Phospholipid Packing Asymmetry in Curved Membranes Detected by Fluorescence Spectroscopy†

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ABSTRACT: There are distinct differences in the molecular packing of phospholipid molecules in the inner and outer membrane monolayers of small lipid vesicles; a small radius of curvature imparts an asymmetry to the interface between thse two monolayers. I have used an amphiphilic fluorescent probe, *N*-[5-(dimethylamino)naphthalenyl-1-sulfonyl]glycine (dansylglycine), to determine if this asymmetry in molecular packing leads to the existence of different environments for fluorescent probes resident in the membrane. Dansylglycine is highly sensitive to the dielectric constant of its environment, and the fluorescence signal from membrane-bound dye is distinct from that in the aqueous medium. When dansylglycine is first mixed with vesicles, it rapidly partitions into the outer monolayer; the subsequent movement of dye into the inner monolayer is much slower. Because of the time lag between the initial partitioning and the subsequent translocation, it is possible to measure the emission spectrum from membrane-bound dye before and after translocation, thus distinguishing the two potential environments for dansylglycine molecules. In the outer membrane monolayer of small dipalmitoylphosphatidylcholine vesicles, dye fluorescence emission is maximal at 530 nm, corresponding to a dielectric constant of 7 for the medium surrounding the fluorophore. For dye in the inner monolayer, emission is maximal at 519 nm, corresponding to a dielectric constant of 4.7. The results suggest that water molecules are excluded more efficiently from the dye binding sites of the inner membrane monolayer than they are from those of the outer monolayer. When similar measurements were made on vesicles with a very large radius of curvature, there was no detectable difference in the wavelength of maximal emission from dye located in the outer or inner monolayers, both locations giving rise to emission at approximately 535 nm, corresponding to a dielectric constant of 10. Thus, it seems that in small lipid vesicles the phospholipid packing geometry of the outer membrane monolayer closely parallels that of a planar bilayer. In contrast, the inner monolayer appears to be subjected to packing constraints unique to systems with small radius of curvature.

When phospholipid molecules are dispersed in aqueous medium, the hydrophilic interaction of the lipid head groups with water, combined with the lack of attraction for water by the hydrophobic surfaces of the lipid acyl chains, results in the spontaneous formation of multilamellar systems (liposomes) coexisting with unilamellar structures (vesicles) (Bangham et al., 1974). Vesicles consist of a simple lipid bilayer in the form of a spherical shell. The bilayer is composed of two symmetrical monolayers of phospholipid, one forming the inside of the vesicle and one forming the external surface. In the case of large vesicles, there is no evidence for, and no reason to suspect, differences in molecular packing of the phospholipids within these two monolayers. Extensive ultrasonic treatment of lipid dispersions however, generates a suspension of very small vesicles, with a relatively narrow size distribution (Huang, 1969). In these small vesicles (30–40-nm diameter), the bilayer has a high radius of curvature, which leads to distinct differences in the molecular packing between the inner and outer monolayers and to an asymmetry in the interface between them, in spite of their identical chemical compositions (Sheetz & Chan, 1972).

Experimental evidence for this asymmetry has been provided principally by NMR[1] studies, which have revealed that the outer monolayer of small DPPC vesicles contains lipids whose acyl chains are indeed more tightly packed and whose head groups are more loosely packed than those in the inner monolayer (Schuh et al., 1982). There still seems to be some dispute over whether the inner (Schuh et al., 1982) or the outer (Brouillette et al., 1982) monolayer of a small vesicle has a phospholipid head group packing density that most closely approximates that of a planar bilayer, but there is general agreement that packing asymmetry must lead to subtle differences in the local environment within each monolayer. In view of the fact that there are a number of fluorescent molecules whose spectral characteristics are influenced to a considerable extent by the structural order of the membrane lipids in which they are embedded, it seemed reasonable to predict that these differences in the environments provided by the two monolayers could be detected by fluorescence spectroscopy. In practice, distinguishing and resolving the signals from fluorescent reporter groups present in the two monolayers present significant practical problems. A conventional experimental approach would be to equilibrate vesicles with a probe that partitions freely between the two monolayers and then record spectral characteristics in the presence or absence of a surface-restricted quenching reagent that will modify or eliminate those signals emanating from only the outer monolayer. This method works reasonably well with some ESR

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron paramagnetic spin resonance; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

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probes but is often less successful when applied in conjunction with optical probes reporting from deep within the bilayer, where there is frequently a requirement for high concentrations of quenching agents whose membrane impermeability is suspect (Chaplin & Kleinfeld, 1983).

Dansylglycine is a weakly acidic amphiphile that we have previously used to monitor transmembrane pH gradients (Drant & Bramhall, 1983; DeGuzman & Bramhall, 1984). The dansyl chromophore is exceptionally sensitive to changes in the dielectric constant of its surrounding medium, showing a dramatic increase in fluorescence quantum yield and an appreciable increase in emission frequency, as the solvent polarity is decreased. Although freely soluble in aqueous media, the dye, when mixed with lipid vesicles, rapidly attains a partition equilibrium between water and the outer monolayer in a fast diffusion-controlled process; this step leads to an increase in fluorescence intensity for the system as the chromophore becomes embedded in the vesicle membrane. In a second, subsequent step, which generates a further increase in fluorescence intensity, the inner monolayer of the vesicle becomes populated with dye. This process involves permeation through the bilayer and is slow. Because, under appropriate experimental conditions, the time interval between initial equilibration of the dye with the outer monolayer and significant population of the inner monolayer can be long (hours), the fluorescence emissions from the two monolayers can be distinguished and analyzed with relative ease. In this paper I demonstrate that in homogeneous small lipid vesicles composed of synthetic lecithin the packing arrangements imposed on a bilayer of small radius of curvature lead to differences between the inner and outer membrane monolayers that can be visualized by taking advantage of this time-dependent redistribution of membrane-bound dansylglycine.

Materials and Methods

Chemicals. Dansylglycine was obtained from Sigma Chemical Co. (St. Louis, MO). It contained traces of the dansyl-free acid and was purified before use by thin-layer chromatography on silica gel in a developing solvent of chloroform/methanol (2:1 v/v). Purified material (R_f 0.24) was eluted from the adsorbent with methanol and stored as a 10^{-2} M solution in absolute ethanol at −20 °C. 1,4-Dioxane (spectrophotometric grade) was supplied by Aldrich Chemical Co. (Milwaukee, WI). The purity of synthetic dipalmitoylphosphatidylcholine (Sigma) was verified by thin-layer chromatography on silica gel in a developing solvent of chloroform/methanol/glacial acetic acid/water (90:40:12:2 v/v); the lipid required no further purification.

Preparation of Lipid Vesicles. Small unilamellar lipid vesicles were prepared by sonication of lipid suspensions in aqueous buffer under an atmosphere of nitrogen. Lipid was dissolved in chloroform/methanol (2:1 v/v), aliquoted into glass tubes, dried under nitrogen at 40 °C, and placed under high vacuum for 18–20 h at 20 °C in order to remove residual chloroform before use. Typically, 50 mg of DPPC was sonicated in 1 mL of buffer (100 mM sodium pyrophosphate adjusted to pH 8.0 with 1 M citric acid). Sonication was performed with a microprobe at a power setting of 30–35 W for a total of 15 min; the temperature of the lipid suspension was maintained at 5 °C above the lipid's melting point (i.e., at 46 °C) throughout the sonication process. The vesicle preparation was cooled to 20 °C, centrifuged (100000*g*, 30 min) to remove structures other than small vesicles, and used immediately.

Large unilamellar vesicles were prepared either by detergent dialysis (Mimms et al., 1981) or by solvent evaporation (Szoka & Papahadjopoulos, 1978). In the first case, 25 mg of DPPC was dissolved in 2 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 116 mg of octyl glucoside. The solution was warmed to 45 °C, bath-sonicated briefly (5 min), and then dialyzed extensively against buffer at 45 °C with a Spectropor 3 membrane (Spectrum Medical Industries, Los Angeles, CA). The vesicle suspension that formed during dialysis was concentrated 5-fold by vacuum dialysis in a collodion bag apparatus; the final vesicle preparation was stored at 4 °C in the presence of 0.02% (w/v) sodium azide.

For the preparation of solvent-evaporation vesicles, 25 mg of DPPC was dissolved in a mixture of isopropyl ether (5 mL) and chloroform (5 mL). To this solution was added 1.5 mL of sodium phosphate buffer, pH 7.2, and the mixture was bath-sonicated at 45 °C in a tightly stoppered 100-mL round-bottomed flask for 20 min. Volatile solvent was removed by rotary evaporation at 45 °C under reduced pressure, yielding an aqueous slurry of large lipid vesicles. The slurry was diluted 2-fold with buffer, transferred to a glass vial, and stored at 4 °C in the presence of azide, as above.

Quantitation of Membrane-Bound Dye. Equilibrium measurements of dye binding to lipid bilayers were performed in plastic multiwell microdialysis chambers with a 200-μL cavity on each side of a semipermeable membrane (M_r 12000 cut-off), as previously described (Bramhall, 1984). A suspension of phospholipid vesicles was placed in one compartment and an equal volume of isotonic buffer, containing 10^{-5} M dansylglycine, placed in the opposite chamber. The cells were rotated at approximately 15 rpm for 18 h at the experimental temperature, and percentage binding of dye was determined from the ratio of the fluorescence intensities of equal-volume samples taken from the vesicle-free (F_a) and vesicle-containing (F_v) compartments [samples diluted in *n*-dioxane/water (1:1), fluorescence emission measured at 554 nm]. The quantity of membrane-bound dye (F_m) was calculated from eq 1.

$$F_m = F_v - F_a \quad (1)$$

The membrane:buffer partition coefficient k_b (binding coefficient) was calculated from eq 2 as the slope of the line

$$k_b = (F_m/F_a)(V_a/V_m) \quad (2)$$

obtained by plotting F_m/F_a against V_m/V_a, where V_m and V_a are the volumes of the membrane and aqueous phases, respectively. V_m was calculated by assuming a partial specific volume for DPPC of 0.98 mL/g.

Fluorescence Studies. Fluorescence and light scattering measurements were performed with a Fluorolog II spectrometer (Spex Industries, Metuchen, NJ) equipped with temperature-control accessories and magnetic stirrer. Sample temperatures were monitored with a platinum-resistance thermometer housed in the optical cuvette. Rapid kinetic measurements were obtained with a conventional stopped-flow apparatus (mixing time <5 ms) equipped with gas-pressured syringes and temperature-control accessories. Thermally induced lipid phase transitions were detected by monitoring the Rayleigh scattering intensity of vesicle suspensions as a function of temperature. Linear temperature gradients were generated with a digital temperature programmer (Neslab Inc., Portsmouth, NH).

Results and Discussion

In view of the extensive use of a wide variety of fluorescent probes in studies involving small lipid vesicles, it seemed appropriate to question whether these differences in molecular packing would lead to corresponding differences in probe

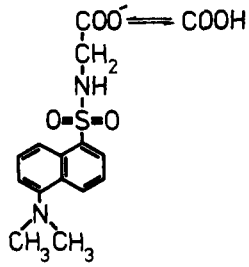

FIGURE 1: Structure of dansylglycine.

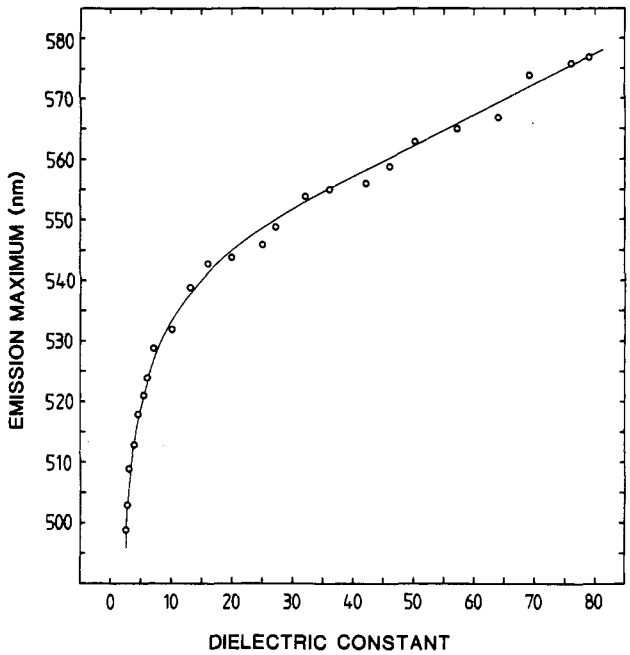

FIGURE 2: Dependence of fluorescence wavelength on solvent dielectric constant. Dansylglycine was dissolved in dioxane/water mixtures of defined dielectric constant to a final concentration of 10^{-6} M. The fluorescence emission spectrum was recorded for each sample over the range 400–700 nm in 1-nm increments. Emission intensity was integrated for 0.5 s at each wavelength position, and the fluorescence spectrometer was used in the ratio mode to eliminate variations in excitation source intensity. The spectra were corrected for frequency-dependent variations in detector sensitivity; emission maxima were located from first-derivative functions of the emission spectra. The excitation wavelength was 350 nm; the temperature was 25 °C.

distribution or environment between the two monolayers. A number of fluorescent dyes have emission characteristics that can be influenced to a significant extent by the structural order of the membrane lipids in which they are embedded, but it is not generally possible to distinguish the spectral responses from the two monolayers.

Influence of Solvent Polarity. Dansylglycine (Figure 1) is freely soluble in water. Aqueous solutions of the dye are weakly fluorescent, showing an emission maximum around 580 nm and an excitation maximum at 350 nm; the excited-state lifetime is approximately 4 ns (Chen & Kernohan, 1967). In keeping with other dyes containing the dansyl chromophore, dansylglycine shows a marked sensitivity toward the dielectric constant of its surrounding medium, showing an extensive blue shift in emission wavelength and increase in quantum yield, with decreases in solvent polarity (Hartley & Massey, 1956; Chen, 1967); these changes are quantitated in Table I. The relationship between emission frequency and environmental dielectric is better illustrated in Figure 2, from which it is apparent that the dependence of emission maximum on dielectric constant becomes severe in media of low polarity. This particular property of dansylglycine, coupled with the ability

Table I: Spectral Characteristics of Dansylglycine in Dioxane/Water Mixtures

% dioxane	e^a	emission max (nm)	Q^b	emission intensity (510 nm)^c
0	79	577	0.07	0.48
2.5	79	577	0.08	0.57
5	78	578	0.09	0.67
7.5	77	577	0.10	0.78
10	76	576	0.11	0.92
15	69	574	0.14	1.23
20	64	567	0.18	1.63
25	57	565	0.22	2.13
30	50	563	0.26	2.61
35	46	559	0.30	3.19
40	42	556	0.34	3.77
45	36	555	0.39	4.34
50	32	554	0.43	4.95
55	27	549	0.47	5.49
60	25	546	0.51	6.08
65	20	544	0.55	6.62
70	16	543	0.58	7.12
75	13	539	0.61	7.52
80	10	532	0.64	7.92
85	7	529	ND^d	ND
90	6	524	0.68	8.54
92	5.2	521	ND	ND
94	4.5	518	0.68	8.87
96	3.7	513	ND	ND
98	3.0	509	ND	ND
99	2.6	503	ND	ND
100	2.2	499	0.66	8.94

[a] Calculated from the data of Farkas (1932), Clemett et al. (1964), and Büttner and Heydtmann (1969). [b] Relative quantum yields determined by integration of the fluorescence emission envelope for each sample. The value of 0.43 for the quantum yield of dansyl amide in dioxane/water (1:1 v/v) was taken from the data in Vaz and Schoellmann (1976). [c] Fluoresence intensity measured at 510 nm, in arbitrary units. [d] Not determined.

of the dye to partition readily into the hydrophobic phase of lipid bilayers, makes the dye an excellent probe of membrane structure.

Influence of Temperature on Dye Translocation Kinetics. We have previously demonstrated (Jähnig & Bramhall, 1982; Bramhall, 1984) how the membrane translocation kinetics of dyes similar in structure to dansylglycine can be monitored by recording the changes in fluorescence characteristics that occur as dye binds to, and subsequently permeates across, vesicle bilayers with rates that are, at any given experimental temperature, inversely proportional to bilayer thickness. Figure 3 shows that the behavior of dansylglycine is qualitatively identical with that seen previously with dansyl galactoside, a neutral amphiphilic dye. Below the phase transition temperature (T_c) of the vesicle bilayer, which is indicated by the sharp change in light scattering profile, there is strong temperature dependence for dye permeation, suggesting a high activation energy for the process; above T_c, the dependence of temperature is less severe. Similar behavior is seen with a variety of saturated lecithins with acyl chain lengths varying from 12 to 18 carbons (Bramhall, 1985). In each case, the change in apparent activation energy that occurs at T_c appears to be very sharp, suggesting a sudden change in the properties of the bilayer at this temperature, however, we have previously demonstrated that high apparent activation energies and sharp discontinuities in Arrhenius functions of membrane processes can arise from the initiation of small progressive changes in membrane properties extending over a relatively broad temperature range (Lee et al., 1985). The two important conclusions to be drawn from Figure 3 are simply that permeation rates for dansylglycine across lecithin bilayers are highly

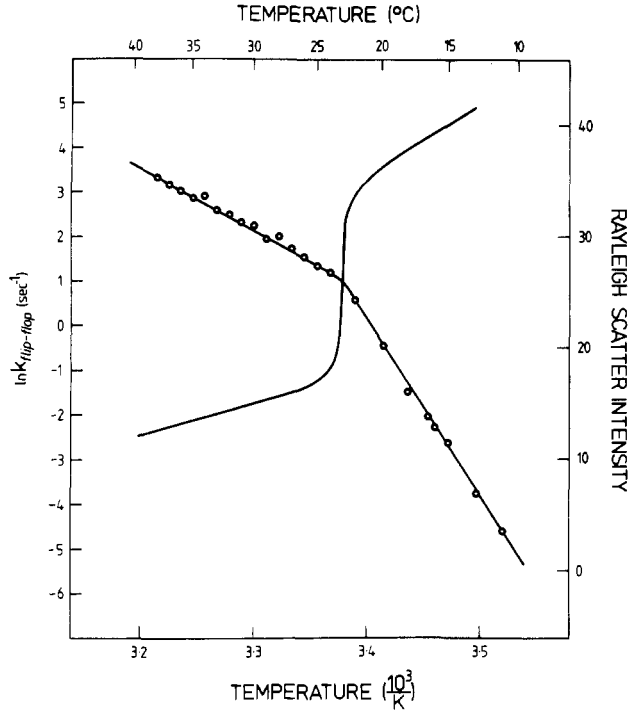

FIGURE 3: Translocation kinetics for movement of dansylglycine from outer to inner monolayer of lecithin vesicles. A dispersion of small unilamellar DMPC vesicles in 50 mM sodium citrate/pyrophosphate buffer (pH 4.0) was mixed rapidly with a solution of dansylglycine, in the same buffer, by a conventional stopped-flow mixing chamber in the sample compartment of a fluorescence spectrometer. Fluorescence emission intensity (520 nm) was monitored continuously during, and after, mixing. The dye molecules rapidly attain a partition equilibrium between buffer and the outer monolayer of the vesicles, a redistribution that resulted in a large, rapid increase in sample fluorescence intensity. In a second step, associated with a further increase in fluorescence intensity, the inner monolayer of the vesicles became populated with dye. The data from this second phase were plotted to a first-order rate equation to generate a rate constant for dye translocation (flip-flop) from outer to inner monolayer. Rate constants were determined over a range of temperatures and are presented here in the form of an Arrhenius function. The final concentration of lipid was 1 mM; the final concentration of dye was 10^{-5} M. A sample of the final mixture of dye plus vesicles was examined to determine the lipid melting characteristics in the presence of dye. The sample was placed in the sample compartment of a fluorescence spectrometer adjusted for detection of 90° Rayleigh scatter at 400 nm. The change in light scattering properties of the sample was recorded as the temperature was increased from 13 to 41 °C at a heating rate of 0.2 °C/min.

temperature-dependent below T_c and that the dye permeates as a neutral species.

Influence of pH. Dansylglycine has two ionizable groups and can exist in both charged and neutral forms. The *N,N*-dimethylamino function of the chromophore can become protonated (p*K* approximately 4) to yield a nonfluorescent compound (Förster, 1951), which thus does not contribute to sample fluorescence intensity to any appreciable extent even though the compound may be membrane bound. The carboxylate function of the glycyl moiety can also dissociate (p*K* approximately 2.5) to generate an anionic form of the dye that will bind to membranes almost as efficiently as the neutral species. At pH 6.0, the ratio of anionic species to neutral species is approximately 3000:1; this ratio varies with external pH, and because the neutral compound permeates across the bilayer so much more rapidly than any dissociated species, the rate of dye flux is strongly pH dependent. As a consequence, dansylglycine will accumulate in the interior of sealed vesicles when the internal pH is higher than that of the external medium. This accumulation of dye leads to an increase in fluorescence intensity that is proportional to the existing pH gradient and that is reversed if the gradient is collapsed, e.g., by the addition of ammonium acetate (Bangham et al., 1967), a feature that allows dansylglycine to be used to measure the size and stability of transmembrane pH gradients (Drant & Bramhall, 1984).

Fluorescence Studies. The two principal experimental variables that regulate the membrane translocation kinetics of dansylglycine are thus temperature and pH; in addition, bilayer composition (acyl chain length) can be manipulated to bring translocation kinetics into experimentally convenient ranges for any given combination of these two variables. Thus, when dansylglycine is mixed with DPPC vesicles suspended in pH 6.0 buffer at 15 °C, the half-time for equilibration of dye between the outer and inner bilayer leaflets is greatly in excess of 100 h; for all practical purposes, the dye is surface-restricted. If the temperature of the system is raised to 36 °C, the equilibration half-time is decreased to 2 min (translocation rate constant $k = 5.19 \times 10^{-3}$ s^{-1}) and the probe can rapidly distribute across the vesicle membrane. Figure 4 shows the changes in fluorescence emission intensity and frequency that accompany this redistribution of dye.

At the start of the experiment, the aqueous solution of dansylglycine showed the fluorescence emission spectrum illustrated in Figure 4A. The emission maximum was 577 nm with a relative quantum yield of 0.07 (Table I). The temperature of the dye solution was 14.5 °C (26 °C below T_c for DPPC) so that when DPPC vesicles were added the dansylglycine instantly equilibrated with the outer membrane monolayers but was prevented from moving to the inner monolayer. Figure 4B shows the emission profile of this membrane-bound dye immediately after the addition of vesicles; the emission maximum at 530 nm indicates a dielectric constant of 7 for the environment of the membrane-bound fluorophore. Note that the fluorescence from the membrane phase was isolated by subtraction of the aqueous phase spectrum after quantitative correction for the decrease in free dye concentration resulting from dye binding to vesicles; the spectrum was also corrected for frequency-dependent variations in detection sensitivity and for changes in light scattering properties created by the presence of vesicles; these corrections and manipulations were performed very simply with the microprocessor-based fluorescence spectrometer used for the studies.

The vesicles added to the dye solution (above) had an internal pH of 8.0; the supporting buffer was pH 6.0. Under these conditions, dansylglycine is accumulated in the lumenal space. At 14.5 °C, the dye moves across DPPC bilayers so slowly that it is, effectively, surface restricted. If the temperature is elevated, however, dye translocation takes place at measurable rates, and the dye can accumulate in the vesicle lumen. The high local concentration of dye in the lumenal space is reflected in a correspondingly elevated dye concentration in the inner membrane monolayer. Figure 4D shows the consequences of incubating the sample at 36 °C (5 °C below T_c) for 20 min; the fluorescence signal from membrane-bound dye increased in both frequency and intensity as dye populated the vacant inner monolayer. Subtraction of outer membrane fluorescence (B) revealed that the dye now resident in this inner monolayer showed an emission maximum at 519 nm, corresponding to immersion in a medium of dielectric constant 4.7 (Figure 4E, Table I).

When the pH gradient existing across the vesicle bilayer is collapsed by the addition of ammonium acetate, the elevated concentration of dye in the lumenal space also collapses and

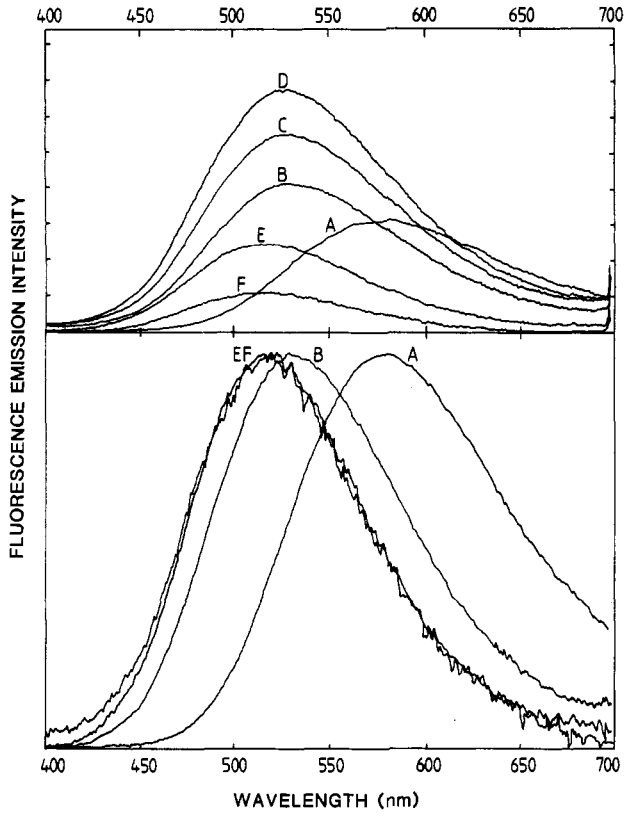

FIGURE 4: Spectral analysis of dansylglycine embedded in small DPPC vesicles. (Upper panel) Two milliliters of 100 mM sodium pyrophosphate, buffered to pH 6.0 with citric acid, was equilibrated to 14.5 °C in the quartz sample cuvette of a fluorescence spectrometer. Ten microliters of a 1 mM ethanolic solution of dansylglycine was added, to give a final dye concentration of 5×10^{-6} M. With an excitation wavelength of 350 nm (2-nm band-pass), the sample emission spectrum was recorded from 400 to 700 nm. Scan speed was 1 nm/s, and the data from five repetitive scans were accumulated (A). To this dye solution was added 50 μL of a 40 mg/mL suspension of small unilamellar DPPC vesicles in 100 mM sodium pyrophosphate buffer, pH 8.0. The sample was maintained at 14.5 °C and was stirred continuously while scans were made as before (B). (At this point, approximately 20% of the dye molecules were membrane-bound but restricted to the outer monolayer of the vesicles.) The contents of the cuvette were then transferred to a heating block equilibrated to 36 °C, incubated at this temperature for 20 min, and then returned to the fluorescence spectrometer and rapidly cooled to 14.5 °C again (at this point, dye was located in both the inner and outer monolayers). The sample was scanned, as before, with continuous stirring at 14.5 °C (D). Ten microliters of 1 M ammonium acetate was added, and the sample was scanned again (D); addition of ammonium acetate had no visible effect at this temperature. The sample was incubated at 36 °C for 20 min before being returned to the fluorometer and scanned, as before, at 14.5 °C (C). (At this point, dye was still resident in both the inner and outer monolayers, but some dye had been lost from the inner monolayer when the pH gradient across the vesicle bilayer was collapsed by the presence of ammonium acetate at 36 °C.) All spectra were collected in the ratio mode and were fully corrected for instrumental response characteristics and for scattered light. The fluorescence contribution from residal aqueous dye has been subtracted from spectra B–D. Difference spectra were obtained by simple subtraction of scan files. Spectrum E (D–B) represents dye resident in the inner monolayer in the presence of a transmembrane pH gradient, inside alkaline; scan F (C–B) represents the inner monolayer dye after the pH gradient has been collapsed. (Lower panel) Difference spectra depicted at a common maximal amplitude to illustrate the change in emission frequency that occurred at the different stages of this experiment.

equilibrates with the aqueous dye concentration of the external medium. Figure 4C shows that there was no change in fluorescence frequency profile when ammonium acetate was added at 14.5 °C (because dye translocation is so slow at this temperature), but when the system was allowed to relax by

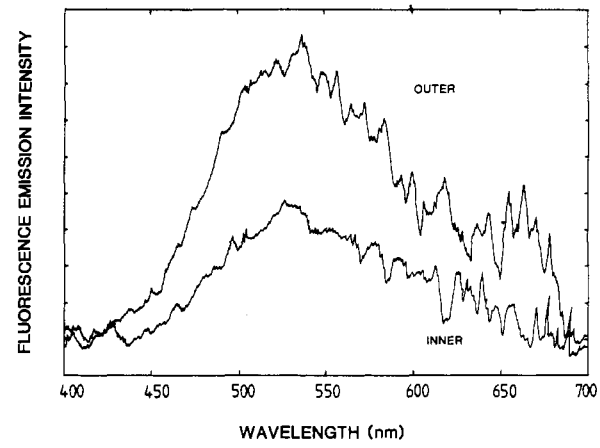

FIGURE 5: Emission characteristics of dye embedded in large diameter vesicles. Two milliliters of 50 mM sodium phosphate (pH 7.2) containing 5×10^{-6} M dansylglycine was equilibrated at 14.2 °C. To this dye solution was added 10 μL of a 10 mg/mL suspension of large unilamellar vesicles (prepared by octyl glucoside dialysis) giving a final lipid concentration of 7×10^{-5} M. Under experimental conditions similar to those described for Figure 4, the emission spectrum from this sample was recorded (10 scans accumulated). The scans were repeated after the sample had been incubated at 34 °C for 20 min and then returned to 14.2 °C. The fluorescence signals originating from the *outer* and *inner* membrane monolayers were isolated and corrected for light scatter and instrumental response characteristics as described for the preceding figure.

incubation at 36 °C, dye was lost from the inner membrane monolayer, resulting in a decrease in fluorescence in membrane-bound dye. At final equilibrium, the emission maximum of dye in the inner monolayer was 519 nm (Figure 4F). The fact that collapse of the pH gradient triggered an intensity reduction but no frequency shift in inner monolayer dye fluorescence confirmed that the low dielectric medium (4.7) was in a region very close to the location of pH changes triggered by the addition of ammonium acetate. Because the pH changes were restricted to the vesicle lumen, this is strong support for the hypothesis that the low dielectric environment represents the inner membrane monolayer. Integration of emission envelopes F and B (Figure 4), followed by correction for variations in fluorescence quantum yield, showed that, at final equilibrium, the outer monolayer accounted for 73% membrane-bound dye whereas the inner monolayer accounted for 27%.

Influence of Vesicle Size. In order to determine that the two distinct environments for membrane-bound dye were a direct consequence of lipid packing constraints imposed by a small radius of curvature in the boundary bilayer of small vesicles, the fluorescence experiments were repeated with phospholipid vesicles made by two separate methods that are known to generate predominantly unilamellar vesicles with a large diameter. The two methods selected were the octyl glucoside dialysis method of Mimms et al. (1981) and the solvent evaporation technique of Szoka and Papahadjopoulos (1978). In both cases, accurate spectral analysis was considerably complicated by the high degree of Rayleigh scatter associated with aqueous suspensions of the large vesicles. This problem was partially overcome by inserting filters (370 nm high band-pass) between the sample and photomultiplier and by using extremely dilute lipid suspensions. The spectra illustrated in Figure 5 demonstrate that, in spite of the low-amplitude signal from the membrane phase (because of the correspondingly small amount of phospholipid present in the optical sample), there was no discernible difference in the wavelength of maximal emission between dye resident in the inner and outer membrane monolayers of large lipid vesicles

produced by detergent dialysis, dye from both environments showing an emission maximum at approximately 535 nm; essentially similar results were obtained with vesicles produced by solvent evaporation.

Direct Evidence for Inner Monolayer Location of Dye. The interpretation of the preceding fluorescence measurements rests on two principal assumptions: (a) that, for practical purposes, dansylglycine cannot traverse the boundary bilayer of small, unilamellar DPPC vesicles at 14 °C but can at 36 °C and (b) that changes in fluorescence intensity/frequency accurately reflect corresponding changes in the distribution of dye between the aqueous and membrane phases. Although the first of these assumptions is given strong support by the translocation kinetics data presented for DMPC in Figure 3, I performed a straightforward experiment to provide direct evidence for restricted translocation at low temperatures. Lipid vesicles, mixed with dansylglycine, were passed down a gel filtration desalting column. Figure 6 shows that when all maneuvers were performed at 8 °C (cold-room temperature), vesicles eluted in advance of dye and were nonfluorescent. If, however, the vesicle/dye mixture was preincubated for 20 min at 36 °C before being chromatographed at 8 °C, then the resulting eluted vesicles showed fluorescence with emission characteristics identical with those associated with the "inner monolayer" dansylglycine of Figure 4. Clearly, when dye is mixed with vesicles at 8 °C (T_c − 33 °C), binding is restricted to the outer (proximal) membrane monolayer, and when this mixture is subsequently passed down a gel filtration column, all the membrane-bound dye is lost as the vesicles move through dye-free buffer to be eluted within the void volume of the column. In contrast, any dye that is allowed to reach the inner membrane monolayer during prior incubation at 36 °C (T_c − 5 °C) is retained during chromatography because of the extremely low dye translocation rate at the separation temperature (8 °C). Under these conditions, eluted vesicles retain dye in the inner monolayer, although all dye is lost from the outer monolayer, and show an emission profile virtually indistinguishable from that obtained from the subtraction spectrum for the inner monolayer presented in Figure 4.

Temperature Dependence of Membrane–Buffer Dye Partition Coefficient. Because of the large changes in fluorescence emission properties that accompany the binding of dansylglycine to lipid vesicles, it is practically straightforward to determine dye binding constants and translocation kinetics by spectral analysis (Figures 3 and 4) rather than resorting to physical separation of bound and free dye (Figure 6). The emission spectrum observed with a mixture of dye, vesicles, and buffer is a linear combination of spectra with different maxima and intensities and will thus show an intermediate maximum whose precise position will depend on the relative contributions of the two species of dye (bound and free). The 10-fold difference in quantum yield between membrane-bound dansylglycine and dye free in solution makes calculation of binding constants from fluorescence data relatively simple:

$$x = (F_{mix} - F_{aq})/9 \quad (3)$$

$$K_b = [x/(F_{aq} - x)](V_a/V_m) \quad (4)$$

F_{aq} and F_{mix} are the integrated fluorescence emission intensities of dansylglycine in the absence and presence (respectively) of lipid vesicles. Figure 4 shows that the emission intensity and maximum of an aqueous solution of dansylglycine both change dramatically in the presence of phospholipid vesicles; Figure 7 shows, in addition, that the emission intensity of the mixture is highly temperature dependent, fluorescence becoming more intense as the temperature is lowered; emission frequency is

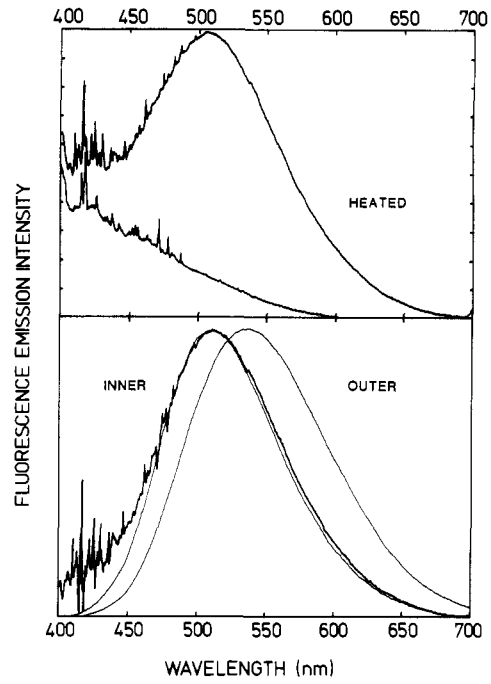

FIGURE 6: Direct isolation of vesicles with dye only in the inner membrane monolayer. (Upper panel) One milliliter of 100 mM potassium phosphate buffer (pH 6.0), containing 1 mg of DPPC in the form of small, unilamellar vesicles, was equilibrated to 8 °C. Ten microliters of a 1 mM ethanolic solution of dansylglycine was added, to give a final dye concentration of 10^{-5} M. The mixture was incubated for 20 min at 8 °C and then applied to the top of a small gel permeation column (0.5 × 17 cm, Bio-Rad P-10 gel equilibrated with 100 mM potassium phosphate, pH 6.0, at 8 °C). Fractions (0.5 mL) were collected as the column was eluted with cold buffer; the fractions containing lipid vesicles (identified by light scattering) were pooled, transferred to a quartz cuvette, and scanned for fluorescence emission over the range 400–700 nm at exciting radiation of 350 nm. No fluorescence was detected; the signals created by light scattering effects are shown above. The entire experiment was repeated, exactly as described above, with fresh vesicles and gel filtration medium. In this second experiment, however, immediately after the addition of the dansylglycine the vesicle suspension was heated to 36 °C and held at this temperature for 20 min before being cooled back down to 8 °C and applied to the gel column. With a temperature of 8 °C being maintained for all subsequent manipulations, the vesicle-containing fractions were again pooled and scanned for fluorescence emission. The resulting curve is identified in the upper panel as "heated". (Lower panel) The spectrum generated by the "heated" membranes is shown with scatter subtracted. The emission spectrum superimposes precisely upon the emission profile for dye inferred to be resident in the inner membrane monolayer (Figure 4), drawn here as a thinner solid line. The wavelength of maximal emission is the same for both spectra (510 nm); the emission spectrum attributed to dye resident in the outer membrane monolayer is also shown for comparison. All spectra shown above are uncorrected for frequency-dependent variations in instrumental response characteristics but were collected in ratio mode.

unaffected. The emission intensity of a corresponding dye solution in the absence of lipid is essentially temperature independent, so the effects in the presence of lipid could result from changes in dye binding efficiency, with more being bound at lower temperatures, or alternatively could result from temperature-dependent changes in fluorescence quantum yield of membrane-bound dye. Table I shows that dansylglycine quantum yield is relatively constant (0.64–0.66) over a range of environmental dielectric constants (10–2) within which there are dramatic shifts in wavelength of maximal emission (532–499 nm) so it is unlikely that temperature could be affecting dye quantum yield simply by forcing dye deeper into the bilayer to regions of even lower dielectric constant. Furthermore, Figure 8 shows that solutions of dansylglycine in 95% dioxane (dielectric constant 4.0) and water (dielectric

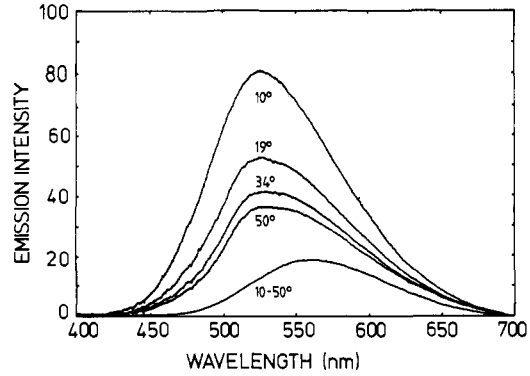

FIGURE 7: Temperature dependence of fluorescence characteristics of membrane-bound dansylglycine. A stirred suspension of small, unilamellar DPPC vesicles suspended in 50 mM sodium phosphate, pH 6.0, containing 5×10^{-6} M dansylglycine was equilibrated to several experimental temperatures. At each temperature (50, 34, 19, and 10 °C), the fluorescence emission spectrum of the sample was determined (using exciting radiation at 350 nm); the lipid concentration was 1.66×10^{-3} M. For comparison, the experiment was repeated with an aqueous solution of the dye containing no lipid vesicles; the emission profile of this sample did not vary over the temperature range 10–50 °C.

Table II: Temperature-Dependent Binding of Dansylglycine to Phospholipid Bilayers

temp (°C)[a]	K_b[b]
10	223
24	80
50	60

[a] Experimental temperature. [b] The membrane:buffer partition coefficient K_b (binding coefficient) was determined by equilibrium binding experiments as described under Materials and Methods. Suspensions of small unilamellar DPPC vesicles in 50 mM sodium phosphate, pH 6.8, were equilibrated to the experimental temperature before being dialyzed against solutions of dansylglycine in the same buffer. Final concentration of dye was 5×10^{-6} M; phospholipid concentrations ranged from 6.65 to 66.5 mM.

constant 79) demonstrate only slight increases in emission intensity (approximately 0.5%·°C^{-1}) as the temperature is lowered, whereas, in the presence of phospholipid vesicles, dye emission intensity increases almost 4-fold over the same temperature range. These differences are not created by light scattering and appear not to be influenced by the major gel–liquid crystalline phase transition of the DPPC bilayers used in the experiment (indicated by the sharp peak of the first derivative Rayleigh light scatter profile that occurs at 41 °C), suggesting that they are not effected by changes in solvent (phospholipid) microscopic viscosity, which can modify vibronic deactivation of the dye excited state and which is believed to change dramatically at T_c (Barratt et al., 1969). The remaining possibility, that binding of dansylglycine to lipid bilayers is itself temperature dependent, is strongly supported by the data in Table II, which shows the results of equilibrium binding measurements made at three different temperatures. Note that at 10 °C the lipid pool available for dye binding is constituted solely by the outer monolayer of the small phospholipid vesicles, which is generally considered to comprise approximately 68% of the total phospholipid (Schullery et al., 1980). There is little doubt that the dye binding efficiencies measured by equilibrium dialysis follow the same temperature profile as the dye fluorescence intensity measurements shown in Figure 8 and that, as a consequence, fluorescence measurements can provide an accurate resolution of the relative amounts of membrane-bound and free dansylglycine associated with a suspension of lipid vesicles.

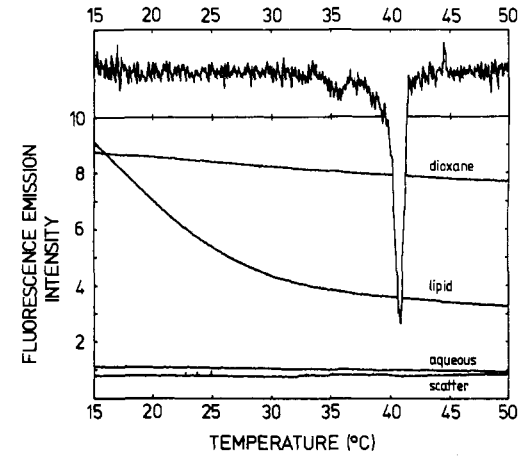

FIGURE 8: Temperature-dependent binding of dansylglycine to phospholipid bilayers. (Lower panel) Fluorescence emission intensity was recorded continuously as the sample temperature was raised from 15 to 50 °C; excitation was at 350 nm, and emission was recorded at 520 nm. The scan labeled "aqueous" was obtained with 50 mM sodium phosphate buffer, pH 6.0, containing 10^{-5} M dansylglycine; the scan labeled "scatter" was obtained with 50 mM sodium phosphate, pH 6.0, containing 1.66×10^{-3} M DPPC in the form of small, unilamellar vesicles. The "lipid" sample was composed of phosphate buffer containing both vesicles and dye mixed together at 45 °C and then cooled to 15 °C and scanned to 50 °C with continuous stirring; the final concentrations of dye and lipid were 10^{-5} M and 1.66×10^{-3} M, respectively. The "dioxane" sample consisted of dioxane/water, 95:5 (v/v), containing 3×10^{-6} M dansylglycine. (Upper panel) First derivative of the Rayleigh light scattering profile of the "scatter" sample (small, unilamellar DPPC vesicles) monitored with both excitation and emission monochromators set to 400 nm over the temperature range 15–50 °C.

Conclusions. It seems clear that differences in molecular packing between the inner and outer membrane leaflets give rise to structural asymmetries that can be detected by dansylglycine. The dye is highly sensitive to subtle changes in the polarity of its environment and can be used either as a nonpermeating or permeant probe according to the choice of experimental conditions. The dye is obviously quite deeply embedded in the membrane because the chromophore reports from a medium of very low dielectric constant; at the same time, the polar carboxylate function is sensitive to the proton concentration in the aqueous medium. Although apparent differences in the polarity of dye binding sites in the inner and outer leaflets could result from modified relaxation processes for the excited-state fluorophores, I favor the alternative explanation which is that they may well be a reflection of variations in the local availability of water. The work of Simon et al. (1982) suggests that the tighter packing of phospholipid head groups in the inner monolayer must lead to a reduced penetration of water molecules into that region, implying that water might penetrate less deeply into the inner monolayer of small vesicles than either the corresponding outer monolayer or both monolayers of planar membranes. An additional effect of this packing asymmetry is the "wedge effect" created by the high radius of curvature in the bilayer: dye molecules populating the inner monolayer are likely to be forced deeper into the membrane by the combined effects of tighter head group packing and looser acyl chain packing. The converse will be true for the outer monolayer, and these local restrictions may well make partitioning into the inner monolayer less favorable than into the outer leaflet. This type of effect is certainly thought to influence the distribution of phospholipids in small vesicles composed of mixtures of lipids (Nordlund et al., 1981), and it is consistent with our observation that, whereas the outside/inside ratio of phospholipid is 2.1 for small

DPPC vesicles (Schullery et al., 1980), the comparable ratio for dye in this system is 2.8, implying a greater concentration of dye in the outer monolayer than in the inner and supporting the notion that phospholipid packing asymmetry causes an asymmetric dye distribution by creating differential constraints on the environments of the dye molecules populating the two halves of the membrane.

The results of this study illustrate the practical utility of dansylglycine as a reporter chromophore in fluorescence studies of membrane structure. In addition, they highlight the importance of maintaining a clear distinction between the two principal environments in small vesicles (the inner and outer leaflets) that are shown to give rise to two different reporter group signals. No doubt a wide variety of membrane probes responds to the differences between these two environments; if no attempt is made to resolve the signals from the two monolayers, a false impression of homogeneity in the membrane environment will be generated. Addition of a carboxylate functional group appears to be an experimentally convenient way to reduce and control the rate at which a fluorescent dye equilibrates with the internal spaces of sealed membrane systems, providing a practical method for effecting this resolution.

Registry No. DMPC, 13699-48-4; DPPC, 2644-64-6; dansylglycine, 1091-85-6.

REFERENCES

Bangham, A. D., de Gier, J., & Greville, G. D. (1967) *Chem. Phys. Lipids 1*, 225–246.

Bangham, A. D., Hill, M. W., & Miller, N. G. A. (1974) *Methods Membr. Biol. 1*, 1–68.

Barratt, M. D., Green, D. K., & Chapman, D. (1969) *Chem. Phys. Lipids 3*, 140–144.

Bramhall, J. (1984) *Biochim. Biophys. Acta 778*, 393–399.

Bramhall, J. (1985) *AOCS Monogr. 13*, 43–50.

Brouillette, C. G., Segrest, J. P., Ng, T. C., & Jones, J. L. (1982) *Biochemistry 21*, 4569–4575.

Büttner, D., & Heydtmann, H. (1969) *Z. Phys. Chem. (Munich) 63*, 316–318.

Chaplin, D. B., & Kleinfeld, A. M. (1983) *Biochim. Biophys. Acta 731*, 465–474.

Chen, R. F. (1967) *Arch. Biochem. Biophys. 120*, 609–620.

Chen, R. F., & Kernohan, J. C. (1967) *J. Biol. Chem. 242*, 5813–5823.

Clemett, C. J., Forest, E., & Smyth, C. P. (1964) *J. Chem. Phys. 40*, 2123–2128.

DeGuzman, R., & Bramhall, J. (1985) *Biophys. J. 47*, 365a.

Drant, S. E., & Bramhall, J. (1984) *Biophys. J. 45*, 332a.

Farkas, P. (1932) *Z. Elektrochem. Angew. Phys. Chem. 38*, 654–665.

Förster, T. (1951) *Fluoreszenz Organischer Verbindungen*, p 45, Vandenhoeck & Ruprecht, Göttingen, FRG.

Hartley, B. S., & Massey, V. (1956) *Biochim. Biophys. Acta 21*, 58–68.

Huang, C. (1969) *Biochemistry 8*, 344–351.

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

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

Lee, B., McKenna, K., & Bramhall, J. (1985) *Biochim. Biophys. Acta 815*, 128–135.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 833–840.

Nordlund, J. R., Schmidt, C. F., Dicken, S. N., & Thompson, T. E. (1981) Biochemistry *20*, 3237–3241.

Schuh, J. R., Banerjee, U., Muller, L., & Chan, S. I. (1982) *Biochim. Biophys. Acta 687*, 219–225.

Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.

Simon, S. A., McIntosh, T. J., & Latorre, R. (1982) *Science (Washington, D.C.) 216*, 65–66.

Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.

Vaz, W. L. C., & Schoellmann, G. (1976) *Biochim. Biophys. Acta 439*, 206–218.