Polar Head Molecular Packing of Dipalmitoylglycerophosphocholine in the Gel State: A Fluorescence Investigation

J. Teissié

Centre de Biochimie et Génétique Cellulaires du CNRS, 31062 Toulouse Cedex, France

Received August 22, 1986; Revised Manuscript Received October 24, 1986

ABSTRACT: Binding of the fluorescent probe 8-anilino-1-naphthalenesulfonate to lecithin monolayers was shown to be dependent on the molecular packing of the lipids. No binding was observed in the gel state. The binding site appeared to be structurally unaffected during the compression of the monolayer, and it was concluded that the site was an assembly of "fluid" lipids. Four lipids are in close contact with the probe, and they are surrounded by 8–10 "fluidized" others. From these observations, the dissociation constant appeared as a good indicator of molecular packing at the polar head level in other lipid assemblies. In the gel state, macrovesicles and multilayers are a tight homogeneous assembly; at the same temperature, microvesicles display defects, in a highly fluid state, which are embedded in a rigid matrix where no binding occurs. Ten percent of the outer layer are in this "nongel" organization.

Molecular packing of the constituents of a biological membrane is considered critical for the various processes occurring at the membranous level as observed for some enzymatic activities (Raison et al., 1971) or in the action of anesthetics (Ueda et al., 1977). "Model membranes", which are different forms of dispersions of lipids in water, are supposed to give a very close approach to the real system as described by the Danielli hypothesis. However, except for the case of multilamellar liposomes (MLV),[1] no definitive conclusion on the molecular organization in lipid dispersions can be obtained. NMR studies are relevant only of the major constituent. They need "a priori" definitions for the necessary computational work (Thayer & Kohler, 1981), and they lack a good sensitivity when the lipids are at a temperature where the gel state is present. Raman studies are only conclusive on the nature of the interactions. ESR and fluorescence are both dependent on the use of probes which may be disturbing for the lipid matrix (Cadenhead et al., 1975). Whatever the method is, the observed organization is strongly dependent on the "model" which is used. A major parameter of the different models appeared to be the radius of curvature of the vesicles. For very small fluid vesicles (SUV), NMR studies showed that these systems were very sophisticated (Chrzeszczyk et al., 1977; Huang & Mason, 1978). It is known that drastic changes occurred during the phase transition (Lichtenberger et al., 1982).

The definition of the molecular packing is straightforward when one considers monolayers of lipids spread at the air/water interface. One would determine the molecular packing for other models by comparing the behavior of a given property, which is molecular packing dependent, with its values in a monolayer of the same lipid(s). As shown in the NMR studies, the packing appears not to be homogeneous along the radius of a vesicle. Thus, if the observed property is well-defined in position, then this approach gives the packing at this particular level. During the last few years, fluorescence spectroscopy has been proven to be a convenient tool for the study of monolayers (Teissié et al., 1976, 1978a,b; Teissié, 1979a,b, 1981). Our results with monolayers can be compared with those of parallel studies with dispersions.

Binding of the amphiphilic dye 8-anilino-1-naphthalenesulfonate (ANS) to phospholipid assemblies was reported to be a sensitive indicator of the lipid assembly (Slavik, 1982). X-ray studies on multilayered systems (MLV) of lecithin have shown that this probe, when bound, is located at the lipid/water interface (Lesslauer et al., 1972). An increase in quantum yield and a blue shift in the emission spectra were observed during the interaction of ANS with various models (Haynes & Stark, 1974; Tsong, 1975). Thus, the monitoring of the interaction is easy and accurate. The binding is mainly due to hydrophobic forces, but it is also determined by the electrostatic interactions arising from the surface charges (Teissié, 1979b; Flanaghan & Heskett, 1973; Bakker & Van Dam, 1974; McLaughlin & Harary, 1976). Furthermore, monolayer studies have shown that the packing of the film modulates the affinity of the probe for the film (Teissié, 1979b) when the ionic strength of the subphase is kept high. ANS binding can thus be used as a probe for the molecular packing of phospholipid assemblies at the level of their polar head.

In this study, the binding parameters of ANS to dipalmitoylglycerophosphocholine (DPPC) "model membranes" (SUV, LUV, MLV, BLM, and monolayers) were systematically investigated. DPPC was choosen for the following reasons: (1) lecithin is the main component in many biological membranes. (2) Since DPPC has a well-defined chemical structure, comparative studies can be made with results obtained in different laboratories. Binding was observed under conditions of high ionic strength (0.1M NaCl) in order to minimize the electrostatic effects. The molecular packing of the host monolayer was changed from the fully expanded down to the gel states. The packing in the models at the polar head level was computed by comparison.

Materials and Methods

Chemicals. DPPC was obtained from Sigma and used without further purification after it was checked for purity by TLC. The magnesium salt of ANS (Kodak) was recrystallized from water after filtration through activated charcoal. NaCl

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; DPPC, dipalmitoylglycerophosphocholine; SUV, small unilamellar vesicles (microvesicles); LUV, large unilamellar vesicles (macrovesicles); MLV, multilamellar vesicles (liposomes); BLM, black lipid membranes (planar); NMR, nuclear magnetic resonance; ESR, electron spin resonance; TLC, thin-layer chromatography.

0006-2960/87/0426-0840$01.50/0

(Normapur grade from Prolabo) was dissolved in quartz double-distilled deionized water, and the solution was filtered through Millipore filters (0.22-μm pore size) to remove any particles. Solutions were always deoxygenated by bubbling nitrogen. The pH of the saline solutions was 5.5.

Microvesicles (SUV). Microvesicles were prepared either by sonication or by injection. The method of Seufert (1970) was followed in the first case with a slight modification which consisted of using bath sonication instead of probe sonication: the bath sonicator (Ultrasons 75 T 801) was operated for more than 2 h. The temperature of the bath was kept above the transition temperature of DPPC (41 °C). Stock solutions of microvesicles were used in the 24 h following preparation, in order to avoid aggregation troubles.

The injection method was used as described by Batzri and Korn (1973). An ethanol solution of DPPC was rapidly injected into a well-stirred 0.1 M NaCl solution at 50 °C. In order to lower the ethanol content, the suspension was then dialyzed overnight.

In either case, residual multilamellar liposomes were centrifuged out in a Spinco L50 operated 10 min at 100000g at 4 °C. This was checked by electron microscopy observation of negatively stained samples.

Liposomes (MLV). Phospholipids were dispersed in the saline solution containing a known concentration of the probe. The homogenization of the mixture was achieved at a temperature above the phase transition temperature and lasted more than 1 h in order to ensure a homogeneous distribution of the probe within the phospholipid layers. Inner filter effects were calibrated by use of a reference solution of ANS in ethanol.

Unilamellar Macrovesicles (LUV). The general procedure described by Enoch and Strittmatter (1979) was followed with some modifications. Small sonicated vesicles were first prepared at a concentration of about 15 mg/mL.

The suspension was kept at 55 °C, and a small aliquot of 0.2 M sodium deoxycholate was added to give a ratio of detergent to phospholipid of 1:3 to 1:4. The mixture was incubated at 55 °C for 30 min. The detergent was removed by a gel chromatography dialysis (Pharmacia Sephadex G25, 1 × 50 cm, 55 °C). The vesicles were concentrated by centrifugation. Electron microscopy studies showed that the size of the vesicles was 100 nm (±30 nm) in diameter; SUV are about 25–30 nm in diameter. The unilamellar character was proved by fluorescence. At a temperature lower than the transition temperature, ANS was added to the aqueous suspension of vesicles and the fluorescence signal recorded. Under such conditions, only external binding sites are accessible. The suspension was then heated at 45 °C for 30 min and then cooled back to the initial temperature. The fluorescence signal was then twice the initial one, proving that the number of binding sites on the inner layer is the same as on the outer one (Tsong, 1975).

Monolayer Fluorescence. These experiments were performed as already described (Teissié, 1979b). A front-face fluorometer of our own design was used. The exciting light (wavelength selected by an interference filter) is focused down on the air/water interface (illuminated spot diameter, 4 mm). The relative angle between the excitation and observation axis is 45°, the observation being performed perpendicularly to the interface. The emission was recorded through an interference filter by a photomultiplier tube. The signal to noise ratio was typically 50.

The observed signal was the sum of the scattered light, the fluorescence of the aqueous dispersed probe, and the fluorescence of the bound probe. The scattered light was first measured without film (we have previously shown that under our experimental conditions, spreading of the film, whatever its packing, does not affect this value). The probe was then injected and the solution stirred. Thus, we obtained the fluorescence of the aqueous phase which was shown to increase linearly with the concentration of the dispersed probe in our concentration range.

The monolayer was then spread, and the increase in emission gave the emission of the bound probe. The number of bound probes was very small as compared to the total number in the subphase; thus, the concentration in the aqueous phase was not affected.

Orientation of the absorption moment of the bound probe was determined as described elsewhere (Teissié, 1979a). This is simply obtained by using linearly incident light and by computing the ratio X/YZ of the fluorescent light emitted when the exciting beam is polarized either parallel (X) or at a 45° angle (YZ) to the monolayer. As shown in (Teissié, 1979a), this ratio, which may vary between 0 and 4, is indicative of the orientation of the absorption moment of the probe relative to the perpendicular to the monolayer. If one takes into account the fluidity of a lipid assembly, the ratio is a function of the mean orientation angle *and* of the fluctuation angle because the probe may oscillate in a cone around its equilibrium position. Any structural rearrangement of the lipid assembly is going to affect one or both of these angles and, as a consequence, this ratio. Taking into account our simulation calculus (unpublished results), which are direct derivations of the equations in Teissié (1979a), it can be concluded that a change must be larger than 10% to be indicative of any significant change in the lipid assembly.

Fluorescence Measurements. They were made on a FICA 55 spectrofluorometer (corrected) working at a resolution of 7.5 nm and coupled to a chart recorder. An Aminco Bowman fluorometer was used in some experiments. The temperature of the cell was kept constant within 0.1 °C.

The range of probe concentration which may be used is limited by the inner filter effect. Such an effect can be corrected only in an empirical way. Thus, we have tried to minimize this effect by using a narrow range of concentration (up to 32 μM, i.e., for an absorption of the sample of 0.15 if the light path is 1 cm). However, for multilayered liposomes (MLV), such a range of concentration cannot be used as the affinity of the probe for this model is very small (see Results). In this particular case, taking into account that the fluorescence of the probe either bound to liposomes or dispersed in ethanol has similar emission properties, the inner filter effect was empirically corrected by the inner filter effect observed in ethanol.

RESULTS

Binding of ANS to DPPC Monolayers. When a DPPC monolayer is spread on the surface of a micromolar ANS aqueous solution (0.1 M NaCl, pH 6, T 20 °C), a strong increase in fluorescence is observed when the film is in the liquid-expanded state. This is a confirmation of our previous observation (Teissié, 1979b). If the monolayer is continuously compressed in order to induce the phase transition of the lipid, the fluorescence emission first increases and then decreases (Figure 1A,B). However, one should take into account that the illuminated area stays constant during the experiment. If, as described in a review paper (Slavik, 1982), the ANS binding site is an assembly of four lipids, we should conclude that the number of potent binding sites present in the illuminated spot increases with the compression. In order to monitor the real

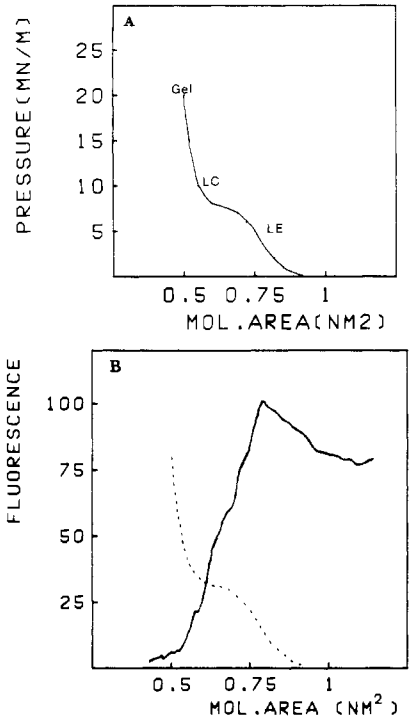

FIGURE 1: Change of bound ANS fluorescence during the compression of a DPPC monolayer. (A) Compression isotherm of a DPPC monolayer on a 0.1 M NaCl subphase with the indications of the different states of the lipid: LE, liquid expanded; LC, liquid condensed; Gel. This compression isotherm is indicated by dotted lines on all other figures concerning studies performed on monolayers. This isotherm is not affected by the presence of the amphiphile in the subphase in the concentration range used in the study. (B) A DPPC monolayer was spread on a 0.1 M NaCl/8 μM ANS, pH 6, subphase at 20 °C. Fluorescence was observed at 476 nm with excitation at 356 nm. The fluorescence of the aqueous phase was first recorded before spreading the film and was set as zero film fluorescence. The lipids were spread, and a 3-min delay was then observed. The film was then automatically compressed with a continuous monitoring of the molecular area, the film pressure, and the fluorescence.

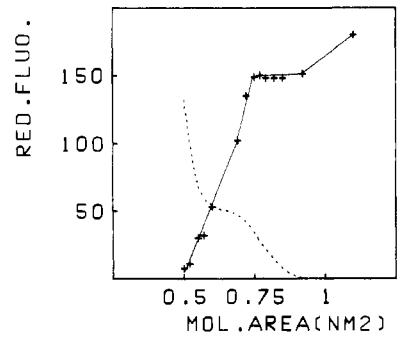

FIGURE 2: Change in "reduced" ANS fluorescence during the compression of a DPPC monolayer. Conditions are as in Figure 1. The reduced fluorescence (ordinate) is the signal emitted by each bound ANS molecule. It is easily shown to be the product of the fluorescence (observed on Figure 1) by the molecular area on the film (see text).

behavior of each bound ANS molecule, we must compute the so-called "reduced" intensity which is the product of the observed intensity by the molecular area per phospholipid. As shown in Figure 2, the reduced intensity is observed to decrease continuously as soon as the surface pressure is larger than 0.2 mN/m.

As described under Materials and Methods, any change in the orientation of the bound probe would be detected by use of polarized incident light. The characteristic ratio X/YZ does not change during the compression (Figure 4) (mean value, 1.046; standard deviation, 0.026).

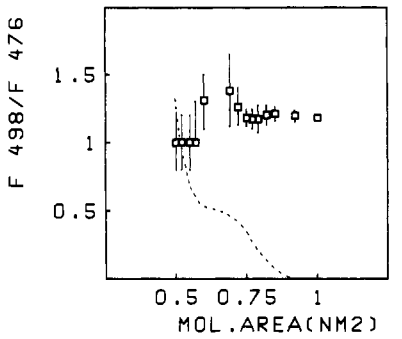

FIGURE 3: Ratio of bound ANS fluorescence observed at two different wavelengths during the compression of a DPPC monolayer. The experiment described in Figure 1 was performed successively at two different observation wavelengths (476 and 498 nm). For each molecular area of DPPC, the ratio of the observed intensities of bound probe was computed and is plotted vs. the molecular area of the host lipid. The dotted line is the compression isotherm of Figure 1A.

Continuous monitoring of the emitted fluorescence was performed during compression of the film. Thus, molecular area, surface pressure, and fluorescence were simultaneously recorded during the compression.

Taking into account the comments we made under Materials and Methods and the work in Teissié (1979a), these results do not show any significant change in the orientation parameter (fluctuations smaller than 10% of the mean value). The value close to 1 indicates that the fluctuation angle is rather large. This high fluidity of a monolayer is in agreement with our data on lateral diffusion (Teissié et al., 1978a; Theretz et al., 1984).

No major structural change affects the ANS binding site (located at the lipid/water interface) during the compression and the associated phase transition plateau. This result can be compared with our results on the orientation of 12-(9-anthroyloxy)stearic acid, which, in contrast, is affected by the phase transition of dipalmitoylglycerophosphoglycerol (Theretz et al., 1984).

One of the striking properties of ANS is the shift of its emission spectrum with changes in the probe environment (Stryer, 1965; Slavik, 1982). To detect such a change, the ratio of the emission observed at two different wavelengths is a good indicator (Teissié et al., 1976). The ratio of the fluorescence observed at 476 nm to that at 498 nm for different DPPC packing is plotted in Figure 3. No significant change is observed during the phase transition (mean value, 1.13; standard deviation, 0.12). This ratio is indeed very sensitive to modifications of the environment of bound ANS as we showed in our previous study on the lecithin monolayer where a small change in the ionic content of the subphase was enough to alter it (Teissié, 1979b). In the present case, we conclude that the environment of the probe remains unchanged. The structure of the binding site stays the same as long as liquid-expanded and gel states coexist. This value of 1.13 is similar to the one we reported in our investigation of the binding of ANS to egg yolk lecithin (Teissié, 1979b).

Dissociation constants were obtained as described in Teissié (1979b). Here again, reciprocal plots of bound ANS fluorescence vs. the concentration of free ANS are straight lines. This provides clear evidence that no electrostatic repulsion between bound probes occurs under our experimental conditions (high ionic strength). Changes in the dissociation constant with the host lipid molecular area is given in Figure 5.

K_D increases with increasing packing, and no binding is observed when the lipid is in the gel state. Extrapolation at

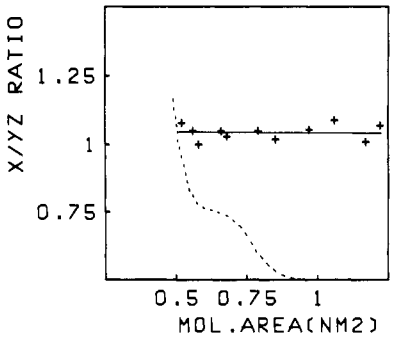

FIGURE 4: Ratio of bound ANS fluorescence observed with crossed excitation light during the compression of a DPPC monolayer. The experiment described in Figure 1 was performed with a Glan polarizer present in the exciting light beam. The light may be polarized parallel to the film (X) or to the incident plane (YZ). For each molecular area of the DPPC monolayer, the polarizer was tilted several times, and the different levels of fluorescence were recorded. The ratio of the fluorescence observed with the polarizer in the X position to that with the polarizer in the YZ position is plotted vs. the molecular area of the host lipid. The dotted line is the compression isotherm of Figure 1A.

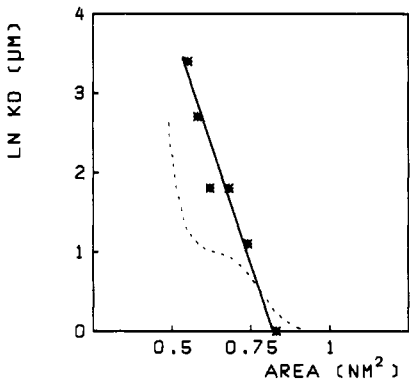

FIGURE 5: Changes as a function of the molecular packing of the lipid of the dissociation constant for the binding of ANS to DPPC monolayers spread at the air/water interface. Constants were obtained from fluorescence measurements. The aqueous subphase was 0.1 M NaCl, pH 5.5, T = 20 °C. The dotted line is the compression isotherm of Figure 1A.

infinite free ANS concentration of the "reduced" fluorescence, I_∞, is observed to decrease with decreasing molecular area and to be null when the lipid is in the gel state. As

$$I_\infty = Kn\phi$$

where n is the number of binding sites per lipid, K is linked to the position of the emitter relative to the monolayer (and monitored by the ratio X/YZ), and ϕ is the quantum yield (and monitored by shifts in the emission wavelengths in the case of ANS).

We have just described the evidence proving that neither K (the ratio X/YZ stays constant upon compression) nor ϕ (no spectral shift is detected) is affected during the compression. As a conclusion, the behavior of I_∞ shows that n decreases when the monolayer is compressed; thus, the density of binding sites decreases.

In an attempt to obtain more information on the structure of the binding site, we have evaluated the number of ANS molecules bound per lipid. This was done by comparing the fluorescence emitted by the film and that arising from the subphase. Taking into account the geometry of the detecting device and the increase in quantum yield between the free and bound probes (from data on bilayers, we assume a 75-fold increase), we obtain for a molecular area of 0.6 nm² (K_D = 10 µM) 25 DPPC molecules for each bound ANS if the free

Table I: Spectroscopic Parameters of ANS Binding

temp (°C)	λ_{max} excitation (nm)	λ_{max} emission (nm)	K_d (µM)
	(A) Microvesicles (SUV)		
20	376 ± 5	485 ± 5	9.7 ± 1
51	376 ± 5	488 ± 5	>15
	(B) Macrovesicles (LUV)		
20	376 ± 5	480 ± 5	23.4 ± 1
51	376 ± 5	486 ± 5	17
	(C) Liposomes (MLV)		
20	376 ± 5	478 ± 2	50 ± 5
50	376 ± 5	484 ± 6	100 ± 10

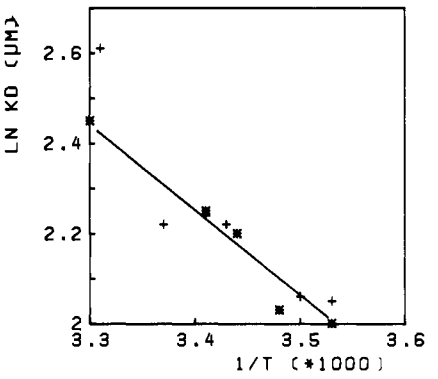

FIGURE 6: van't Hoff plot of the dissociation constant for ANS binding to DPPC microvesicles (SUV). Constants were obtained from fluorescence measurements with phospholipid concentrations varying in the range from 50 to 300 µM. The solution was 0.1 M NaCl, pH 5.5. Microvesicles were obtained either by sonication (asterisks) or by injection (+).

dye concentration is equal to K_D (i.e., half of the binding sites occupied). As a conclusion, 12 DPPC molecules surround one bound ANS molecule.

Binding of ANS to DPPC Microvesicles (SUV). Mixing a suspension of DPPC microvesicles (SUV) with an aqueous solution of ANS induces a sharp and instantaneous increase in the fluorescence signal whatever the temperature of the sample. This observation has already been reported several times [for a review, see Slavik (1982)]. Both scattered light and the emission of bound ANS are part of the signal, but under our experimental conditions (vesicle concentration less than 300 µM in lipid, ANS concentration less than 10 µM), the first effect was determined to be negligible. For a given lipid concentration, double-reciprocal plots of fluorescence intensity vs. probe concentration are linear and do not extrapolate to the origin. The parameters of the binding reaction are computed from these plots and are given in Table I. In the gel state (T = 20 °C), a binding site corresponds to an assembly of 200 DPPC molecules (on the average) whichever way the SUV are prepared (injection or sonication).

By contrast with observations at 20 °C, for which the fluorescence signal was stable with time under our experimental conditions, above the phase transition temperature (41 °C) (Jacobson et al., 1976), the enhancement was clearly biphasic. The process shows a fast phase and a slower one with an amplitude of about 50% of the former one. The time constant of the fluorometer (8 s) did not allow a clear separation of the kinetics. Such behavior has already been described and related to the transport of ANS through the bilayer and to its binding on the inner layer (Tsong, 1975; Jacobson et al., 1976).

In the temperature range from 10 to 30 °C, i.e., below the phase transition temperature, the emission properties remain

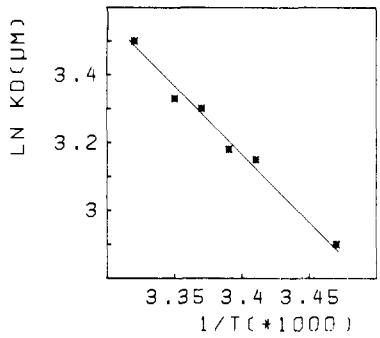

FIGURE 7: van't Hoff plot of the dissociation constant for ANS binding to DPPC unilamellar macrovesicles (LUV). Constants were obtained from fluorescence measurements with phospholipid concentrations varying between 10 and 100 μM. The solution was 0.1 M NaCl, pH 5.5.

unaffected (λ_{max}^{exc} = 377 nm, λ_{max}^{em} = 485 nm). The maximal intensity of emission decreases with increasing temperature. As shown in Figure 6, K_D only slightly increases with increasing temperature. For temperatures between 10 and 25 °C, where the van't Hoff plot of the dissociation constant is linear, ΔH was 10 kJ/mol and ΔS was −76 J/(mol·K). The results are the same with injected and sonicated SUV.

For temperatures above the phase transition temperature, binding sites located on both sides of the bilayer are accessible to the probe. As explained above, instrumental limitations made it impossible to determine accurately the binding parameters for each side. The emission spectra show a small but significant red shift as compared to those observed near 20 °C (Table I). Here again, no difference is noted between microvesicles prepared by injection and by sonication.

Binding of ANS to Liposomes of DPPC (MLV). When ANS is added to an unsonicated dispersion of DPPC at 20 °C, only a small increase in fluorescence is observed. By contrast, if the sample is first heated to 50 °C in the presence of the probe and then cooled back to 20 °C, a large fluorescence increment occurs. Specific problems are encountered when working with liposomes. In particular, large signals due to light scattering need to be calibrated for subtraction from the apparent emitted light. Moreover, high concentrations of ANS need to be used in order to saturate the binding sites, and under such conditions, a correction for high filter effects must be made (Trauble & Overath, 1973).

The emission maximum occurs at 478 nm with a maximum for excitation around 377 nm. The apparent dissociation constant K_D is determined to be 50 μM (±10%) at 20 °C. The density of binding sites is calculated to be about 1 for every 15 phospholipid molecules.

Binding of ANS to DPPC Unilamellar Macrovesicles (LUV). The results are similar to those described for the other lipid dispersions (Table I). The binding sites are assemblies of about 30 DPPC molecules. The van't Hoff plot of the dissociation constant gives a straight line between 15 and 30 °C (Figure 7). ΔH is 34 kJ/mol, and ΔS is −30 J/(mol·K). All these values are relative to the outer layer.

Comparison of the Inner and Outer Layers. As described in Tsong (1975), the permeability of phospholipid dispersions (SUV, MLV) to ANS is known to increase considerably at the phase transition (i.e., around 42 °C for DPPC). As suggested by the "cluster" model of the phase transition (Kanehisa & Tsong, 1978), this is indeed observed to be the case for macrovesicles (LUV). Thus, by heating the suspension up to the phase transition temperature, it is possible to induce the penetration of the probe across the lipid bilayer and as a consequence to make accessible to ANS molecules those binding sites which are located at the inner layer. By using saturating concentrations of ANS, we have been able to evaluate the ratio of binding sites on both sides of the membrane (assuming that the affinity is the same on both sides and that no irreversible changes are induced during the phase transition). The values for this ratio are 1 to 2 for SUV and 1 to 1 for LUV (inner to outer). As described in Pohl and Teissié (1975), the ratio was 1 to 1 for the two faces of a BLM. Taking into account the onionlike structure of MLV, such an approach was not possible in that case.

DISCUSSION

On the basis of X-ray (Lesslauer et al., 1972) and NMR (Podo & Blasie, 1977) data, the location of bound ANS is known to be at the level of the phospholipid polar heads. By means of fluorescence lifetime measurements, it was shown that the environment of ANS bound to egg lecithin vesicles was a highly mobile liquidlike system (Demchenko & Scherbatska, 1985). As emphasized in the introduction, the electric repulsion linked to the sulfonate group was avoided in our experiments since we used a high ionic content aqueous phase.

The major conclusion from our experiments with monolayers at the air/water interface is that the binding sites for ANS remain structurally unchanged when the DPPC monolayer is compressed although the dissociation constant is affected. This conclusion stems from the observations that no reorientation occurs (the ratio X/YZ remains constant) (Figure 4) and that no environmental change is detected (no shift in the emission spectrum of ANS for the I_{476}/I_{498} ratio stays constant) (Figure 3).

Taking into account the high fluidity of the environment of the bound probe, the observed value of the orientation ratio (close to 1) is indicative of a large fluctuation angle (Teissié, 1979b). This feature of the binding site is not affected by the compression of the host monolayer. We can conclude that the structure of the environment of the bound ANS is not altered by the modification of the monolayer packing. However, at the same time, the number of binding sites per lipid is observed to decrease. We may conclude by extrapolation that no binding site is present when the lipid is fully in the gel state (high surface pressure) (see Figure 2). Structurally, the binding site appears to be an assembly of DPPC molecules in the liquid-expanded state. This conclusion is provided by the observations that these binding sites are already present when the lipids are in the liquid-expanded state, that they are unaffected by the compression, that their spectral ratio I_{476}/I_{498} is the same as for egg yolk lecithin, and that a coexistence of the gel and the liquid states is known to be present during the phase transition in monolayers.

Another observation is that although the binding sites are not affected structurally, the dissociation constant is strongly dependent on the packing of the lipids. A tentative interpretation of the dependence of the dissociation constant on the host lipid molecular packing may be proposed as follows. In a recent paper, Jaehnig and Bramhall (1982) described a thermodynamic interpretation of the incorporation of amphiphilic molecules in lipid layers. The free energy for binding is the sum of two contributions: one is due to the amphiphilic properties of the ligand; the second is linked to a fluidization of the lipids around the binding sites. As explained by the authors, this fact is due to the cooperative nature of the lipid phase. From our results, we conclude that two kinds of DPPC molecules are affected in the binding of ANS: the lipids in direct contact with the probe and an annulus of "fluidized" lipids. The first ones (four lipids; Slavik, 1982) remain

Table II: Dissociation Constants for the Binding of ANS to DPPC Assemblies at 20 °C

type of assemblies	obsd K_d (μM)	calcd molecular area (nm^2)[e]	ref
monolayer	NB[f]	<0.5	this paper
microvesicles (SUV)	9.7 ± 1[a]	0.63	this paper
	10.8[a]	0.63	Jacobson (1976)
macrovesicles (LUV)	24 ± 1	0.56	this paper
liposomes (MLV)	50[a]	0.49	this paper
	50[b]	0.49	Jacobson (1976)
spherical monolayer	1000[a,c]	<0.5	Haynes (1974)
BLM	10–40[a,d]	0.51–0.63	Pohl (1975)

[a] Constants were obtained by fluorescence measurements and refer to 20 °C. [b] Constants were obtained by equilibrium dialysis at 20 °C. [c] Temperature was 15 °C. [d] Large inaccuracies affect the determination of K_d. [e] Molecular areas were calculated from the K_d by use of the plot on Figure 5. [f] No binding.

structurally unaffected with decreasing host lipid molecular area. From a thermodynamic point of view, this means no change in the amphiphilic properties; however, as the K_D increases with decreasing molecular area, we must conclude that the free energy for melting required for fluidization of the "annulus" is strongly dependent on the packing. If as suggested by Jaehnig and Bramhall, we assume that the lipids should be in the fluid state for the incorporation of one ANS molecule, our results simply indicate that more and more lipids must be fluidized along the phase transition. This is a direct consequence of the transition process where with the compression more and more lipids are in the gel state and coexist with fluid lipids (Kanehisa & Tsong, 1978).

Binding of ANS to SUV of pure DPPC is consistent with a simple model of interaction with preexisting sites where the equilibrium constant is practically the same whatever the method used for their preparation. In the temperature range below the phase transition, only binding sites located on the outer layer of the SUV are accessible (Tsong, 1975; Jacobson et al., 1976). ANS being bound at the polar head level, our observation confirms that both injection and sonication methods give identical particles with respect to the structure of the polar heads of the outer layer in this temperature range. As no shift in the emission spectra was observed whatever the probe concentration used, the detected binding sites can be considered as homogeneous.

For SUV, the van't Hoff enthalpy (ΔG) of binding (between 10 and 25 °C) is small (about 4 kJ), and the ΔS is quite large, of the order of −20 eu. These values are very similar to those obtained for the analogous probe *N*-phenylnaphthylamine to the fluid egg lecithin SUV (Ting & Salomon, 1975) and fairly typical of hydrophobic interactions. This behavior can be explained by the so-called "hydrophobic effect" [as defined in Tanford (1973)] in the solvation of the ANS molecules when they leave the hydrophobic matrix.

As suggested in the introduction, the dissociation constant of ANS can be used to monitor the molecular packing of DPPC polar heads in the different models. In order to eliminate artifacts linked to the thermodynamic parameters of this interaction, we must compare data obtained at 20 °C (temperature of the monolayer experiments). These molecular areas are those at the level of the polar heads of the lipids of the outer layer where ANS is bound (Lesslauer et al., 1972).

Results are given in Table II. These are obtained by use of the curve in Figure 5 and of the observed K_D at 20 °C. For comparison, it should be remembered that for DPPC in the gel state a molecular area value of 0.48 nm^2 was found for MLV (Chapman et al., 1967), 0.45 nm^2 for BLM, and 0.67 nm^2 for SUV (Teissié et al., 1978). The curvature of these

Table III: Number of Lipids per Bound ANS under Saturating Conditions[a]

system	lipids per ANS
monolayer (0.6 nm^2)	12
microvesicles (SUV)	130
macrovesicles (LUV)	15
multilayers (MLV)	15

[a] These lipids are the binding site and the annulus of "fluidized" ones. The outer layer alone is taken into account in this determination performed at 20 °C. The data were computed from the number of DPPC molecules per bound ANS. In bilayered "models" (SUV and LUV), as binding was operated only on the external side, the experimental data shown in the table were corrected by the relative number of lipids on each layer (1/2 in SUV, 1/1 in LUV).

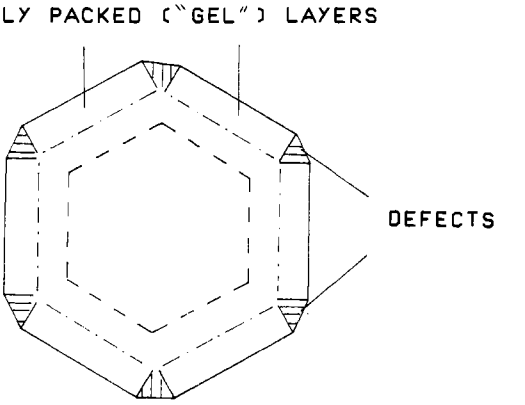

FIGURE 8: Cross section of a DPPC microvesicle (SUV) at 20 °C as suggested by our measurements and the X-ray studies (Blaurock & Gamble, 1979). The dotted line on the inner side is present to indicate that this layer was not investigated in the present study.

models will not significantly affect the structure of the binding site even with SUV. Assuming that four lipids are in contact with one bound ANS (Slavik, 1982), the deviation from planar of such a site can be calculated to be 0.2% of the radius of a SUV. In other words, this geometrical difference is negligible.

The comparatively low dissociation constant we have found when using microvesicles is in keeping with the relationship observed between the ANS dissociation constant and the packing density of phospholipids in monolayers. The high affinity of ANS for the outer layer of DPPC microvesicles (at 20 °C) would thus indicate a large molecular area for the lipids involved in the binding of this amphiphile, in agreement with previous observations (Teissié et al., 1978b). However, we should take into account the surprisingly low number of binding sites as compared with those involved in the binding with macrovesicles (Table III) (15 molecules per binding site with macrovesicles, 130 molecules for microvesicles; those values have been corrected for the outer to inner ratio).

We have calculated that 12 lipids build an ANS binding site in DPPC monolayers when its K_D is equal to 10 μM as for a SUV at 20 °C. The value we obtained for SUV (130 lipids) should be considered as indicative that in fact only very few lipids are involved in this affinity binding of ANS to DPPC SUV. Only small patches of lipids are specific for this interaction. A recent X-ray study of DPPC microvesicles (Blaurock & Gamble, 1979) has shown that this system may be faceted. The microvesicle, when the lipid is in the gel state, is not a sphere but a juxtaposition of planar bilayers (Figure 8). The necessary junction between those facets was thought to be an unordered assembly. From the K_D values observed in the present study, the lipids which are present in those junction areas appear to be in a "fluid"-like state. The facets, on the other hand, are highly ordered and tightly packed, and

no ANS binding can be detected at their level (the value of K_D being too high). Those "defects" (as compared to the regular assembly of lipids in the "gel state") would be specific targets for the binding of amphiphiles as described in this work and in Teissié et al. (1978b). Macrovesicles are shown in the present study to be more tightly packed (molecular area of about 0.5 nm^2) than the defects present in SUV; they appear to be homogeneous (15 lipids per bound ANS). The observation that the packing in macrovesicles is a little less tight than in the gel-state monolayer (surface pressure larger than 15 mN/m) may be connected to the curvature present in the vesicles, this being an average property of the lipid layer. It should be emphasized that the defects present in SUV are not found in LUV.

The presence of these defects was proposed to explain the difference of behavior of DPPC organized either in SUV or in LUV toward the hydrolysis by pancreatic phospholipase A2 (Menasche et al., 1986; Lichtenberg et al., 1986). Our observation that in SUV the polar heads are in the same packing as in monolayers with a surface pressure of 10 mN/m is in complete agreement with the published results of the optimum pressure for phospholipase activity (10 mN/m) (Zographi et al., 1971). Of course, as no defects are present with LUV as for MLV in the gel state, hydrolysis would not occur in these systems.

By comparing the number of binding sites for SUV, LUV, and monolayers (for the same K_D), we propose that about 10% (15/130 = 0.11) of the lipids are involved in the defects of SUV. It should be kept in mind that our observation is valid only insofar as polar heads of the outer layer of a DPPC microvesicle are concerned. These conclusions should not be extended to the inner layer because it was recently shown that an asymmetry of packing existed between the two layers by use of dansylglycine fluorescence spectroscopy (Bramhall, 1986).

ACKNOWLEDGMENTS

We thank G. Chandler for her help in writing the manuscript and the "unknown" reviewer for his helpful commentaries.

Registry No. DPPC, 2644-64-6; ANS, 82-76-8.

REFERENCES

Bakker, E. P., & Van Dam, K. (1974) *Biochim. Biophys. Acta 339*, 157–163.

Batzri, S., & Korn, E. D. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.

Blaurock, A. E. & Gamble, R. C. (1979) *J. Membr. Biol. 50*, 187–204.

Bramhall, J. (1986) *Biochemistry 25*, 3479–3486.

Cadenhead, D. A., Kellner, B. J., & Muller-Landau, F. (1975) *Biochim. Biophys. Acta 382*, 253–259.

Chapman, D., Williams, R. M., & Ladbrooke, B. D. (1967) *Chem. Phys. Lipids 1*, 445–475.

Chrzesczyk, A., Wishnia, A., & Springler, C. S. (1977) *Biochim. Biophys. Acta 470*, 161–169.

Demchenko, A. P., & Shcherbatska, N. V. (1985) *Biophys. Chem. 22*, 131–143.

Enoch, H. G., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145–149.

Flanaghan, M. T., & Hesketh, T. R. (1973) *Biochim. Biophys. Acta 298*, 535–545.

Haynes, D. H., & Stark, H. (1974) *J. Membr. Biol. 17*, 313–340.

Huang, C., & Mason, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 308–310.

Jacobson, K., & Papahadjopoulos, D. (1976) *Biophys. J. 16*, 544–560.

Jaehnig, F., & Bramhall, J. (1982) *Biochim. Biophys. Acta 690*, 310–313.

Kanehisa, M. I., & Tsong, T. Y. (1978) *J. Am. Chem. Soc. 100*, 424–432.

Lesslauer, W., Cain, J. E., & Blasie, J. K. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1499–1503.

Lichtenberg, D., Felgner, P. L., & Thompson, T. E. (1982) *Biochim. Biophys. Acta 684*, 277–281.

Lichtenberg, D., Romero, G., Menashe, M., & Biltonen, R. L. (1986) *J. Biol. Chem. 261*, 5334–5340.

MacLaughlin, S., & Harary, H. (1976) *Biochemistry 15*, 1941–1948.

Menashe, H., Romero, G., Biltonen, R. L., & Lichtenberg, D. (1986) *J. Biol. Chem. 261*, 5328–5333.

Podo, F., & Blasie, J. K. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1032.

Pohl, W. G., & Teissié, J. (1975) *Z. Naturforsch, C: Biosci. 30C*, 147–151.

Raison, J. K., Lyons, J. M., Melhorn, R. J., & Keith, A. D. (1971) *J. Biol. Chem. 246*, 4036–4040.

Seufert, W. D. (1970) *Biophysik (Berlin) 7*, 60–73.

Slavik, J. (1982) *Biochim. Biophys. Acta 694*, 1–25.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.

Tanford, C. (1973) *The Hydrophobic Effect*, Chapter 4, Wiley, New York.

Teissié, J. (1979a) *Chem. Phys. Lipids 25*, 357–368.

Teissié, J. (1979b) *J. Colloid Interface Sci. 70*, 90–96.

Teissié, J. (1981) *Biochemistry 20*, 1548–1554.

Teissié, J., Tocanne, J. F., & Baudras, A. (1976) *FEBS Lett. 70*, 123–126.

Teissié, J., Tocanne, J. F., & Baudras, A. (1978a) *Eur. J. Biochem. 73*, 77–85.

Teissié, J., Tocanne, J. F., & Pohl, W. G. (1978b) *Ber. Bunsen-Ges. Phys. Chem. 82*, 875–876.

Thayer, A. M., & Kohler, S. J. (1981) *Biochemistry 20*, 6831–6834.

Theretz, A., Teissié, J., & Tocanne, J. F. (1984) *Eur. J. Biochem. 142*, 113–119.

Ting, P., & Salomon, A. K. (1975) *Biochim. Biophys. Acta 406*, 447–451.

Trauble, H., & Overath, P. (1973) *Biochim. Biophys. Acta 307*, 491–512.

Tsong, T. Y. (1975) *Biochemistry 14*, 5409–5414.

Ueda, I., Tashiro, C., & Arakaua, K. (1977) *Anesthesiology 46*, 327–332.

Verger, R., Mieras, M. C. E., & De Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023–4034.

Zografi, G., Verger, R., & De Haas, G. H. (1971) *Chem. Phys. Lipids 7*, 185–206.