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Partitioning of Exchangeable Fluorescent Phospholipids and Sphingolipids between Different Lipid Bilayer Environments†

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ABSTRACT: Exchangeable phospho- and sphingolipid probes (phosphatidylcholine, -ethanolamine, -serine, and -glycerol, phosphatidic acid, sphingomyelin, cerebroside, and sulfatide) have been synthesized in which one acyl chain is substituted with a fluorescent bimanyl, 7-(dimethylamino)coumarin-3-yl, or diphenylhexatrienyl group. The distribution of these probes between two different populations of lipid vesicles can be readily monitored by fluorescence intensity measurements, as described by Nichols and Pagano [Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720-1726], when one of the vesicle populations contains a low mole fraction of a nonexchangeable quencher, (12-DABS)-18-PC. The probes examined in this study exchange between phospholipid vesicles on a time scale of minutes, with kinetics indicating that the transfer process takes place by diffusion of probe monomers through the aqueous phase. As expected, lipid probes with different charges differ markedly in their equilibrium distributions between neutral and charged lipid vesicles. However, probes with different polar headgroups differ only modestly in their relative affinities for vesicles composed of "hydrogen-bonding" lipids (PE and PS) vs "non-hydrogen-bonding" lipids (PC and PG or *O*-methyl-PA). Probes with different headgroups also show modest, albeit reproducible, differences in their relative affinities for cholesterol-containing vs cholesterol-free PC/PG vesicles. Our results suggest that lipids with different headgroup structures may mix more nearly ideally in liquid-crystalline lipid bilayers than would be predicted from previous analyses of the phase diagrams for binary lipid mixtures.

The nature of the lateral interactions between different lipid species can strongly influence the physical properties and the organization of bilayer membranes that contain multiple lipid components. Because of this fact, numerous studies have examined the interactions between different types of polar lipids in bilayers, using a variety of physical techniques [for reviews, see Lee (1977), Gaffney and Chen (1977), Mabrey and Sturtevant (1978), Melchior and Steim (1979), McElhaney (1984), Keough and Davis (1984), Thompson and Tillack (1985), Brauner and Mendelsohn (1986), and Curatolo (1987)]. To date, most thermodynamic information regarding the interactions between different lipids in bilayers has been derived from analyses of the phase diagrams for various binary mixtures of phospho- and sphingolipids. The principles of regular solution theory are often applied in such analyses to estimate quantitatively how differences in the headgroups and/or the acyl chains of different lipid species affect their free energy of mixing (Lee, 1977; van Dijck et al., 1977; Von Dreele, 1978; Cheng, 1980; Keough & Davis, 1982).

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The above approach to examine the thermodynamics of lipid mixing in bilayer membranes suffers from several significant limitations, particularly for the analysis of systems more complex than binary mixtures or for the study of lipid mixtures that exhibit complex solid-phase polymorphism. Alternative approaches can be useful to provide information about the thermodynamics of mixing between different lipid species in such systems, for which a full characterization of the phase diagram is not desirable or feasible. One such alternative approach is the measurement of the equilibrium distribution of exchangeable lipid species between different types of lipid vesicles, a method that can detect differences in the free energy of the exchangeable species in different lipid environments. This approach has been exploited previously to examine the equilibrium partitioning of radiolabeled cholesterol (Lange et al., 1979; Wattenberg et al., 1983; Fugler et al., 1985; Rujanavech & Silbert, 1986; Yeagle & Young, 1986) and of fluorescent phosphatidylcholine and phosphatidic acid probes (Nichols & Pagano, 1982) between different types of lipid vesicles.

In this study, we have adapted the resonance energy transfer based method described by Nichols and Pagano (1982) to examine the partitioning of a variety of exchangeable fluorescent lipids between lipid vesicles of various compositions. In this method, which requires no physical separation of different vesicle populations, the distribution of a fluorescent probe between two populations of vesicles is made readily measurable by including in one vesicle population a nonexchangeable quencher of the probe fluorescence. Using this approach, we have evaluated how the structure of the polar headgroup of a lipid molecule affects its partitioning between different lipid bilayer environments, which vary in their surface charges, lipid polar headgroup compositions, and/or sterol contents.

Materials and Methods

Materials. 1-Palmitoyl-2-[8-(bimanylthio)octanoyl]phosphatidylcholine [(8-bimane)-PC][1] and the corresponding species with an 11-(bimanylthio)undecanoyl chain at the 2-position [(11-bimane)-PC] were synthesized as described previously for the synthesis of the latter compound (Silvius et al., 1987). The intermediate phosphatidylcholine species in the above syntheses were in all cases purified by preparative thin-layer chromatography. 1-Palmitoyl-2-[11-[[[7-(dimethylamino)coumarin-3-yl]carbonyl]methylamino]undecanoyl]phosphatidylcholine [(11-DMCA)-PC] was prepared by labeling 1-palmitoyl-2-[11-(methylamino)undecanoyl]phosphatidylcholine, synthesized as described previously (Silvius et al., 1987), with a slight excess of 7-(dimethylamino)-coumarin-3-carboxylic acid *N*-hydroxysuccinimide ester (Molecular Probes, Junction City, OR) in 8:2:0.1 $CHCl_3$/CH_3OH/triethylamine for 6 h at 25 °C. The labeled phosphatidylcholine was purified by preparative thin-layer chromatography, using 65:35:2.5:2.5 $CHCl_3/CH_3OH/H_2O$/concentrated NH_4OH as the developing solvent. 1-Decanoyl-2-[3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]-propanoyl]-*sn*-glycero-3-phosphocholine was prepared by acylation of 1-decanoyllysophosphatidylcholine with the anhydride of 3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]-propanoic acid in the presence of 4-pyrrolidinopyridine (Mason et al., 1981).

The fluorescent phosphatidylcholines from the above preparations were converted to the corresponding PE, PS, PG, and PA derivatives by phospholipase D catalyzed transphosphatidylation or hydrolysis, as described by Comfurius and Zwaal (1977). Efficient transphosphatidylation of these probes could be achieved with incubation times or phospholipase concentrations that were 10–40-fold less than those required for the transphosphatidylation of longer chain PC's. The transphosphatidylated probes were extracted from the reaction mixtures and purified by preparative thin-layer chromatography.

N-[11-(Bimanylthio)undecanoyl]sphingomyelin and -cerebroside were prepared by coupling sphingosinephosphocholine (Cohen et al., 1984) or galactosylpsychosine [Radin (1974) as modified by Koshy and Boggs (1982)] to 11-[(mercaptomethyl)thio]undecanoic acid for 6 h at 37 °C in the presence of di-2-pyridyl disulfide and triphenylphosphine (Kishimoto, 1975). The intermediate *N*-[11-[(mercaptomethyl)thio]undecanoyl] sphingolipids from the above reactions were deprotected with excess dithiothreitol and labeled with a slight excess of monobromobimane (Silvius et al., 1987). *N*-[11-(Bimanylthio)undecanoyl]sulfatide was prepared in a similar manner from sulfogalactosylpsychosine (Koshy & Boggs, 1982), but the acylation step was carried out with the acyl chloride derivative of 11-[(mercaptomethyl)thio]undecanoic acid (Koshy & Boggs, 1983). All of these sphingolipid probes, as well as the intermediate protected mercaptoacyl compounds, were purified by preparative thin-layer chromatography (developing solvents: 65:25:4 $CHCl_3/CH_3OH$/concentrated NH_4OH for cerebroside, 80:20:5:3 $CHCl_3/CH_3OH/CH_3COOH/H_2O$ for sulfatide, and 50:40:10:6 $CHCl_3/CH_3OH/CH_3COOH/H_2O$ for sphingomyelin). 12-(Bimanylthio)octadecanoic acid (Silvius et al., 1987) was converted to the choline ester by reaction with choline iodide, dicyclohexylcarbodiimide, and 4-pyrrolidinopyridine (10, 5, and 1 molar equiv, respectively) for 16 h at 25 °C in the dark. The labeled choline ester was purified, after partitioning between $CHCl_3$ and 1:1 CH_3OH/H_2O, by thin-layer chromatography in 50:15:5:5:2 $CHCl_3$/acetone/$CH_3OH/CH_3COOH/H_2O$. All fluorescent labeling reactions were carried out with exclusion of light and under nitrogen.

Egg yolk phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL), and phosphatidylethanolamine and phosphatidylglycerol were prepared from it by enzymatic transphosphatidylation (Comfurius & Zwaal, 1977). Dioleoylphosphatidylserine and 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) were synthesized as described previously (Silvius & Gagné, 1984; Stamatatos et al., 1988). Cholesterol was obtained from Nu-Chek Prep (Elysian, MN) and was twice recrystallized from ethanol. All common inorganic chemicals were of reagent grade or better. All organic solvents were redistilled before use; diethyl ether

[1] Abbreviations: 8-bimane, 8-(bimanylthio)octanoyl; (8-bimane)-PC, -PE, -PG, -PA, and -PS, 1-palmitoyl-2-[8-(bimanylthio)octanoyl]-PC, -PE, -PG, -PA, and -PS; (12-DABS)-18-PC, 1-hexadecanoyl-2-[12-[[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]octadecanoyl]-*sn*-glycero-3-phosphocholine; 11-DMCA, 11-[[[7-(dimethylamino)coumarin-3-yl]carbonyl]methylamino]undecanoyl; (11-DMCA)-PC, -PE, -PG, -PA, and -PS, 1-palmitoyl-2-[11-[[[7-(dimethylamino)coumarin-3-yl]carbonyl]methylamino]undecanoyl]-PC, -PE, -PG, -PA, and -PS; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane; DPHP, 3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]propanoyl; EDTA, ethylenediaminetetraacetic acid trisodium salt; egg PE and egg PG, phosphatidylethanolamine and phosphatidylglycerol prepared by transphosphatidylation of egg yolk PC; egg PC, phosphatidylcholine from egg yolk; LUV, large unilamellar vesicle(s); *O*-methyl-PA, 1,2-diacyl-*sn*-glycero-3-phosphomethanol; PA, 1,2-diacyl-*sn*-glycero-3-phosphate; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PG, 1,2-diacyl-*sn*-glycero-3-phosphoglycerol; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; Tes, tris(hydroxymethyl)-methanesulfonic acid sodium salt; TNBS, trinitrobenzenesulfonic acid sodium salt.

used for vesicle preparations was redistilled, stabilized with 1% water, and stored shielded from light at 4 °C.

Methods. Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation and filtered through 0.1-μm pore size Nucleopore membranes (Wilschut et al., 1980). The buffer used to prepare the vesicles was 290 mM sucrose, 0.5 mM Tes, and 0.1 mM EDTA, pH 7.4, for experiments in which a low ionic strength was desired and 150 mM NaCl, 2 mM Tes, and 0.2 mM EDTA, pH 7.4, in all other experiments. Measurements of the trapped volumes of such vesicles, carried out with carboxyfluorescein as an internal marker (Wilschut et al., 1980), gave values of 3–4 μL/μmol of lipid, consistent with average vesicle diameters of ca. 850–1150 Å in these preparations.

Vesicles were labeled with exchangeable lipid probes according to either of two protocols. "Symmetrically" labeled vesicles were prepared by including 0.5–2 mol % of the fluorescent probe into the lipid mixture dispensed for the initial preparation of vesicles. "Asymmetrically" labeled vesicles were prepared by incubating preformed, unlabeled vesicles with sonicated dispersions of the probe in buffer, normally at vesicle and probe concentrations of 1 mM and 5–20 μM, respectively, for 1–2 h at 37 °C.

The partitioning of exchangeable lipid probes between different types of lipid vesicles was measured by incubating a fixed amount of probe-labeled donor vesicles (7.5–15 nmol) with varying amounts of a second, unlabeled population of acceptor vesicles (0–300 nmol) in a total volume of 3 mL. One of the two vesicle populations in these mixtures contained 2 mol % (12-DABS)-18-PC, a nonexchangeable quencher of the probe fluorescence (Silvius et al., 1987). The fluorescence signal recorded from these samples was measured before and after the addition of 1% Triton X-100; the latter fluorescence reading allowed the original fluorescence reading to be corrected, where necessary, for possible small variations in the amount of probe from sample to sample (Nichols & Pagano, 1982).

To estimate the fraction of the total lipid that was exposed to the external aqueous medium in preparations of large unilamellar PC/PG, PC/DOTAP, and PC/PG/cholesterol vesicles, similar vesicles were prepared containing 5 or 10 mol % PE. The percentage of total PE exposed at the outer surfaces of these vesicles was determined by the TNBS labeling assay of Nordlund et al. (1981). As PE has been shown to adopt a random transbilayer distribution in lipid vesicles with large radii of curvature (Nordlund et al., 1981), this value provides an estimate (typically with a standard error of less than 10% of the estimated value) of the fraction of the total vesicle lipid that is exposed at the external surface.

Phospholipid concentrations were determined by the method of Lowry and Tinsley (1974), with the modification that the sample digestion time was extended to 4 h. Concentrations of glycosphingolipid probes were determined by measurements of probe fluorescence in methanolic solution, using the corresponding labeled sphingomyelin as a standard.

Results

The structures of the phospho- and sphingolipid probes used in this study are shown in Figure 1. In phosphatidylcholine vesicles, the bimane-, DMCA-, and DPHP-labeled species show excitation (emission) maxima at 390 nm (468 nm), 400 nm (460 nm), and 364 nm (435 nm), respectively. The distribution of these probes between two populations of lipid vesicles can be readily monitored by fluorescence intensity measurements when one of the vesicle populations contains a small mole fraction of (12-DABS)-18-PC, a quencher of the

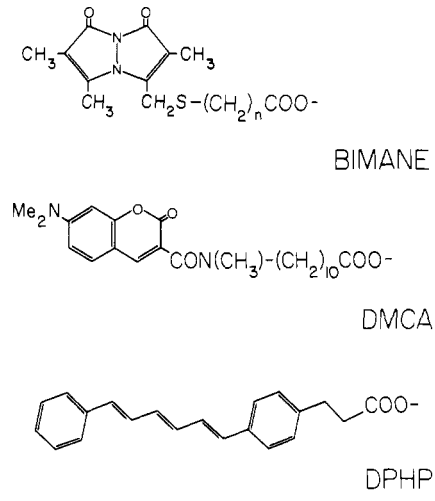

FIGURE 1: Structures of the fluorescent fatty acyl chains attached to the exchangeable lipid probes used in this study. For bimane- and DMCA-labeled phospholipid probes, the labeled acyl chain was attached to the 2-position of a phospholipid carrying a palmitoyl chain at the 1-position. For DPHP-labeled probes, the labeled chain was attached to the 2-position of a phospholipid carrying a decanoyl group at the 1-position.

probe fluorescence (Nichols & Pagano, 1982; Silvius et al., 1987). The spontaneous exchange of this quencher between lipid vesicles is too slow to affect the results of the experiments described here (Silvius et al., 1987).

In Figure 2A are shown time courses of fluorescence observed when large unilamellar donor vesicles (80:20 egg PC/egg PG), symmetrically or asymmetrically labeled with (8-bimane)-PC as described under Materials and Methods, are mixed with a large (20-fold) excess of acceptor vesicles prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18-PC. Upon addition of the acceptor vesicles, the fluorescence signal recorded from either sample rapidly falls to a new plateau value, which remains stable when the vesicles are further incubated for times up to at least a few hours. A much greater fraction of the probe is readily exchangeable (>95% vs ~55% in the example shown in Figure 2A) when asymmetrically rather than symmetrically labeled donor vesicles are used. A still smaller fraction of (8-bimane)-PC (<20%) was readily exchangeable from (vortexed) multilamellar vesicles which were symmetrically labeled with the probe (not shown). It thus appears that only probe molecules that are present at the outer surfaces of the donor vesicles can exchange between vesicles on the time scale of our experiments and that virtually all of the probe molecules in asymmetrically labeled vesicles become incorporated into the vesicles' outer surfaces (Pagano et al., 1981). Similar results were obtained with all of the other exchangeable fluorescent probes examined in this study. Asymmetrically labeled vesicles were therefore used in all further experiments described in this paper in order to maximize the fraction of the probe that was readily exchangeable.

Kinetics of Lipid Probe Exchange between Vesicles. In Figure 2B are shown the time courses of the fluorescence changes observed when a fixed amount of large unilamellar 80:20:2 egg PC/egg PG/(12-DABS)-18-PC vesicles, asymmetrically labeled with (8-bimane)-PE to a final level of 1 mol %, was incubated with varying amounts of acceptor vesicles prepared from 80:20 egg PE/DOPS. It can be seen that while the amplitudes of the fluorescence changes observed depend strongly on the amount of acceptor vesicles added, the half-time for equilibration of the probe between the vesicle populations is constant over a wide range of acceptor vesicle concentrations. This result suggests that the mechanism of probe

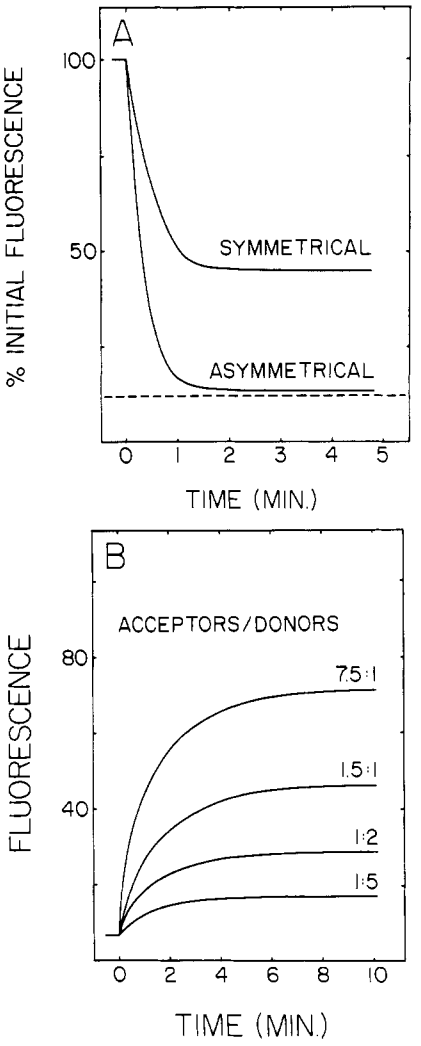

FIGURE 2: (A) Time courses of fluorescence changes observed when large unilamellar 80:20 egg PC/egg PG vesicles (2.5 μM), labeled symmetrically or asymmetrically with 2 mol % (8-bimane)-PC as described under Materials and Methods, were mixed at time zero with bath-sonicated 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles (100 μM). The dashed horizontal line represents the fluorescence measured for an equal amount of (8-bimane)-PC incorporated into 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles. (B) Time courses of fluorescence changes observed when 80:20:2 egg PC/egg PG/(12-DABS)-PC donor LUV (5 μM), asymmetrically labeled with 1 mol % (8-bimane)-PE, were mixed at time zero with 80:20 egg PE/DOPS acceptor LUV at the indicated ratios of donor to acceptor vesicles. All traces have been plotted to a common fluorescence scale.

transfer does not involve collisions between vesicles but rather proceeds by the transfer of probe molecules through the aqueous phase, at an overall rate that is limited by the rate of desorption of the probe from the donor vesicles (Roseman et al., 1980; Nichols & Pagano, 1981, 1982). Under these conditions, the initial slopes and the amplitudes of fluorescence time courses like those shown in Figure 2B can be used to calculate the unimolecular rate constant k_{off} for desorption of the probe from the donor vesicle surface [see Nichols and Pagano (1982)].

In Table I we summarize the rate constants, estimated as just described, for the desorption of various fluorescent phospho- and sphingolipid probes from 80:20 egg PC/egg PG LUV or 80:20 egg PE/DOPS LUV in the presence of a large excess of acceptor vesicles. In all cases, we established that variation of the acceptor vesicle concentration over at least a 5-fold range did not significantly affect the half-time for the probe equilibration process. The length of the labeled acyl

Table I: Rate Constants for Dissociation of Phospho- and Sphingolipid Probes from Large Unilamellar Phospholipid Vesicles[a]

probe headgroup	donor vesicles	k_{off} (min^{-1}) when labeled acyl chain is		
		8-bimane	11-bimane	11-DMCA
PC	PC/PG	1.49	0.070	0.060
PE	PC/PG	0.53	0.033	0.020
PS	PC/PG	2.88	0.144	0.116
PG	PC/PG	1.33	0.080	0.082
PA	PC/PG	2.82	0.093	0.065
sphingomyelin	PC/PG		1.088	0.756
cerebroside	PC/PG		0.149	0.105
sulfatide	PC/PG		0.725	
PC	PE/PS	1.27	0.085	0.072
PE	PE/PS	0.51	0.040	0.028
PS	PE/PS	2.06	0.128	0.112
PG	PE/PS	1.62	0.101	0.080
PA	PE/PS	1.67	0.047	0.043
sphingomyelin	PE/PS		0.608	0.464
cerebroside	PE/PS		0.100	0.095
sulfatide	PE/PS		0.611	

[a] LUV prepared from either 80:20:2 egg PC/egg PG/(12-DABS)-18-PC or 80:20 egg PE/DOPS were asymmetrically labeled with the indicated probes at a level of 1 mol %, as described under Materials and Methods. The fluorescence of the labeled donor vesicles (2.5 μM) was continuously monitored upon the addition of acceptor vesicles [typically 10–50 μM; 80:20 egg PE/DOPS vesicles were used as acceptors with 80:20:2 egg PC/egg PG/(12-DABS)-18-PC donor vesicles and vice versa]. The initial slopes and the maximum values of the resulting fluorescence changes were measured and were used to calculate the apparent first-order rate constant for the probe exchange process. Since this apparent rate constant was in all cases independent of the acceptor vesicle concentration from 10 to 50 μM, the rate constants measured were taken as estimates of the unimolecular rate constant k_{off} for dissociation of the probes from the bilayer surface.

chain strongly affects the rate of exchange for a given type of probe: 11-bimane-labeled probes typically desorb from PC/PG or PE/PS vesicles at rates 15–30 times slower than their 8-bimane-labeled counterparts. The structure of the lipid headgroup also has a substantial effect on the exchange rates measured for different probes with the same labeled acyl chain. PE and cerebroside probes, for example, desorb from phospholipid vesicles much more slowly than do the corresponding PC or sphingomyelin probes, respectively, while anionic lipid probes generally desorb relatively rapidly from the vesicle surfaces.

The desorption rate constants measured for anionic lipid probes could be significantly affected by the surface charge of the "donor" vesicles. To evaluate the importance of this effect, we compared the rates of desorption of (11-bimane)-PC and -PA from bath-sonicated vesicles with different surface charges. The values of k_{off} measured for desorption of (11-bimane)-PA from bath-sonicated vesicles prepared from egg PC, 80:20 egg PC/egg PG, and 80:20 egg PC/DOTAP were 0.29 min^{-1}, 0.59 min^{-1}, and 0.14 min^{-1}, respectively, under the conditions used in the experiments described above. By contrast, the k_{off} values measured for desorption of (11-bimane)-PC were much more closely comparable for these three preparations of vesicles (0.33 min^{-1}, 0.36 min^{-1}, and 0.31^{-1}, respectively). We can estimate from these results that, under the conditions of the experiments summarized in Table I, electrostatic effects will accelerate the desorption rates measured for anionic lipid probes by a factor of roughly 2-fold over the rates expected in the absence of such effects.

To examine the possible pH dependence of the desorption rates for probes with titratable polar headgroups, we measured the rate constants for desorption of (11-bimane)-PA, -PE, -PS, and (as a control) -PC from bath-sonicated egg PC/egg PG (80:20) vesicles at various pH values. As shown in Figure 3,

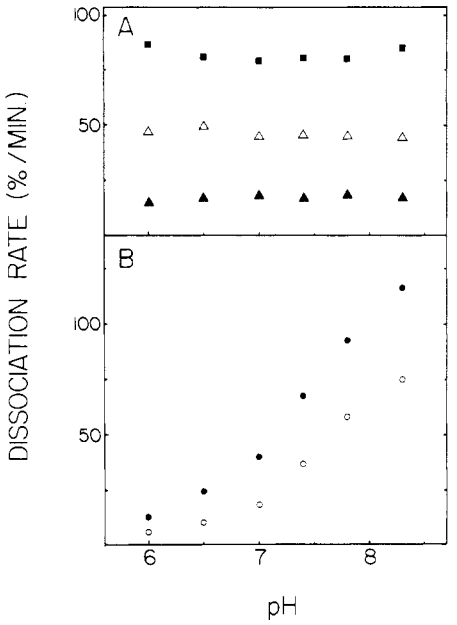

FIGURE 3: (A) pH dependence of the initial rate of dissociation of (8-bimane)-PC (△), -PS (■), and -PE (▲) from bath-sonicated 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles upon addition of a 20-fold excess of 80:20 egg PC/egg PG vesicles. (b) pH dependence of the initial rate of dissociation of (8-bimane)-PA from bath-sonicated 98:2 egg PC/(12-DABS)-PC vesicles (○) or 80:20:2 egg PC/egg PG/(12-DABS)-PC vesicles (●). Initial rates of probe dissociation from the donor vesicles upon addition of the acceptors were determined from time courses similar to those shown in Figure 2B, dividing the initial slope by the maximum fluorescence change observed.

the k_{off} values measured for the PE, PS, and PC probes did not vary significantly from pH 6.0 to pH 8.3, while that measured for (11-bimane)-PA varied by more than 10-fold over this pH range. The observed variation of k_{off} with pH for the PA probe can be well described by assuming that it represents the titration of a single proton-binding group with an effective pK_a of 7.4–7.6. A very similar pH dependence is observed for the desorption of this PA probe from egg PC vesicles, although in this case the k_{off} values measured at any given pH are roughly half those measured with egg PC/egg PG donor vesicles.

Partitioning of Lipid Probes between Different Vesicle Populations. (A) Effects of Surface Charge. As described in the Appendix, measurements of the fluorescence intensity for a lipid probe in the presence of varying proportions of donor and acceptor vesicles can be analyzed to determine an effective partition coefficient (SK_p) describing the distribution of the probe between the two types of vesicles. In a first series of experiments, we used this approach to examine the partitioning of anionic, cationic, and neutral lipid probes between lipid vesicles with various surface charges. These measurements were useful to evaluate the potential importance of electrostatic effects in determining the partitioning of charged lipid probes between populations of lipid vesicles that could exhibit somewhat different surface potentials.

In Figure 4 are plotted the fluorescence changes measured when egg PC/(12-DABS)-18-PC (98:2) donor vesicles, labeled with either (8-bimane)-PA (panel A) or the choline ester of 12-(bimanylthio)octadecanoic acid (panel B), were incubated with varying amounts of cationic or anionic acceptor vesicles. Each of the curves shown in Figure 4 is well described by a hyperbolic relationship of the form given in eq 2. The effective partition coefficients (SK_p) estimated from these curves, and from similar curves measured at low ionic strength, are given in the legend to Figure 4. It is apparent that the PA probe

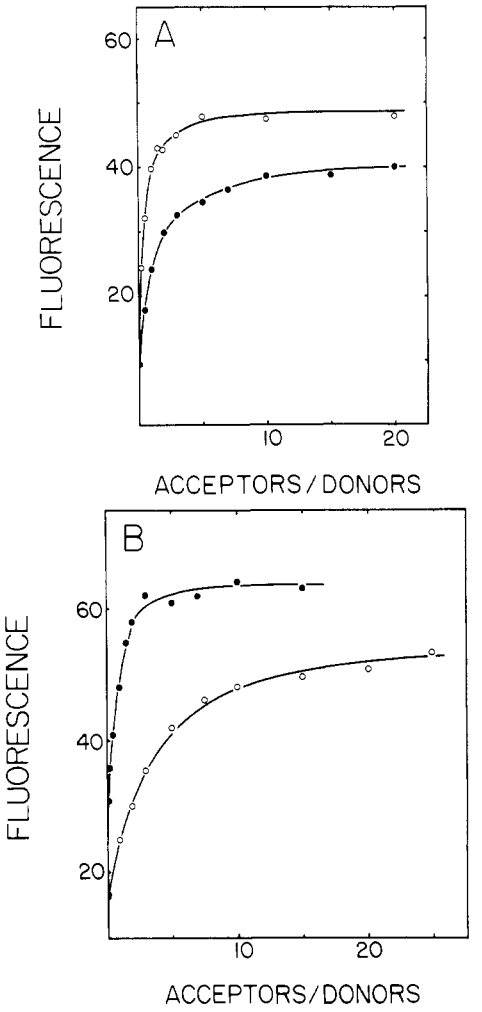

FIGURE 4: Fluorescence values measured when a fixed concentration of bath-sonicated 98:2 egg PC/(12-DABS)-PC vesicles (4 μM lipid), asymmetrically labeled with 1 mol % (8-bimane)-PA (panel A) or 12-(bimanylthio)stearic acid choline ester (panel B), was incubated with variable amounts of bath-sonicated anionic 85:15 egg PC/egg PG vesicles (filled circles) or cationic 85:15 egg PC/DOTAP vesicles (open circles). After 20-min incubation, the fluorescence of each sample was measured before and after the addition of 1% Triton X-100; the latter reading allowed correction for small variations in the amount of probe from sample to sample. The values of SK_p measured from these curves and from the results of similar experiments carried out in a buffer of low ionic strength (290 mM sucrose, 0.5 mM Tes, 0.1 mM EDTA, pH 7.4; values shown in parentheses) were as follows: for (8-bimane)-PA, SK_p = 0.66 (0.20) or 2.44 (3.77) with anionic or cationic acceptor vesicles, respectively; for 12-(bimanylthio)stearic acid choline ester, SK_p = 2.75 (5.59) or 0.26 (0.14) with anionic or cationic acceptor vesicles, respectively.

associates with cationic vesicles with greater avidity than it does with anionic vesicles, while the opposite is true for the labeled choline ester. As expected, the effects of the acceptor vesicle surface charge on the partitioning of these charged probes are more pronounced at lower ionic strength.

In a second, related set of experiments, we compared the partitioning of (8-bimane)-PA and -PC between bath-sonicated egg PC/(12-DABS)-18-PC donor vesicles and large unilamellar acceptor vesicles with different surface charges. The results of these experiments are summarized in Table II. The effective partition coefficients (SK_p) measured for (8-bimane)-PA in these experiments do not clearly show the expected dependence on the surface charges of the acceptor vesicles. At the same time, however, the effective partition coefficients for (8-bimane)-PC are found to vary considerably for the different types of acceptor vesicles examined. When

Table II: Partitioning of Bimane-Labeled PA and PC between PC Vesicles and Vesicles with Varying Surface Charges[a]

acceptor vesicles	SK_p (8-bimane)-PC	SK_p (8-bimane)-PA	K_p(PA)/K_p(PC)
PC/PG (95:5)	0.40	0.37	0.93
PC/PG (85:15)	0.71	0.51	0.71
PG	6.66	0.88	0.175
PC/DOTAP (85:15)	0.43	1.09	2.54

[a] Egg PC vesicles, prepared by bath-sonicated egg PC dispersions in buffer for 10 min under nitrogen, were asymmetrically labeled with (8-bimane)-PC or -PA (1 mol %) as described under Materials and Methods. The labeled vesicles (3 or 5 μM) were incubated with varying concentrations (0.5–60 μM) of large unilamellar acceptor vesicles of the indicated compositions. Effective partition coefficients (SK_p), describing the distributions of the probes between the donor and acceptor vesicle populations, were determined from fluorescence measurements on these samples as described in the Appendix.

the partition coefficient measured for the PA probe in each experiment is normalized to that measured for the PC probe in the same experiment (Table II, last column), the effects of the acceptor surface charge on the partitioning of the anionic PA probe become clear.

The results of TNBS labeling assays (results not shown) showed that acceptor vesicles similar to those used in the above experiments, but containing small amounts of egg PE, exposed a similar proportion of their total lipids at their outer surfaces (53–60%) regardless of whether they contained 15% PG, 95% PG, or 15% DOTAP as the charged component. This result suggests that the different types of lipid vesicles examined in Table II genuinely differ in their affinities even for the uncharged (8-bimane)-PC probe, possibly reflecting differences in the surface packing densities of the lipids in different types of vesicles. It is apparent that the ratio of K_p values for two probes may be a more informative parameter than is the absolute K_p value for either probe in experiments that are designed to examine the effect of the probe headgroup on partitioning between different lipid environments.

(B) Effects of Phospholipid Composition. Many of the physical properties of polar lipids have been suggested to depend strongly on the abilities of the lipid headgroups to participate in hydrogen-bonding interactions with the headgroups of neighboring lipids in the membrane bilayer [for reviews, see Boggs (1980, 1987)]. To evaluate whether the hydrogen-bonding ability of a lipid headgroup can affect its tendency to associate with "hydrogen-bonding" lipids in preference to "non-hydrogen-bonding" lipids,[2] we measured the partitioning of various phospho- and sphingolipid probes between large unilamellar vesicles which were composed either of egg PE and DOPS or of egg PC and egg PG or *O*-methyl-PA. Amino phospholipids such as PE and PS can serve both as hydrogen-bond donors and acceptors and are considered to be good hydrogen-bonding species. By contrast, lipids such as PC can serve only as hydrogen-bond acceptors and are considered to be poor hydrogen-bonding species (Boggs, 1980, 1987; Hauser et al., 1981).

In Table III we summarize the results of a series of measurements of the partitioning of various lipid probes between

[2] For simplicity, in this discussion we define as "hydrogen-bonding" lipids those species that can serve as *donors* of hydrogen bonds to adjacent lipids (e.g., amino phospholipids) and as "non-hydrogen-bonding" lipids those species that cannot (e.g., PC or *O*-methyl-PA). Species of the latter class may of course still serve as acceptors (e.g., via carbonyl or phosphoryl groups) in hydrogen-bonding interactions with adjacent lipid molecules.

Table III: Partitioning of Phospho- and Sphingolipid Probes between PC/PG and PE/PS Large Unilamellar Vesicles[a]

probe headgroup	labeled chain	K_p(probe)/K_p(PC probe)[c]
PE	bimanyloctanoyl	0.68 ± 0.06
PE[b]	bimanyloctanoyl	0.59 ± 0.08
PE	DMCA-undecanoyl	0.68 ± 0.09
PE	DPHP	0.83 ± 0.03
PG	bimanyloctanoyl	0.90 ± 0.07
PG	DMCA-undecanoyl	0.79 ± 0.06
PS	bimanyloctanoyl	1.17 ± 0.05
PS	DMCA-undecanoyl	1.42 ± 0.19
PA	bimanyloctanoyl	1.76 ± 0.07
PA[b]	bimanyloctanoyl	1.82 ± 0.16
PA	DMCA-undecanoyl	1.73 ± 0.24
PA	DPHP	1.40 ± 0.15
sphingomyelin	bimanylundecanoyl	1.24 ± 0.06
sphingomyelin	DMCA-undecanoyl	1.04 ± 0.05
cerebroside	bimanylundecanoyl	0.81 ± 0.08
cerebroside	DMCA-undecanoyl	0.66 ± 0.08
sulfatide	DMCA-undecanoyl	0.94 ± 0.05

[a] Large unilamellar vesicles, prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18-PC or from 80:20 egg PE/DOPS, were asymmetrically labeled with the indicated lipid probes at 1 mol %. The fluorescence of labeled donor vesicles (3 or 5 μM) was measured after incubation (30 min for bimanyloctanoyl-labeled species; 2 h for other probes) in the presence of varying concentrations of acceptor vesicles [typically 0.5–60 μM; egg PE/DOPS vesicles were used as acceptors with egg PC/egg PG/(12-DABS)-PC donor vesicles and vice versa]. The fluorescence measurements obtained were used to determine effective partition coefficients (SK_p) as described in the Appendix. For presentation in this table, the effective partition coefficient measured for a given probe in each experiment has been divided by the effective partition coefficient measured for the corresponding PC probe in the same experiment. [b] 80:20:2 egg PC/*O*-methyl-PA/(12-DABS)-18-PC vesicles were used in place of egg PC/egg PG/(12-DABS)-18-PC vesicles in these experiments. [c] Values are presented as the mean ± SEM from at least three separate determinations.

80:20:2 (molar proportions) egg PC/egg PG/(12-DABS)-18-PC LUV and 80:20 egg PE/DOPS LUV. In this table, the relative affinity of each probe for PE/PS vesicles vs PC/PG vesicles is expressed as the ratio of the partition coefficient K_p for the probe to the partition coefficient measured in the same experiment for the corresponding PC probe. Data were collected both by labeling PC/PG/(DABS-PC) vesicles with the exchangeable probe and titrating with PE/PS vesicles, as illustrated in Figure 4, and in the reciprocal experiment in which PE/PS vesicles were initially labeled with the probe and were titrated with PC/PG/(DABS-PC) vesicles. The results obtained with either method generally agreed very well, and the data shown in Table III summarize results obtained from both types of measurements.

Several important points are evident from the data presented in Table III. First, the partition coefficients for the various lipid probes tested in this system all lie within a factor of 2 of the values determined for the corresponding PC probes. [For reference, the effective partition coefficients (SK_p) measured for the PC probes in these experiments averaged 0.66 for (8-bimane)-PC, 0.49 for (11-DMCA)-PC, and 0.85 for DPHP-PC.] Second, the effects of a probe's headgroup structure on its relative affinity for PE/PS vs PC/PG vesicles are quite consistent regardless of whether the probe carries a bimane-, a coumarin-, or a DPH-labeled acyl chain. This quantitative consistency of the partitioning results obtained with three different families of probes is noteworthy, since the structures of the fluorescent groups and the flexibilities of the labeled acyl chains differ markedly for the three probe types.

Table IV: Partitioning of Lipid Probes between Large Unilamellar PC/PG Vesicles and PC/PG/Cholesterol Vesicles[a]

probe	K_p(probe)/K_p(PC)
N-(11-DMCA)sphingomyelin	1.99 ± 0.18
N-(11-DMCA)sulfatide	1.31 ± 0.09
N-(11-DMCA)cerebroside	1.79 ± 0.09
(11-DMCA)-PE	0.77 ± 0.14

[a] Large unilamellar vesicles, prepared from 80:20:2 egg PC/egg PG/(12-DABS)-18-PC or 48:12:40 egg PC/egg PG/cholesterol, were asymmetrically labeled with the indicated probes at 1 mol %. The partitioning of the probes between these two types of vesicles was determined as described for the experiments summarized in Table III. The partition coefficient for a given probe is defined as the ratio of the affinity of the probe for the cholesterol-containing vesicles over that for the cholesterol-free vesicles. For presentation in this table, the partition coefficient measured for each probe has been divided by that measured for (11-DMCA)-PC in the same experiments. Values are presented as the mean ± SEM from at least three separate experiments.

Third, the selectivity of a given probe for PE/PS vesicles vs PC/PG vesicles cannot be systematically correlated with the hydrogen-bonding ability of the probe headgroup. For example, PE and monoionized PA are both hydrogen-bonding species while PC is not (Boggs, 1980, 1987), yet PE probes show a lower relative affinity and PA probes a greater relative affinity for PE/PS vesicles (in competition with PC/PG vesicles) than do PC probes. Essentially identical results were obtained using PC/*O*-methyl-PA vesicles in place of PC/PG vesicles in these experiments (see Table III).

(*C*) *Effects of Cholesterol.* In a final series of experiments, we examined the partitioning of several bimane-labeled phospho- and sphingolipid probes between LUV composed of either 80:20 egg PC/egg PG/(12-DABS)-18-PC or 48:12:40 egg PC/egg PG/cholesterol. The results of these experiments are summarized in Table IV, where the effective partition coefficient measured for each probe has been normalized to that measured for the corresponding PC probe in the same experiment. The relative affinities of various probes for cholesterol-containing vs cholesterol-free vesicles decrease in the order sphingomyelin > cerebroside > sulfatide ≈ PC > PE. Again, however, the different probes vary only modestly in their relative affinities for cholesterol-containing vs cholesterol-free vesicles. The partition coefficient measured for the anionic sulfatide probe in these experiments may be slightly elevated, in favor of the cholesterol-containing vesicles, by the fact that the surface charge density is somewhat lower in the cholesterol-containing than in the cholesterol-free vesicles. However, from results like those shown in Table II, we can estimate that this effect will be small (enhancing K_p by no more than 20–25%) under the conditions of our experiments.

The value of the effective partition coefficient (SK_p) measured for the partitioning of (11-bimane)-11-PC between cholesterol-containing and cholesterol-free vesicles in the above experiments averaged 0.191 ± .027 in six independent determinations. The results of TNBS labeling experiments, carried out with similar vesicles containing 5 mol % egg PE as described under Materials and Methods, indicated that essentially the same fraction of the vesicle lipid was exposed at the outer surfaces of both cholesterol-containing and cholesterol-free vesicles (i.e., that $S \approx 1$). It thus appears that cholesterol-containing vesicles exhibit a substantially (3–6-fold) lower affinity for all of the probes studied here than do similar vesicles that do not contain cholesterol.

Discussion

Previous analyses of phase diagrams for various binary mixtures of lipids with different headgroups (Shimsick & McConnell, 1973; Wu & McConnell, 1975; Mabrey & Sturtevant, 1976; Arnold et al., 1981; Ruocco et al., 1983; Maggio et al., 1985; Curatolo, 1986, 1987) have suggested that such lipids often show significantly nonideal mixing even in liquid-crystalline lipid bilayers. These results have important implications for understanding the microscopic organization of multicomponent lipid membranes, including such aspects as the lateral distributions of different lipid species (Von Dreele, 1978; Knoll et al., 1985; Thompson & Tillack, 1985; Somerharju et al., 1985), the energy required to create and to maintain transmembrane lipid asymmetry (Tenchov & Koynova, 1985), and the local formation of nonlamellar structures in mixtures of "bilayer-forming" and "non-bilayer-forming" lipids (Vasilenko et al., 1982; Tilcock et al., 1982; Boni & Hui, 1983; Siegel, 1984; Eriksson et al., 1985; Tate & Gruner, 1987). It therefore seems desirable to utilize complementary approaches, such as direct measurements of lipid partitioning between different lipid environments (Lange et al., 1979; Nichols & Pagano, 1982; Wattenberg et al., 1983; Fugler et al., 1985; Rujanavech & Silbert, 1986; Yeagle & Young, 1986), to confirm the conclusions of the above studies and to extend them to more complex systems (e.g., mixtures of lipids with heterogeneous acyl chain compositions, or mixtures of lipids and sterols), for which a rigorous determination of the phase diagram may not be feasible.

Our measurements of the kinetics of equilibration of fluorescent lipid probes between different lipid vesicles show that the headgroup structure and the acyl chain lengths of a lipid probe affect its rate of dissociation from lipid bilayers in a manner similar to that observed previously with other types of exchangeable lipid analogues (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982; Massey et al., 1982a,b; Correa-Freire et al., 1982; Frank et al., 1983; Ferell et al., 1985; Nichols, 1985). Of most importance for present purposes is the observation that variations in the headgroup structures of the lipid probes examined here substantially affect the rates of probe dissociation from lipid bilayers. PE probes, for example, dissociate from PC/PG or PE/PS bilayers two to three times more slowly than do PC probes with the same acyl chains, and cerebroside probes dissociate from such bilayers five to eight times more slowly than do sulfatide or sphingomyelin probes with the same *N*-acyl groups. These results may reflect an ability of the PE and cerebroside probes to form hydrogen bonds to the headgroups of neighboring lipids (Hitchcock et al., 1974; Pascher & Sundell, 1977; Hauser et al., 1981), an interaction that would be expected to retard the desorption of such molecules from lipid bilayers (Massey et al., 1982b). Likewise, the results shown in Figure 3 indicate that a PA probe in its monoanionic form, which can hydrogen bond to neighboring lipids, dissociates at least 10 times more slowly from the surface of a PC or a PC/PG bilayer than does the doubly ionized form of the probe, which cannot form such hydrogen bonds (Boggs, 1980, 1987). These findings indicate that the exchangeable lipid probes studied here interact with the surrounding bilayer in a manner that is strongly affected by the properties of the probe headgroup.

In view of the kinetic results just noted, it is rather surprising to find that lipid probes with a variety of different headgroups vary only modestly in their relative affinities for different types of phospholipid bilayers. This result is also surprising because, as already noted, previous studies of the behavior of binary lipid mixtures have suggested that the mixing of lipids with different polar headgroups can be markedly nonideal even in the liquid-crystalline state. As a case in point, we can estimate from previously reported results (Lee, 1977; Arnold et al.,

1981) that the excess enthalpy of mixing of a low mole fraction of dimyristoyl- or dipalmitoyl-PE with the corresponding PC in a liquid-crystalline bilayer will be on the order of 1.0 kcal mol^{-1}. If the exchangeable PE and PC probes examined in this study were to show a similar discrimination in their interaction with PE bilayers vs PC in lipid bilayers, we would predict that a PE probe would show roughly a 5-fold greater relative affinity for PE-rich vesicles, in competition with PC-rich vesicles, than would the corresponding PC probe. In fact, we find that exchangeable PE probes show a slightly *lesser* preference for PE-rich vesicles vs PC-rich vesicles than do the corresponding PC probes.

The observation that PE probes fail to discriminate in favor of a hydrogen-bonding lipid environment (PE/PS vesicles) over a non-hydrogen-bonding environment (PC/PG or PC/*O*-methyl-PA vesicles) is not inconsistent with the suggestion that the amino group of PE may form hydrogen bonds to neighboring phospholipid molecules in lipid bilayers. The PE amino group can in principle hydrogen bond to the phosphoryl group of any phospholipid, including species such as PC that lack hydrogen bond donating groups. The transfer of PE molecules from a PE-rich to a PC-rich environment, either within one bilayer or between distinct lipid vesicles, thus need not decrease the *total* number of hydrogen bonds between lipid headgroups in the system as a whole. The same is true for the transfer of PC molecules from a PC-rich to a PE-rich environment. There thus need be no inherent energetic "cost", at least in terms of a loss of hydrogen-bonding interactions, that accompanies the intermixing of PE and PC molecules in lipid bilayers.

Our measurements of the partitioning of various lipid probes between PC/PG vesicles and PC/PG/cholesterol vesicles (Table IV) indicate that the structure of the probe headgroup has a significant, if modest, influence on the relative affinity of the probe for cholesterol-containing vs cholesterol-free bilayers. The relative affinities of different phospholipid probes for cholesterol-rich bilayers decrease in the order sphingomyelin > PC > PE, which coincides with the order of affinities of these three lipids for cholesterol as deduced from previous calorimetric studies (van Dijck et al., 1976, 1979; Demel et al., 1977) and some cholesterol-partitioning measurements [Wattenberg et al., 1983; Fugler et al., 1985; Yeagle & Young, 1986; for a contrasting report, see Lange et al. (1979)]. In this system also, however, it is noteworthy that different lipid probes differ only modestly in their discrimination between a cholesterol-free and a cholesterol-containing lipid bilayer environment.

In any study involving the use of fluorescent-labeled lipids to elucidate the behavior of unlabeled lipids, it is important to ensure that the results obtained with the fluorescent lipid adequately reflect the behavior of the corresponding unlabeled species. For two major reasons, we believe that this condition is fulfilled for the thermodynamic experiments described here. First, we note that our major conclusions in this study are based on *comparisons* of the behavior of probes with different headgroups but the same fluorescent-labeled acyl chains. Such comparisons should serve to factor out the possible effects of the fluorescent group per se on the partitioning of lipid probes between different bilayer environments. In support of this suggestion, we note that such comparisons reveal consistent effects of the probe headgroup structure on the partitioning of three different families of fluorescent probes, with quite different structures of their labeled acyl chains, between PC/PG and PE/PS vesicles. Second, as discussed above, our kinetic results demonstrate that the headgroup structure of a lipid probe significantly affects its rate of desorption from a lipid bilayer, in a manner that is consistent for the various families of probes examined in this study as well as for a previously studied series of pyrene-labeled phospholipids (Massey et al., 1982a). It thus appears that the presence of fluorescent reporter groups on the lipid probes examined here does not eliminate the possibility of headgroup-specific interactions between the probes and the surrounding lipid molecules. For these reasons, we feel that the results obtained in this study provide a valid basis to evaluate possible selectivities in the interactions between lipids with different polar headgroups in liquid-crystalline lipid bilayers.

Our findings suggest that the intermixing of lipids with different headgroups in liquid-crystalline bilayer membranes may be more nearly ideal than would be infered from previous analyses of phase diagrams for binary lipid mixtures [see, e.g., Lee (1977), Arnold et al. (1981), and Maggio et al. (1985)]. However, the apparent discrepancies between some of our present conclusions and those derived from studies of the type just cited may rest on certain key differences in the experimental systems and analyses employed in each case. First, our experiments have used samples composed of (noninteracting) unilamellar lipid vesicles, while phase diagrams for multicomponent lipid mixtures are typically obtained with samples in which different bilayers can readily interact at close range (e.g., dispersions of multilamellar vesicles). It is possible that the mixing of different phospholipids may be appreciably different in large unilamellar vs multilamellar structures. Second, quantitative analyses of the phase diagrams for binary lipid mixtures (e.g., using regular solution theory) require simultaneous evaluation of the mixing of the lipid species in the solid (gel) and liquid-crystalline phases. Even simple lipid mixtures often exhibit multiple coexisting solid phases (Luna & McConnell, 1977, 1978; Stewart et al., 1979; Graham et al., 1985; Silvius, 1986), a fact that is not usually allowed for in simple regular-solution analyses. It may be difficult to analyze the phase diagrams for such systems rigorously to extract information about the mixing of lipids in the liquid-crystalline state. Finally, most of the binary lipid systems for which phase diagrams are presently available consist of lipids with saturated or trans-unsaturated acyl chains. It is possible that the effects of headgroup structure on the intermixing of other types of lipids (e.g., 1-saturated 2-cis-unsaturated species, or species with heterogeneous acyl chain compositions) may be rather different from those observed in the systems just noted.

In conclusion, the results presented in this study indicate that lipids with different headgroups may intermix in liquid-crystalline bilayers in a manner that is more nearly ideal than some previous studies have suggested. One major advantage of the partitioning technique described here (and one possible source for the discrepancies between our present conclusions and those derived from previous thermodynamic studies of binary lipid mixtures) is the fact that the partitioning approach can be applied even to systems that contain a number of different molecular components. This approach may thus be useful not only to investigate lipid–lipid interactions in simple vesicle systems but also to test for possible selectivity in lipid–lipid and lipid–protein interactions in more complex systems, including biological membranes.

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APPENDIX

Equilibrium Partitioning of Labeled Probes between Lipid Vesicles. The simplest model of partitioning of an exchangeable lipid probe L* between two populations of lipid vesicles can be described by

$$X_{L^*}(\text{acceptor})/X_{L^*}(\text{donor}) = K_p \quad (1)$$

where X_{L^*}(acceptor) and X_{L^*}(donor) represent the mole fractions of the probe in the external surfaces of the acceptor and donor vesicles, respectively, and K_p is a partition coefficient that is determined by the free energy of transfer of the probe from the donor to the acceptor vesicles. Applying the above definition and simple considerations of mass balance to describe the distribution of an exchangeable fluorescent probe between two different types of lipid vesicles, a straightforward derivation gives us the more directly useful equation

$$F_{norm} = F_D + \Delta F_{max}\frac{SK_p[\text{acceptors}]}{[\text{donors}] + SK_p[\text{acceptors}]} \quad (2)$$

where

$$F_{max} = (F_{Ac} - F_D)f_{exch} \quad (3)$$

In the above equations, the normalized fluorescence F_{norm} is the fluorescence measured for a given probe-containing sample divided by the fluorescence measured after the addition of excess Triton X-100 (which provides a direct measure of the amount of probe present). F_D is the normalized fluorescence of the probe when present in the donor vesicles, F_{Ac} is the normalized fluorescence of the probe when present in the acceptor vesicles, and f_{exch} is the fraction of the total probe that is readily available for exchange. Finally, S represents the fraction of total lipids in the acceptor vesicles that is exposed to the external medium divided by the corresponding value for the donor vesicles.

In our experiments, values for the parameters SK_p and ΔF_{max} were estimated by applying eq 2 to sets of fluorescence data obtained by incubating a small and fixed amount of a fluorescent probe with a fixed concentration of donor vesicles and variable concentrations of acceptor vesicles. Plots of F_{norm} vs the acceptor vesicle concentration were fit directly to eq 2, using a nonlinear least-squares fitting routine employing a Marquardt algorithm, to yield F_D, ΔF_{max}, and SK_p as estimated parameters. In a typical experiment, 20–22 data points were collected per curve, and the mean errors of estimation for ΔF_{max} and SK_p were of the order of 1–2% and 3–5% of the estimated values, respectively. Since F_D and F_{Ac} can be determined directly, it is possible to estimate f_{exch} as well from the estimated value for ΔF_{max}.

The analysis just described can estimate an effective partition coefficient (SK_p) but not K_p itself. This limitation can be overcome in two ways. First, we can estimate the value of S by an independent experiment: in this study, we did so by applying the TNBS labeling assay of Nordlund et al. (1981) to estimate the fractions of lipids exposed at the outer surfaces of various types of vesicles that contained low mole fractions of PE (see Materials and Methods). Second, by comparing the SK_p values measured for the partitioning of two different probes between two populations of vesicles, we can calculate the *ratio* of the K_p values for the partitioning of the two probes between different lipid environments.

Registry No. Cholesterol, 57-88-5.

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Evidence for a tRNA/rRNA Interaction Site within the Peptidyltransferase Center of the *Escherichia coli* Ribosome†

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ABSTRACT: A nine-base oligodeoxyribonucleotide complementary to bases 2497–2505 of 23S rRNA was hybridized to both 50S subunits and 70S ribosomes. The binding of the probe to the ribosome or ribosomal subunits was assayed by nitrocellulose filtration and by sucrose gradient centrifugation techniques. The location of the hybridization site was determined by digestion of the rRNA/cDNA heteroduplex with ribonuclease H and gel electrophoresis of the digestion products, followed by the isolation and sequencing of the smaller digestion fragment. The cDNA probe was found to interact specifically with its rRNA target site. The effects on probe hybridization to both 50S and 70S ribosomes as a result of binding deacylated $tRNA^{Phe}$ were investigated. The binding of deacylated $tRNA^{Phe}$, either with or without the addition of poly(uridylic acid), caused attenuation of probe binding to both 50S and 70S ribosomes. Probe hybridization to 23S rRNA was decreased by about 75% in both 50S subunits and 70S ribosomes. These results suggest that bases within the 2497–2505 site may participate in a deacylated tRNA/rRNA interaction.

In an attempt to elucidate the mechanisms of protein biosynthesis, considerable attention has been focused on the interactions of transfer RNA (tRNA) with the ribosome. Recent evidence suggests that the ribosome possesses three tRNA binding sites (Rheinberger et al., 1981) designated the P-site (for peptidyl), A-site (for aminoacyl), and E-site (for exit). While the stoichiometry of tRNA binding has now been defined, the portions of the ribosome involved in tRNA/ribosome interactions have not yet been delineated.

Evidence is accumulating which suggests that ribosomal RNA (rRNA) may participate in tRNA/ribosome interactions. A-site-bound tRNA derivatives have been cross-linked to 16S rRNA (Schwartz et al., 1975; Gornicki et al., 1985) while P-site-bound tRNA derivatives have been cross-linked to 23S rRNA (Barta & Kuechler, 1983; Sonenberg et al., 1978) and 16S rRNA (Schwartz & Ofengand, 1978; Prince et al., 1982). Shielding of certain rRNA bases from chemical modification has been shown to occur upon the binding of

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