3 mM.

simply be a linear sum of the contributions of the two separate phases, and the number of binding sites per lipid molecule would be given as:

$$[B]_t = (1-f) * [B]_a + f * [B]_b \tag{9}$$

where $[B]_a$ and $[B]_b$ represent the number of binding sites per lipid molecule for membranes of pure "*a*" and "*b*", respectively, and where f is the mole fraction of the lipid type *b*. Table 3 shows that the decrease in the measured number of binding sites is much smaller than that calculated on the basis of nonmixing according to analysis by Eq. (9). The PC and PA are therefore at least partially mixed on the microscopic scale. The behavior expected for the case of ideal mixing depends upon the assumptions made about the way the presence of lipids of type *b* affects the *a* binding sites. If it is assumed that the degree of mixing is not affected by the ANS^- binding, and that the presence of one or more *b* molecules in a four-membered binding site renders this site incapable of contributing to ANS binding (*cf.* Fig. 2-III), then the number of binding sites in the mixture will be given as:

$$[B]_t = [B]_a * (1-f).^4 \tag{10}$$

Table 2 shows good agreement between the values for the number of binding sites per lipid molecule experimentally determined and the number calculated from Eq. (10). The assumption that the incorporation of a PA molecule renders each of the nearest neighboring lecithin molecules incapable of contributing to ANS binding thus seems to be adequate to explain the results, and it is concluded that the lecithin and PA are mixed ideally on the microscopic scale.[4] The relatively small decrease in the binding constant and quantum yield can be explained on the basis of next to nearest neighbor interactions of the PA which tend to disorder the PC polar head groups within the binding site, making the ANS more accessible to water molecules and decreasing the hydrophobic interaction energy with the ANS^-.

Table 3 shows the effect of PA content and ionic composition of the medium on *tau*. PA addition produces a small decrease in *tau*, with the maximal effect about 33%. Sequential addition of KCl and $CaCl_2$ to final concentrations of 0.3 M and 8 mM, respectively, results in an increase of *tau* above that of the value for low ionic strength. This effect is 15% or smaller. Expansion and contraction of the membrane as a function of the membrane surface potential is a possible explanation for this effect. Supporting this interpretation is the observation that the effects of PA addition and ionic strength decrease with increasing temperature. As noted above, thermal expansion of the membrane results in a lower *tau* value for the bound species. A more careful look at the dependence of *tau* on the PA content and ionic composition of the medium may provide important information about the nearest neighbor interactions of PA with the choline head-groups of the lecithin binding sites.

Phosphatidyl Ethanolamine

We could find no evidence for ANS^- binding to dimyristoyl phosphatidyl ethanolamine (PE). Attempts were made at temperatures between 5 and 70 °C, pH values between 3.0 and 10.0, and ANS^- and lipid concentrations up to 10^{-3} M. The phase transition temperature of dimyristoyl PE has been reported as about 77 °C (*cf.* [25]), a value 54 degrees higher than that of its PC analogue. This could be explained as the result of strong interaction between the polar head groups involving a folded polar head group conformation [20]. Possible formation of intermolecular hydrogen bonds between the amine group at the end of the polar head group and the phosphate residue of a neighboring polar head group would tend to stabilize

4 There was no evidence for ANS^- species with short lifetimes which might be identified with binding to four-membered sites containing one or more PA molecules.

Table 4. Effect of temperature and dimyristoyl PE content on ANS binding parameters for dimyristoyl lecithin monolayer vesicles

Ratio mole ratio PE/PC	T (°C)	K_{10} ($\times 10^{-1}$ M^{-1})	$[B]_t$	$K_{10}*[B]_t$ ($\times 10^{-3}$ M^{-1})	Q	*tau* (nsec)
10:0	15	9.5	0.25	2.37	0.37	9.0
	30	7.5	0.25	1.87	0.31	7.5
	50	6.5	0.25	1.62	0.24	5.7
			(0.164) [a]			
9:1	15	4.0 ± 0.5	0.22 ± 0.07	0.88 ± 0.28	0.37 ± 0.05	—
	30	7.0 ± 1.0	0.23 ± 0.07	1.6 ± 0.5	0.27 ± 0.05	—
	55	5.0 ± 0.5	0.22 ± 0.07	1.1 ± 0.3	0.12 ± 0.02	—
			(0.060) [a]			
7:3	15	1.2 ± 0.2	0.15 ± 0.01	0.180 ± 0.012	0.37 ± 0.05 [b]	—
	30	6.9 ± 0.3	0.084 ± 0.020	0.579 ± 0.130	0.27 ± 0.05 [b]	—
	55	6.5 ± 0.1	0.098 ± 0.020	0.637 ± 0.130	0.12 ± 0.02 [b]	—
			(0.0064) [a]			
4:6	15	9.7 ± 0.7	0.0028 ± 0.008	0.027 ± 0.002	0.395	ca. 9.5 [c]
	30	7.3 ± 0.5	0.010 ± 0.003	0.073 ± 0.005	0.310	ca. 7.5 [c]
	50	3.4 ± 0.4	0.026 ± 0.006	0.088 ± 0.010	0.240	ca. 5.8 [c]
	60	4.9 ± 0.5	0.016 ± 0.003	0.078 ± 0.008	0.240	ca. 5.8 [c]

Experimental conditions were identical to those of Table 1.

[a] Calculated for the case of ideal mixing according to Eq. (10).

[b] Determined by the method of lipid titration.

[c] Small degrees of binding and the resultant small signals produced about a 20% uncertainty in *tau*.

the condensed state of the membrane. If all of the polar head groups were hydrogen bonded the polar head group region would be much thinner (*cf.* [20]) than for membranes made of the corresponding lecithin, and there would be less opportunity for hydrophobic interaction and shielding of ANS^-. Furthermore, cross-linking between the polar head groups in the four-membered binding sites might structurally hinder ANS^- binding. Attempts to make PE a better host for ANS^- by adding cholesterol to the membrane were unsuccessful.

Experiments were performed to test the effect of PE addition on ANS^- binding to lecithin vesicles. Table 4 shows that PE incorporation in PC membranes does not appreciably reduce the quantum yield values of the bound species, indicating that the shielding of ANS^- in these binding sites is about the same as in PC binding sites, and that the amine groups of the PE do not quench the excited state of ANS^-. The major effect of PE in-

corporation is to decrease the degree of ANS$^-$ binding, as indicated by the decrease in the product $K_{10}*[B]_t$, compared with pure dimyristoyl PC. Above the phase transition temperature of the host lecithin, both the value of K_{10} and $[B]_t$ are decreased by the PE incorporation. The number of binding sites per lipid molecule is decreased, to an extent greater than predicted by Eq. (9), indicating appreciable mixing of the two types of lipid. However, the value of $[B]_t$ is significantly greater than that predicted by Eq. (10) under the assumption that one PE molecule destroys one binding site. There are three avenues of interpretation for this: (a) The assumptions of Eq. (10) hold and the PE and PC are not ideally mixed. (b) The PC and PE are ideally mixed, but the incorporation of PE within a PC binding site does not always destroy the ability of this site to bind ANS. (c) The PC and PE may be initially ideally mixed, but their degree of mixing (or polar head group orientation) is influenced by the ANS$^-$ binding reaction itself. The second and third possibilities are shown schematically in Fig. 2-IV. The assumption that polar head group orientation or hydrogen bonding directing the polar group away from the quadratic binding site leaves the binding site able to act as a host would produce positive deviations from Eq. (10). If the probability of any given PE polar head group being directed away were 0.5 (random orientation), then f in Eq. (10) would be replaced by $(0.5*f)$, and good qualitative agreement with the second interpretation is obtained. However, the decreases in K_{10} observed in some cases, as a result of PE incorporation indicate that the degree of mixing or polar head group orientation may itself be influenced by the ANS$^-$ binding. The above three interpretations cannot be distinguished for PC – PE mixtures on the basis of the present equilibrium data, but will be discussed in conjunction with the kinetic behavior of ANS$^-$ binding in these mixtures in the last paper of this series.

Low quantum yields (0.015 to 0.025) have been reported for ANS bound to vesicles prepared from a lipid mixture containing 82% PE, 11.1% cardiolipin and 7.2% phosphatidyl glycerol with saturated and unsaturated hydrocarbon chains of a variety of lengths [28]. In the present study with dimyristoyl PC and PE the lifetime of the bound species is high, and there was no evidence for ANS$^-$ species with lifetimes shorter than those given in Table 4.

Cholesterol-Lecithin Mixtures

The effect of cholesterol on lecithin membranes has been investigated in numerous electron spin resonance (ESR) and NMR studies. (*cf.* [4, 8, 23] and references therein). The important findings in these studies are that

Table 5. Effect of cholesterol addition on the *tau* value of the long-lived species in dimyristoyl and dipalmitoyl lecithin vesicles

Lecithin	Mole ratio cholesterol/PC	$tau_{15°}$ (nsec)	$tau_{30°}$ (nsec)	$tau_{50°}$ (nsec)
Dimyristoyl	0.00	9.0	7.5	5.7
	0.13	9.7	7.0	6.4
	0.26	9.4	6.9	5.7
	0.50	7.0	6.0	5.5
	1.00	5.8	5.1	5.1 ± 0.5 (5.8)[a]
Dipalmitoyl	0.00	6.8	5.8	5.7
	0.13	8.4	7.0	6.4
	0.26	7.2	8.7	5.6
	0.50	9 ± 2[b]	7.0	6.4
	1.00	9 ± 2[b]	6.2	9 ± 2[b]

Experiments were performed in solution with 6.8×10^{-4} M lipid, 10 mM imidazole, pH 7.3, 0.1 M KCl and 5.3×10^{-1} M ANS. The experimental uncertainty in *tau* is 10% unless otherwise indicated.

[a] In the presence of 0.3 M $MnCl_2$.

[b] Low signal intensities resulted in a greater uncertainty in *tau*.

above the phase transition temperature, cholesterol reduces the motion of the hydrocarbon chains and below the phase transition temperature, it increases the motion. These conclusions are supported by a Raman study [19] in which the probability of *gauche* and *trans* conformations in the hydrocarbon chains was evaluated. The conclusion is that cholesterol is incorporated in the hydrocarbon chain region and exerts its major effects there [4, 8, 16, 23]. NMR studies have shown that the effect on the mobility of the polar head groups is small [4, 5]. On the other hand, ANS^- fluorescence is very sensitive to incorporation of cholesterol into lecithin membranes.

Addition of cholesterol to dimyristoyl or dipalmitoyl PC results in a decrease in ANS fluorescence. We have found that the fluorescent signal in cholesterol lecithin mixtures is always dominated by that of a long-lived species, and that increasing the cholesterol concentration decreases the population of this species. Table 5 shows the effect of cholesterol addition on the lifetime of the long-lived species. Cholesterol addition to dimyristoyl PC produces a slight ($\leqq 35\%$) decrease in the *tau* value, with the magnitude of this effect decreasing with increasing temperature. This could be attributed to a loosening of the polar head groups of the binding site as the

result of nearest-neighbor interactions with the cholesterol. With dipalmitoyl PC, increases in *tau* up to 60% were observed upon cholesterol addition, but this was not a monotonous function of the cholesterol content. It thus seems that the influence of cholesterol on the shielding of the ANS bound in the four-membered binding sites depends upon the length of the lipid hydrocarbon chain region in which the cholesterol is buried.

The analysis of the binding behavior of the long-lived species (Table 6) indicates that the degree of binding is decreased to a greater extent than would be expected from the decrease in mole fraction of lecithin. This indicates that cholesterol tends to mix ideally with the lecithin. The greater than 50% decrease in the degree of binding for a mole ratio of cholesterol of 0.135 indicates that there is little tendency for the cholesterol to form separate lecithin phases with a 1:1 lecithin cholesterol stoichiometry. Since the effect of cholesterol addition is to influence both the number of binding sites and the binding constant K_{10} (particularly for dimyristoyl PC), it is not possible to analyze quantitatively the degree of mixing with Eq. (10). The observed decreases in K_{10} resulting from cholesterol incorporation can be explained if it is assumed that the lecithin and cholesterol are originally well mixed, and that ANS^- binding tends to separate the lecithin from the cholesterol. In this case, the variable of interest is the degree of binding, given as the product $K_{10} * [B]_t$. Analysis of the product $K_{10} * [B]_t$ as a function of cholesterol content gave only qualitative agreement with the expectations of Eq. (10) under the assumption that intercalation of a planar cholesterol molecule between two lecithin molecules within a binding site (*cf.* Fig. 2-I) would destroy the site. Thus the ANS^- experiments tell us that the cholesterol and lecithin molecules are mixed, on the microscopic scale, but it is not possible to state if the mixing is perfectly random.

In several experiments, not shown here, there was evidence for formation of two phases, a lecithin and a 1:1 lecithin cholesterol phase [8], based on the ANS^- binding criteria discussed above. This was observed either when the dispersions were initially poor, or when the samples were aged. Poor dispersions (by the criterion of 90° light scattering) were obtained when the duration of the sonication was too short, or when the temperature was below T_t for either of the components. Aging the preparations several days, especially below the phase transition temperature, results in an irreversible aggregation (criterion: 90° light scattering) with an accompanying separation of the components within a vesicle or redistribution of material between the vesicles. However, in fresh preparations the constituents are more or less randomly mixed and there was no evidence for the formation of two phases upon cholesterol addition.

Table 6. Effect of cholesterol on the binding-site behavior of dimyristoyl and dipalmitoyl lecithin membranes

Lipid	Ratio cholesterol/PC	T (°C)	K_{10} ($\times 10^{-3}$ M)	$[B]_t$	$K_{10}*[B]_t$ ($\times 10^{-3}$ M)	Q
Dimyristoyl	0.00	15	9.5	0.25	2.37	0.37
	0.00	30	7.5	0.25	1.87	0.31
	0.00	50	6.5	0.25	1.62	0.24
				(0.148) [a]		
Dimyristoyl	0.135	15	3.2	0.053	0.169	0.40
	0.135	30	3.8	0.050	0.190	0.29
	0.135	50	4.8	0.0945	0.453	0.27
				(0.0999) [a]		
Dimyristoyl	0.270	15	0.8	ca. 0.25	0.20	0.39
	0.270	30	1.00	ca. 0.86 [b]	0.86	0.29
	0.270	50	9.99	ca. 0.25	2.50	0.24
				(0.0493) [a]		
Dimyristoyl	0.50	15	3.9	0.026	0.100	0.29 (0.032) [c]
	0.50	30	2.8	0.027	0.076	0.25 (0.032) [c]
	0.50	50	5.5	0.022	0.120	0.23 (0.032) [c]
				(0.0156) [a]		
Dimyristoyl	1.0	15	4.0	0.021	0.084	0.24 (0.011) [c]
	1.0	30	4.0	0.024	0.096	0.21 (0.011) [c]
	1.0	50	—	—	—	0.21 (0.011) [c]
Dipalmitoyl	0.00	15	1.0 ± 0.3	0.25	0.25	0.28
	0.00	30	5.0	0.25	1.25	0.24
	0.00	50	4.1	0.25	1.02	0.24
				(0.148) [a]		
Dipalmitoyl	0.135	15	3.62	0.15	0.543	0.35
	0.135	30	3.23	0.19	0.613	0.29
	0.135	50	3.04	0.20	0.608	0.27
				(0.0999) [a]		
Dipalmitoyl	0.270	15	9.6	0.012	0.115	0.30
	0.270	30	ca. 6	ca. 0.02	0.12	0.36
	0.270	50	ca. 1.1	ca. 0.61 [b]	0.67	0.23
				(0.0493) [a]		
Dipalmitoyl	0.500	15	4.2	0.021	0.088	0.37
	0.500	30	2.6	0.045	0.117	0.29
	0.500	50	1.7	0.062	0.105	0.27

Experimental conditions were identical to those of Table 1.

[a] For ideal mixing, calculated according to Eq. (10).

[b] Although the value of $[B]_t$ calculated by the computer analysis is higher than 0.25, the theoretical maximum, the highest degree of ANS binding experimentally observed was 0.10/lipid, or less than half of the theoretical maximum.

[c] Values in parentheses were determined by the procedure of lipid titration, reporting the average quantum yield for the total ANS bound.

The destruction of the binding site for the long-lived ANS$^-$ species upon the addition of cholesterol is probably the result of the spacing-out of the polar head groups by the insertion of the cholesterol between the hydrocarbon chains. The modified binding site would afford less shielding from the solvent water, resulting in diminished *tau* and *Q* values for the bound ANS$^-$. There are three types of evidence for these shorter-lived bound species: (a) Comparisons of the amplitude of the fluorescent decay curve in the time domain of the excitation pulse were made for ANS$^-$ in water and for ANS$^-$ in the presence of 0.6 and 1.0 mole ratio cholesterol-lecithin mixtures. These showed that as much as 1/3 of the Fl_t under these conditions, arises from species with lifetimes shorter than 1 nsec. (b) Dipalmitoyl PC-cholesterol mixtures with mole ratios of 0.6 and 1.0 showed evidence for a spectrum of short-lived species with lifetimes up to about 2 nsec, in addition to the long-lived species. (c) Steady-state titrations of a constant concentration of ANS$^-$ with increasing concentrations of dimyristoyl PC-cholesterol vesicles (mole ratio 0.6 and 1.0) indicate the presence of short-lived species. For dimyristoyl lecithin cholesterol with mole fraction 0.6, the quantum yield of this form is about 0.03, corresponding to a fluorescence lifetime of about 0.6 nsec. For a mole fraction of 1.0, the quantum yield and calculated lifetime are 0.01 and 0.2 nsec, respectively. Thus, ANS$^-$ can bind to sites containing cholesterol but in a much less shielded form.

Quenching with Mn^{2+}

Addition of Mn^{2+} to solutions of ANS$^-$ in water, methanol and ethanol results in quenching of the fluorescence. The high diffusion-controlled bimolecular rate constants of about 10^{10} M^{-1} sec^{-1} calculated for this process (Table 7) indicate that inner sphere coordination is not necessary for quenching, and that the quenching proceeds upon formation of an

Table 7. Paramagnetic quenching of ANS$^-$ by Mn^{2+}

Solvent	K_q[a] $(=k_1/k_f)$ (M^{-1})	*tau* (nsec)	k_1[b] (M^{-1} sec^{-1})
H_2O	1.25×10^1	0.55	2.3×10^{10}
MeOH	8.8×10^1	6.05	ca. 1.4×10^{10}
EtOH	15.8×10^1	8.85	ca. 1.8×10^{10}

In MeOH and EtOH, the maximal quenching at high concentrations of Mn^{2+} is only 50%. This will be discussed in a future publication.

[a] Calculated as the Mn^{2+} concentration giving half maximal quenching.

[b] Calculated as $k_1 = K_q/tau$.

encounter complex. The mechanism of quenching most probably involves paramagnetic interaction with the excited singlet state of ANS^- to produce a nonfluorescent triplet state, but energy transfer to the Mn^{2+} cannot be ruled out. Further investigations are planned to resolve this question. However, even without a knowledge of the exact quenching mechanism, it is possible to use the quenching reaction to study the interaction of the bound form of ANS^- with cations.

Reference to Tables 1, 3 and 5 shows that the addition of Mn^{2+} to a final concentration of 0.1 or 0.3 M has no effect on the lifetime of the excited state of the long-lived ANS^- species in dimyristoyl PC or its mixtures with PA or cholesterol. From the lack of effect we were able to calculate that the bimolecular rate constant (k_1) for reaction of Mn^{2+} with the bound form of ANS^- is lower than 6×10^7 M^{-1} sec^{-1}, a value which is two orders of magnitude lower than the rate constant for a diffusion-controlled bimolecular collision shown in Table 7. Thus, the polar head groups shield the ANS^- well from the Mn^{2+} in the aqueous phase, even in the dimyristoyl PC-cholesterol mixtures with a mole ratio of 1.0. It is shown in the second paper of this series that the alkali and alkali earth cations have little tendency to bind to the phosphate groups of lecithin. The divalent cations (but not the monovalents) bind with fairly high affinity to the phosphate groups of phosphatidic acid groups. But even when Mn^{2+} is bound to PA molecules neighboring the four-membered lecithin sites, the ANS^- is not quenched. The lack of quenching with Mn^{2+} thus serves to emphasize the properties of ANS^- as a probe for lecithin hydrophobic binding sites.

Effects of Local Anesthetics

It was shown previously [13] that semi-polar molecules can either enhance or compete with ANS^- binding depending on their molecular size. Procaine and benzyl alcohol can enhance ANS fluorescence at low concentrations. The data of Table 8, which show only minor effects of these compounds on the quantum yield, indicate that the enhancement was due to an increase in binding. This is probably due to an alteration of the polar head group packing resulting from binding in the sites neighboring the ANS^- binding sites.

Above the phase transition temperature, procaine and benzyl alcohol at concentrations of 2.5 mM produce essentially no change in the *tau* value of ANS^- in dipalmitoyl PC monolayer vesicles. Below the phase transition temperature, they produce small decreases (13% and 5%, respectively) in the *tau* value, indicating that they increase the mobility of the polar head

Table 8. Effect of valinomycin and local anesthetics on *tau*

Lipid	Addition	T (°C)	*tau* (nsec)
Dimyristoyl lecithin, monolayer	none	15	9.0
	valinomycin (2×10^{-5} M)	15	6.2
Dimyristoyl lecithin, monolayer	none	31	7.2
	valinomycin (2×10^{-5} M)	31	5.7
Dipalmitoyl lecithin, monolayer	none	15	6.8
	benzyl alcohol (2.5 mM)	15	6.5
	procaine (2.5 mM)	15	5.9
Dipalmitoyl lecithin, monolayer	none	51	5.7
	benzyl alcohol (2.5 mM)	51	6.0
	procaine (2.5 mM)	51	5.8

Experimental conditions: 4.0×10^{-4} M lipid, 0.3 M KCl, 10 mM imidazole buffer, pH 7.3, 5.3×10^{-5} M ANS.

groups of the ANS$^-$ binding sites. The influence of the local anesthetics on the rate of ANS$^-$ binding will be discussed in a future publication.

With larger molecules, such as 2′-amino-*p*-toluene-sulfonanilide, fluorescein and acridine orange competition with ANS$^-$ is observed suggesting that ANS$^-$ can be used as a general method for testing for the binding of organic molecules.

Valinomycin

Table 8 shows that the addition of valinomycin to a final concentration of 2×10^{-5} M to dimyristoyl PC monolayer vesicles ([lipid] = 4×10^{-4} M) produces a modest decrease in *tau* (9.0 to 6.2 nsec) below the phase transition. This indicates that binding of valinomycin to the membrane surface [14] at a concentration of one molecule per 20 lipid molecules, can cause a certain degree of disorder in the polar head group region. Above the phase transition temperature where the valinomycin can penetrate the hydrocarbon chain region, the effect on the *tau* value is smaller.

Conclusions

ANS$^-$ is a probe for the structure in the polar head group region of membranes. It binds to lecithin membranes in sites composed of four polar

head groups, and reports the mobility and accessibility of water to this region through the variation of its quantum yield and binding constant. PA, which lacks a suitable polar head group, and PE, whose polar head group is apparently in an unfavorable conformation, do not support ANS^- binding in the pure form. This property has been exploited to measure the degree of mixing of PA and PE with PC. The analysis indicates that PA and PC are mixed ideally and that PE and PC are mixed more or less ideally under all conditions of temperature and concentration. Cholesterol and PC seem to mix ideally at all mole ratios with a reduction of $[B]_t$ for the long-lived species. At high cholesterol concentrations, at least one new type of ANS^- binding site is created in which the polar head groups are spaced out and the ANS^- is less shielded from the solvent water.

The effect of cholesterol to loosen the packing of the polar head groups and decrease the surface concentration of these semipolar groups at the membrane surface may account for the stabilizing effect of cholesterol on the vesicles in regard to aggregation. The reduction of ANS^- binding (i.e. binding of semipolar molecules and permeants) resulting from PE or PA addition to PC vesicles may have consequences for nonspecific permeation, an important process in drug transport and charge transport with uncouplers of oxidative phosphorylation. A model for nonspecific permeation has been proposed in which binding to the sites on the polar head group region is the first step in the permeation sequence [13].

Our observations indicate that ANS^- can be used as an indicator for the structure of the polar head group region of phospholipid membranes containing only one type of polar head group, and that the probe can be used to measure the degree of mixing of lipids with different types of polar head groups. We predict that the fluorescent signals arising from ANS^- bound to complicated lipid mixtures [28] and to biological membranes will be to a large part due to binding in the four-membered sites formed from extended (lecithin?) polar head groups. The fluorescence changes observed in these systems may be the result of changes in the number or character of these sites. The phospholipid systems studied here are very far removed, in terms of their properties and relevance, from biological membranes. But in the present study we were motivated by the thought that one often has to come far away from the problem at hand to understand it [10].

We wish to thank Bernd Frederichs, Jörg Ronnenberg and Reinhard Sädtler for valuable technical assistance and the Journal referees for valuable criticism. D.H.H. was supported by a post-doctoral fellowship from the Damon Runyon Fund for Cancer Research.

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