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Partitioning of Fluorescent Phospholipid Probes between Different Bilayer Environments. Estimation of the Free Energy of Interlipid Hydrogen Bonding†

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ABSTRACT: Fluorescence spectroscopy has been used to monitor the partitioning of a series of exchangeable neutral phospholipid probes, labeled with carbazole, indolyl or diphenylhexatrienyl moieties, between large unilamellar vesicles containing 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) or *N*-hexadecyl-*N*-(9-octadecenyl)-*N*,*N*-dimethylammonium chloride (HODMA). Phosphatidylethanolamine (PE) probes desorb from POPC-containing vesicles at markedly slower rates than do phosphatidylcholine (PC) probes with the same acyl chains. The rate of probe desorption from such vesicles is progressively enhanced by successive *N*-methylations of the amino group but not by methylation of C-2 of the ethanolamine moiety, a modification that leaves unaltered the hydrogen-bonding capacity of the polar headgroup. By contrast, the rates of desorption of different probes (with the same acyl chains) from HODMA or from DOTAP vesicles are much more comparable and reflect no clear systematic influence of the headgroup hydrogen-bonding capacity. Equilibrium-partitioning measurements indicate that the relative affinities of different probes for PC-rich vesicles, in competition with HODMA or DOTAP vesicles, increase with increasing hydrogen-bonding capacity of the probe headgroup in the order PC < *N*,*N*-dimethyl PE < *N*-methyl PE < PE ≈ phosphatidyl-2-amino-1-propanol. From such partitioning data, we estimate that interlipid hydrogen-bonding interactions (in competition with lipid–water interactions) contribute roughly −300 cal mol^{-1} to the free energy of a PE molecule in a hydrated liquid-crystalline phospholipid bilayer; this free-energy contribution is somewhat smaller, but still significant, for *N*-mono- and dimethylated PE's.

A number of biologically important membrane lipids, including the aminophospholipids, glycolipids, and sphingolipids, possess the ability to act as hydrogen-bond donors. Interactions between such molecules and like or unlike species that act as hydrogen-bond acceptors may play an important role in determining membrane stability, surface properties, and lipid lateral organization (Boggs, 1980, 1987; Curatolo, 1986; Thompson & Tillack, 1985; Seddon, 1990).

While it is virtually certain that membrane lipids with hydrogen-bond-donating abilities participate in intermolecular hydrogen bonds of some type in lipid bilayers, the relative strengths of lipid–lipid and lipid–solvent hydrogen-bonding interactions in such systems are by no means clear. The distinctive thermotropic behavior of lipid species such as phosphatidylethanolamine (PE)[1] (Chang & Epand, 1983; Mantsch et al., 1983; Seddon et al., 1983; Wilkinson & Nagle, 1984; Seddon, 1990) and various glycolipids (Bunow, 1979; Freire et al., 1980; Sen et al., 1981; Ruocco et al., 1981; Curatolo, 1982, 1986; Koshy & Boggs, 1983; Maggio et al., 1985; Hinz et al., 1985; Curatolo & Jungawala, 1985; Mannock et al., 1988, 1990) has been attributed at least in part to the hydrogen-bond-donating abilities of their polar headgroups, and X-ray diffraction studies have provided evidence for hydrogen bonding between the headgroups of adjacent lipid molecules in anhydrous crystals of PE (Hitchcock et al., 1974) and of cerebroside (Pascher & Sundell, 1977). However, no direct estimate of the strength of such interlipid hydrogen bonds has been obtained to date. The strength of such interactions is particularly difficult to estimate a priori for the case of hydrated lipid bilayers, where any potential interlipid hydrogen bonds must compete with hydrogen-bonding interactions between the lipid polar headgroups and water.

We have previously used exchangeable phospholipid analogues, labeled with fluorescent reporter groups, to evaluate

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[1] Abbreviations: 8/(11-Carbazole)-PC (-PE), 1-octanoyl-2-[11′-(carbazol-9″-yl)undecanoyl]-*sn*-glycero-3-phosphocholine (-*sn*-glycero-3-phosphoethanolamine); (12-DABS)-18 PC, 1-palmitoyl-2-[12-[[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]octadecanoyl]-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; 10/(3-DPH)-PC (-PE), 1-decanoyl-2-[3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]propanoyl]-*sn*-glycero-3-phosphocholine (-*sn*-glycero-3-phosphoethanolamine); HODMA, *N*-hexadecyl-*N*-(*cis*-9-octadecenyl)-*N*,*N*-dimethylammonium chloride; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; POPG, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoglycerol; (16-TNP)-16 PC, 1-hexadecanoyl-2-[16-(trinitrophenylamino)hexadecanoyl]-*sn*-glycero-3-phosphocholine.

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the partitioning of various phospho- and sphingolipids between different phospholipid environments (Gardam et al., 1989). In this study, we extend this approach to compare the partitioning of analogues of different neutral phospholipids between bilayers composed mainly of phosphatidylcholine, which can serve as an acceptor of interlipid hydrogen bonds, and bilayers composed entirely of *N*-hexadecyl-*N*-(9-octadecenyl)-*N*,*N*-dimethylammonium chloride (HODMA), a quaternary ammonium amphiphile that can serve neither as a donor nor as an acceptor of hydrogen bonds. Our findings indicate that neutral phospholipid probes whose headgroups can serve as hydrogen-bond donors (e.g., PE) show an increased affinity for PC-rich vesicles, in competition with HODMA vesicles, when compared to probes with lesser or no hydrogen-bond-donating ability (e.g., PC). By analyzing this behavior quantitatively, we obtain estimates of the contribution of hydrogen bonding to the total free energy of interaction of neutral amino phospholipids with neighboring phospholipid molecules in a hydrated liquid-crystalline lipid bilayer.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-acyllysophosphatidylcholines were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Fluorescent PCs were prepared by acylation of lysophosphatidylcholines with the anhydrides of fluorescent fatty acids (2-fold molar excess) in dry chloroform in the presence of 4-pyrrolidinopyridine as described previously (Mason et al., 1981). The labeled PCs were purified either by flash chromatography on silica gel 60 (Still et al., 1978), eluting with 65:25:4 $CHCl_3/CH_3OH/H_2O$, or by preparative thin-layer chromatography on silica gel 60, using 50:20:10:10:5 $CHCl_3$/acetone/$CH_3OH/CH_3COOH/H_2O$ as the developing solvent. Other labeled phospholipids were prepared from these fluorescent PCs by phospholipase D mediated base exchange as described previously (Comfurius & Zwaal, 1977; Gardam et al., 1989) and were purified by flash chromatography on Bio-Sil A, with $CHCl_3/CH_3OH/NH_4OH$ mixtures as the eluting solvents.

11-Carbazoleundecanoic acid and 3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]propionic acid were purchased from Molecular Probes (Eugene, OR.). 8-Indolyloctanoic acid was prepared by a modification of the method of Guida and Mathre (1980), as follows. 18-Crown-6 (120 mg) and potassium *tert*-butoxide (1.5 g) were first stirred for 30 min in 12 mL of dry ether. This and all subsequent reaction steps were carried out at room temperature under a dry nitrogen atomosphere. Indole (1 g) was added dropwise in 4 mL of dry ether, and the mixture was stirred for a further 45 min. A solution of 8-bromooctanoic acid (0.67 g) in 4 mL of dry ether was then added, and the mixture was stirred for 48 h at 25 °C. The mixture was cautiously mixed with five volumes of water, acidified with HCOOH, and extracted with five volumes of hexane. The organic layer was washed three times with 1:1 water/methanol and concentrated in vacuo. The residue was incubated overnight at 65 °C with 5 mL of 30% methanolic dimethylamine to derivatize unreacted bromo acid, then concentrated under nitrogen, and the products were partitioned between 1 M aqueous HCOOH and 1:1 (v/v) hexane/diethyl ether. After concentrating the organic layer, the products were purified by column chromatography on Bio-Sil A, eluting first with 10 column volumes of 50:50:0.2 hexane/$CHCl_3$/triethylamine (discarded) and then with 20 column volumes of 50:50:0.2 hexane/$CHCl_3$/acetic acid (pooling fractions found pure by thin-layer chromatography). The ^1H NMR spectrum of this compound was that expected for 8-(indol-1′-yl)octanoic acid; significantly, resonances with chemical shifts expected for (indol-3′-yl)-$C\underline{H}_2$ or indoly-N$\underline{H}$ protons were absent, indicating that contamination with 8-(indol-3′-yl)octanoic acid was negligible.

HODMA was synthesized by reacting hexadecyl mesylate (Nu-Chek Prep, Elysian MN; 330 mg) with 5 mL of 30% methanolic dimethylamine in a sealed vessel for 16 h at 65 °C, then drying thoroughly in vacuo and reacting the residue with 2 equiv of *cis*-9-octadecenyl mesylate in 2.5 mL of 2:1 chloroform/acetonitrile for 48 h at 60 °C. After evaporation of the solvent under nitrogen, the products were partitioned between chloroform and 1:1 methanol/1 M aqueous NaCl, and the recovered organic phase was washed five times with the same methanol/aqueous mixture. The crude HODMA was purified on a column of Bio-Sil A, eluting with a 2–10% gradient of methanol in chloroform containing 0.1% (v/v) ammonium hydroxide to yield the pure product (343 mg) at 7–8% methanol. Aqueous dispersions of HODMA, prepared as described below, showed no endothermic phase transitions between 10 and 90 °C when examined by differential scanning calorimetry as described elsewhere (Silvius & Gagné, 1984).

Methods. As homogeneous preparations of HODMA could not be obtained consistently by the reverse-phase evaporation procedure (Wilschut et al., 1980), all of the vesicle preparations used in this study were prepared by the following alternative method. Well-dried lipid dispersions were initially bath sonicated in buffer (normally 25 mM NaCl, 10 mM sodium acetate, pH 5.6) to near clarity (3 min for HODMA and DOTAP dispersions; 10 min for PC/DOTAP dispersions), then three times frozen in a dry ice/ethanol bath and allowed to thaw at room temperature. The resulting dispersions were finally filtered through 0.1-μm Nucleopore filters. For technical reasons, the trapped volumes of these cationic lipid dispersions could not be determined by conventional solute-retention assays. However, light-scattering measurements and measurements of the fractional exchangeability of fluorescent short-chain PCs incorporated in such dispersions during their preparation (Gardam et al., 1989) suggested them to be very similar in size and lamellarity to POPC/POPG dispersions prepared by the above procedure, for which an average vesicle diameter of ca. 1200 Å was determined by trapped-volume measurements (Wilschut et al., 1980).

Donor lipid vesicles for exchange measurements were "asymmetrically" labeled with exchangeable fluorescent lipid probes by incubation with a bath-sonicated dispersion of the probe species, at a 1:250–1:100 molar ratio of probe to vesicle lipids, for 2 h at 37 °C under nitrogen and in the dark. Fluorescence measurements indicated complete transfer of all probes into the vesicles within this incubation time. For measurements of the kinetics of intervesicle probe transfer, quencher-free "donor" vesicles (5–10 μM lipid), labeled as just described, were mixed at time zero with quencher-containing "acceptor" vesicles (100 μM lipid except where otherwise noted) while the probe fluorescence was continuously monitored (Nichols & Pagano, 1982; Gardam et al., 1989). For measurements of the equilibrium partitioning of lipid probes between different vesicle populations, a fixed amount of donor vesicles (10–60 nmol total lipid, depending on the experiment) was preincubated with varying amounts of acceptor vesicles (0–300 nmol total lipid) for the times indicated in the text before measuring the fluorescence of duplicate aliquots dispersed into buffer and, for normalization, into either 1% aqueous Triton X-100 (for DPH-labeled lipids) or methanol (for carbazole-labeled lipids). Preincubation conditions (time and ionic strength) were chosen to allow redistribution of the

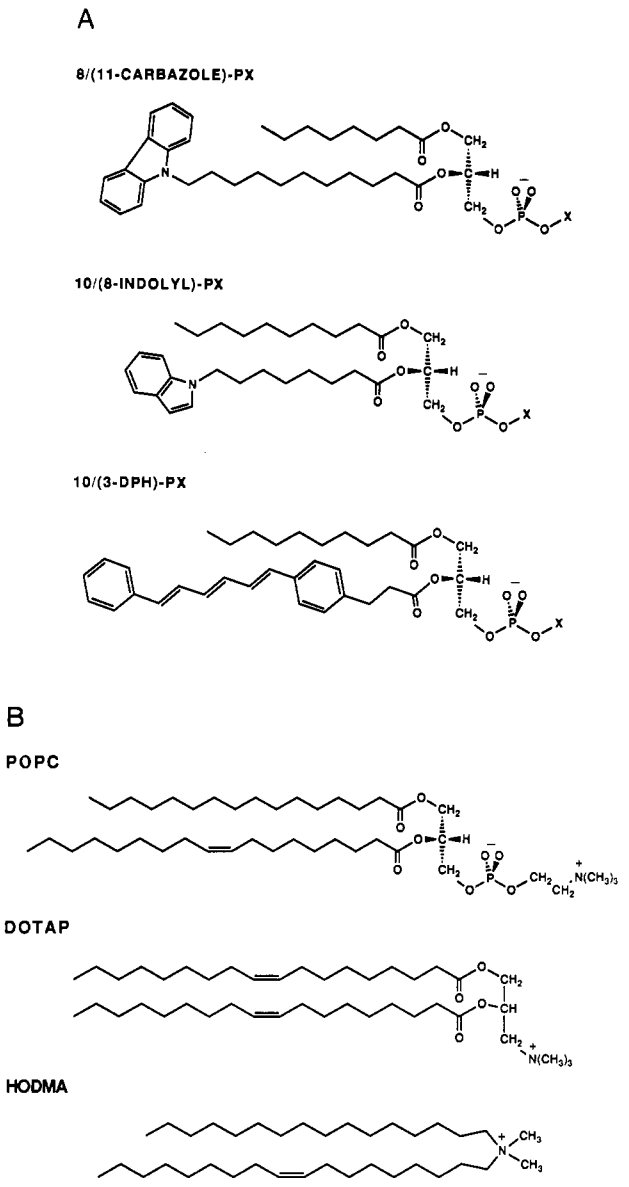

FIGURE 1: (A) Structures of the exchangeable lipid probes used in this study. The residue X in the polar headgroup represents an ethanolamine, N-methylated ethanolamine or DL-2-amino-1-propanol moiety. (B) Structures of the vesicle-forming amphiphiles used in this study.

fluorescent probes to equilibrium between the outer surfaces of lipid vesicles while avoiding significant intermixing of other lipids between donor and acceptor vesicles. A small amount of intermixing of nonexchangeable lipid probes between PC/DOTAP and HODMA vesicles was detected when they were incubated for substantially longer times or at higher ionic strengths.

RESULTS

Kinetics of Intervesicle Probe Exchange. In Figure 1 are shown the structures of the exchangeable lipid probes used in this study. Probes with these labeled acyl chains were chosen to minimize the possibility of any polar interactions between the fluorescent moieties and the polar headgroups. The fluorescence of DPH-labeled species is efficiently quenched when low levels of the nonexchangeable lipid probe 1-palmitoyl-2-[12′-(DABSylmethylamino)octadecanoyl]-PC [[12-DABS]-18 PC (Silvius et al., 1987)] are incorporated in the same lipid vesicle. Similarly efficient quenching of the fluorescence of the carbazole- and indolyl-labeled species is achieved by using low mole fractions of 1-palmitoyl-2-[16′-(trinitrophenylamino)palmitoyl]-PC [(16-TNP)-16 PC; Silvius et al., 1987) or 1-palmitoyl-2-(pyrenyldecanoyl)-PC [(10-pyrenyl)-10 PC], respectively (data not shown).

As described previously, the kinetics of intervesicle probe exchange can be monitored though fluorescence intensity measurements when a population of probe-labeled donor vesicles is mixed with a second population of acceptor vesicles containing a nonexchangeable quencher of the probe fluorescence (Nichols & Pagano, 1982). The initial rates of transfer of various lipid probes between large unilamellar vesicles of various compositions, determined by this approach as described previously (Nichols & Pagano, 1982; Gardam et al., 1989), are summarized in Table I. In all cases, it was verified that when the donor/acceptor ratio was neld constant, the half-time for intervesicle probe exchange was independent of the lipid concentration (over at least a 10-fold range), suggesting that probe exchange does not proceed through intervesicle collisions but rather is rate-limited by the desorption of the probe from the donor vesicle surface (Nichols & Pagano, 1982).

While absolute rates of intervesicle transfer vary widely between different families of fluorescent probes, a consistent pattern is apparent if we compare the behavior of species with different headgroups within any one family. PC probes desorb from 80:20 POPC/DOTAP vesicles roughly 2–3 times faster than do PE probes with the same acyl chains. The ratio of the rates of desorption of 8/(11-carbazole)-PC and of 8/(11-carbazole)-PE from 80:20 POPC/DOTAP vesicles at 37 °C was essentially identical with the corresponding ratio measured for the desorption of the same two probes from 80:20 POPC/POPG vesicles (result not shown). For all families of probes examined, the rates of probe desorption from 80:20 POPC/DOTAP vesicles varied in the order PC > *N*,*N*-dimethyl PE > *N*-methyl PE > PE $\gtrsim$ phosphatidyl-2-amino-1-propanol. By contrast, the various members of a given family of lipid probes desorb from HODMA or DOTAP vesicles at generally similar rates, which show no clear systematic variation with either headgroup size or degree of *N*-methylation (Table I). In other experiments, 10/(3-DPH)- or 8/(11-carbazole)-labeled PE's were in fact found to desorb slightly faster from sonicated HODMA donor vesicles than were the corresponding PC probes (not shown).

Equilibrium Partitioning Measurements. The above results suggest that the hydrogen-bond-donating ability of neutral aminophospholipid probes significantly effects their interactions with PC-containing vesicles but (as expected) not with HODMA vesicles. We therefore next examined the equilibrium partitioning of different neutral phospholipid probes between POPC-rich vesicles and HODMA vesicles. In these experiments, a fixed amount of probe-labeled donor vesicles (PC/DOTAP or HODMA) was preincubated with various amounts of acceptor vesicles of the opposite composition (HODMA or PC/DOTAP); again, one of the vesicle populations also contained a quencher of the probe fluorescence. After equilibration, the probe fluorescence was monitored as a function of the acceptor/donor vesicle ratio. As described previously (Nichols & Pagano, 1982; Gardam et al., 1989), quantitative analysis of such data yields an "effective" partition coefficient (SK_p), which represents the true partition coefficient of the probe between the acceptor and donor vesicles (K_p) multiplied by a parameter S that represents the ratio of the externally exposed surface areas (per molar unit of lipid) for the acceptor vs the donor vesicle populations. In turn, the *ratio* of the effective partition coefficients (SK_p) measured for two

Table I: Rate Constants for Desorption of Neutral Phospholipid Probes from 80:20 POPC/DOTAP, DOTAP or HODMA Vesicles[a]

probe	initial rate (%/min) of probe desorption from POPC/DOTAP (20 °C)	POPC/DOTAP (37 °C)	DOTAP (37 °C)	HODMA (37 °C)
10/(3-DPH)-PC	56	129	10.6	2.7
10/(3-DPH)-*N*,*N*-dimethyl PE	31	98	8.7	2.7
10/(3-DPH)-*N*-methyl PE	24	66	8.7	3.5
10/(3-DPH)-PE	18	51	8.2	2.4
10/(3-DPH)-phosphatidyl-2-amino-1-propanol	18	52	8.5	2.4
8/(11-carbazole)-PC	223	1128	45	6.5
8/(11-carbazole)-*N*,*N*-dimethyl PE	119	453	32	7.3
8/(11-carbazole)-*N*-methyl PE	96	430	38	5.8
8/(11-carbazole)-PE	92	370	38	5.0
8/(11-carbazole)-phosphatidyl-2-amino-1-propanol	85	360	44	5.2

[a] Exchange rates were determined as described in the text, by using donor and acceptor vesicles of like composition save for the inclusion of a nonexchangeable fluorescence quencher in the acceptor vesicles. All data presented in a given column were obtained from triplicate measurements (standard deviations ≤5%) in a single representative experiment, using the same preparation of donor vesicles. While absolute rates of exchange for a given probe and donor vesicle type varied by up to 30% between experiments using different donor vesicle preparations (three separate experiments for each donor vesicle type), the relative rates of exchange (i.e., the ratios of the measured exchange rates) for different probes varied by at most 10%, and usually by ≤5%, between experiments.

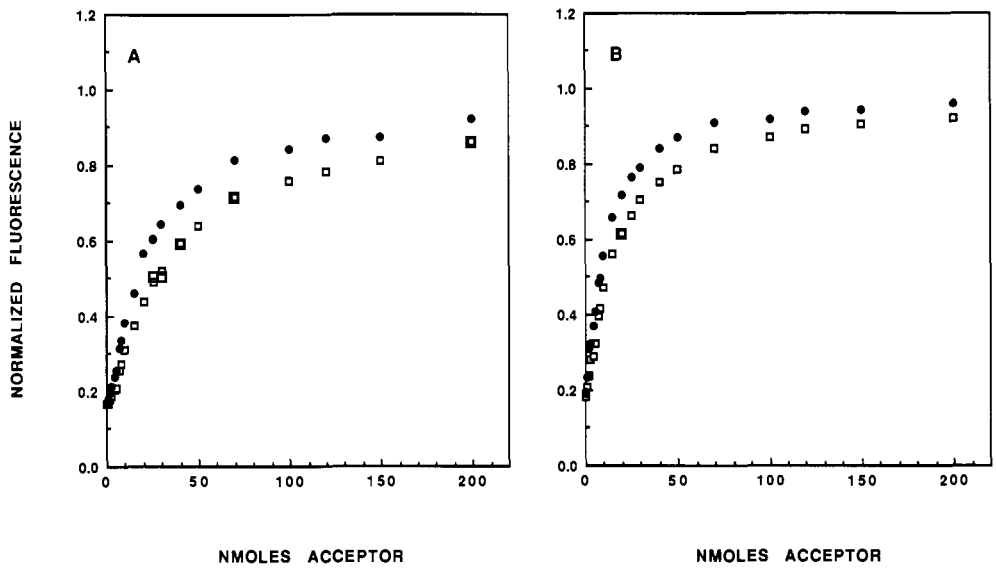

FIGURE 2: (A) Partitioning of 8/(11-carbazole)-PC (filled circles) and -PE (open circles) between 80:20:1 (molar proportions) POPC/DOTAP/(12-DABS)-18 PC donor vesicles (50 nmol) and HODMA acceptor vesicles. To facilitate comparison of the two curves, fluorescence values have been normalized to the value representing complete transfer of the probe from the outer surfaces of donor vesicles to the acceptor vesicles [determined by nonlinear least-squares analysis as described in Gardam et al. (1989)]. Other experimental details are described in the text. (B) Partitioning of 8/(11-carbazole)-PC (filled circles) and -PE (open circles) between 80:20:1 (molar proportions) POPC/DOTAP/(12-DABS)-18 PC donor vesicles (30 nmol) and DOTAP acceptor vesicles. Other experimental details were as for panel A.

different probes A and B when using the same sets of donor and acceptor vesicles is equal to K_p(A)/K_p(B), the ratio of the true partition coefficients for the two probes.

In Figure 2A are shown representative results from an experiment comparing the partitioning of 8/(11-carbazole)-PC and -PE between 80:20:2 POPC/DOTAP/12-TNP-PC donor vesicles and HODMA acceptor vesicles. HODMA vesicles serve as relatively efficient acceptors for both probes, giving considerably more than 50% exchange at equimolar ratios of the two types of vesicles. This behavior appears to be a general property of highly charged vesicles [see also Gardam et al. (1989)]. As well, however, at a given acceptor/donor ratio, a greater proportion of the PC than of the PE probe has exchanged from the PC/DOTAP to the HODMA vesicles. As indicated in Table II, the ratio of partition coefficients K_p[8/(11-carbazole)-PE]/K_p[8/(11-carbazole)-PC] estimated from a series of such experiments was 1.63 ± 0.11, indicating that the relative preference of the PE probe for the PC-containing vesicles (in competition with the HODMA vesicles) is stronger than that of the PC probe by this factor.

Table II: Relative Partition Coefficients K_p(PX*)/K_p(PC*) for the Distribution of Neutral Phospholipid Probes between 80:20 POPC/DOTAP and HODMA Vesicles

probe (PX*)	K_p(PX*)/K_p(PC*)[a]	*N*[b]
8/(11-carbazole)-PE	1.63 ± 0.11	4
8/(11-carbazole)-*N*-methyl PE	1.38 ± 0.05	3
8/(11-carbazole)-*N*,*N*-dimethyl PE	1.32 ± 0.03	3
8/(11-carbazole)-phosphatidyl-2-amino-1-propanol	1.62 ± 0.10	2
10/(8-indoyl)-PE	1.51 ± 0.03	2
8/(11-carbazole)-PE (DOTAP acceptor vesicles)	1.66 ● 0.03	2

[a] Relative partition coefficients were determined as described in the text; a ratio greater than 1.0 indicates that the probe indicated shows a *greater* relative affinity for POPC/DOTAP vesicles, in competition with the HODMA (or DOTAP) vesicles, than does the corresponding PC probe. [b] Number of independent experiments.

Data from quantitative analyses of experiments such as those shown in Figure 2, using a series of neutral phospholipid probes, are summarized in Table II. In this table, a ratio of partition coefficients [K_p(PX*)/K_p(PC*)] greater than 1.0

indicates that the "test" probe PX* shows a higher relative affinity for the PC/DOTAP vesicles (in competition with HODMA vesicles) than does the PC probe (PC*) with the same acyl chains. Of the probe species studied, PE and phosphatidyl-2-amino-1-propanol, with unsubstituted amino groups, show the highest relative affinities for the PC-rich vesicles. The magnitude of this relative preference for PC-rich over HODMA vesicles decreases progressively as the amino group is increasingly methylated.

In control experiments (not shown), it was established that the ratio of partition coefficients for 8/(11-carbazole)-PE and -PC between PC/DOTAP and HODMA vesicles was not significantly affected by the following alterations of the experimental conditions: use of donor and acceptor vesicles that were freeze-thawed but not Nucleopore-filtered; increasing the buffer NaCl concentration from 25 to 50 mM; reducing the pH from 5.6 to 4.5; adjusting the PC/DOTAP molar ratio in the PC-containing vesicles from 80:20 to 90:10; reversing the donor and acceptor vesicle compositions; and varying the mole fraction of the carbazole-labeled probe in the donor vesicles from 0.2 to 1 mol %. In another control experiment, the ratio of partition coefficients of 8/(11-carbazole)-PC and -PE between 90:10:2 POPC/DOTAP/(16-TNP)-16 PC vesicles and 100% POPC vesicles was found to be 1.06 ± 0.05, indicating that the two types of vesicles are essentially equivalent in the relative strengths of their interactions with PE vs PC probes.

As shown in Table II, the ratio of partition coefficients of 10/(8-indolyl)-PE and -PC between 80:20 POPC/DOTAP and HODMA vesicles was comparable to the corresponding ratio measured for 8/(11-carbazole)-PE and -PC. Equilibration of 10/(3-DPH)-PC and -PE between POPC/DOTAP and HODMA vesicles was relatively slow compared to that of the other probes examined, and it was necessary to use lower ionic strengths (≤5 mM) to avoid bulk lipid mixing between POPC/DOTAP and HODMA vesicles on the time scale of the equilibration process. Under these conditions, HODMA vesicles showed some tendency to adsorb to the tubes used for incubations, complicating quantitative analysis of the partitioning results as described above. However, with efforts to minimize and/or to correct for these complications, we could crudely estimate the ratio K_p[10/(3-DPH)-PE]/K_p[10/3-DPH)-PC] between POPC/DOTAP and HODMA vesicles to lie in the range 1.30–1.80, similar to that for the other PC and PE probes examined. In a preliminary description of the experimental approach used here (Gardam & Silvius, 1990), we obtained a higher estimate for this quantity. However, we believe that this discrepancy reflects the difficulties of resolving completely the complications noted above and that the behavior of the 10/(3-DPH)-phospholipid probes, in regard to the effects of headgroup structure on probe interactions with neighboring lipid molecules, is fundamentally similar to that observed for the faster-exchanging 8/(11-carbazole) and 10/(8-indolyl) probes, whose use largely circumvents the above complications.

In a final series of experiments, we compared the partitioning of 8/(11-carbazole)-PC and -PE between 80:20 POPC/DOTAP vesicles and 100% DOTAP vesicles. As shown in Table II, the value measured for the ratio K_p[8/(11-carbazole)-PE]/K_p[8/(11-carbazole)-PC] in the donor/acceptor system (POPC/DOTAP vesicles)/(DOTAP vesicles) was very similar to that measured with HODMA vesicles in place of DOTAP vesicles.

Discussion

While the potential ability of polar lipids such as phosphatidylethanolamine, sphingolipids, and glycolipids to act as hydrogen-bond donors in lipid bilayers is incontestable, it is difficult to estimate a priori the strength of such hydrogen bonds between these species and neighboring lipid molecules. While dilauroyl-PE crystals grown in organic solvents show extensive hydrogen bonding between adjacent lipid headgroups (Hitchcock et al., 1974), at least three factors militate against the formation of similar networks of strong, stable interlipid hydrogen bonds in liquid-crystalline bilayers: the high degree of lipid rotational mobility in such systems, an average intermolecular spacing that may be too large to allow formation of extended networks of intermolecular hydrogen bonds (Boggs, 1987), and the omnipresence of water as a competing hydrogen-bond donor and acceptor. While a variety of NMR and vibrational-spectroscopic studies have examined possible hydrogen-bonding interactions between lipids in anhydrous and hydrated lipid bilayers (Yeagle et al., 1975; Yeagle & Martin, 1976; Schmidt et al., 1977; Bush et al., 1980; Mantsch et al., 1981; Mushayakahara et al., 1982; Smaby et al., 1983; Bertoluzza et al., 1984; Wong & Mantsch, 1988; Blume et al., 1988; Wong et al., 1989), it has generally not been possible to differentiate lipid–lipid from lipid–water hydrogen bonding in hydrated systems by these approaches. Moreover, vibrational-spectroscopic studies of hydrated PE and PC bilayers have not revealed qualitative differences in the carbonyl or phosphoryl resonances of the two species that can be specifically attributed to interlipid hydrogen-bonding in PE bilayers (Mantsch et al., 1981; Blume et al., 1988).

Several previous studies (Massey et al., 1983; Homan & Pownall, 1988; Gardam et al., 1989) have found that exchangeable phospholipid probes whose polar headgroups can serve as hydrogen-bond donors (e.g., PE and monoionized phosphatidic acid) desorb from liquid-crystalline phospholipid vesicles considerably more slowly than do related probes (with like acyl chains) whose headgroups cannot serve as hydrogen-bond donors (e.g., PC and diionized phosphatidic acid). This difference has been attributed to a retarding effect of interlipid hydrogen bonds on the desorption of lipids of the former type from phospholipid bilayers. The present data favor this interpretation over the alternative possibility that headgroup size is the key determinant of the rate of interbilayer probe exchange. Neutral phospholipid probes with different polar headgroups but the same acyl chains desorb at very similar rates from HODMA vesicles, with no clear systematic effect of headgroup size on the desorption kinetics. Moreover, while N-methylated derivatives of PE probes desorb faster from PC/DOTAP vesicles than do the parent PE species, (*ethanolamine*-C_2)-methylated PE probes desorb from such vesicles no more rapidly than do the corresponding PE probes. These differences are difficult to explain on the basis of headgroup size but correlate well with the hydrogen-bonding abilities of the probe headgroups and their vesicle lipid environments.

Analysis of the equilibrium partitioning of different neutral phospholipid probes reveals that species that can serve as hydrogen-bond donors show a higher relative affinity for PC/DOTAP vesicles, in competition with HODMA or DOTAP vesicles, than does PC. The measured values of K_p(PX*)/K_p(PC*) for different carbazole-labeled aminophospholipid probes PX* (PC* = carbazole-labeled PC) increase progressively as the extent of methylation of the amine group decreases and are very similar for PE and phosphatidyl-2-amino-1-propanol probes. As for the kinetic observations discussed above, the partitioning of the neutral lipid probes between HODMA and PC/DOTAP vesicles appears to be influenced by the hydrogen-bonding ability of the probe

headgroup but not by the headgroup size per se. This finding is not inherently unreasonable, as the POPC/DOTAP and the HODMA bilayers may not be greatly different in their acyl chain or interfacial packing but clearly must differ in their intrinsic abilities to serve as acceptors of hydrogen bonds from the intercalated hydrogen-bonding amphiphiles. Interestingly, DOTAP vesicles appear to be very similar to HODMA vesicles in their degree of discrimination (or lack of discrimination) between neutral phospholipid probes with different headgroups. This result suggests that aminophospholipids hydrogen-bond more strongly to the phosphodiester than to the carboxyester moieties of neighboring phospholipids, since DOTAP shares the general backbone structure of PC but lacks its phosphodiester moiety.

On the basis of the above conclusions, we can interpret differences in the partitioning of different phospholipid probes between POPC/DOTAP and HODMA vesicles as reflecting differences in the free energies of hydrogen bonding of the various probe headgroups to neighboring phospholipid molecules in the POPC-containing vesicles. The quantity $-RT \ln [K_p(PX^*)/K_p(PC^*)]$ for an aminophospholipid probe PX* and the corresponding PC probe (PC*) in this mixed-vesicle system thus provides an estimate of the *net* contribution of PX*–POPC hydrogen bonding (in competition with hydrogen-bonding of these species to interfacial water) to the overall free energy of the species PX* in a POPC-rich bilayer. For a PC probe, this free-energy contribution is taken to be zero, since no direct hydrogen-bonding interactions are possible between a PC probe and neighboring PC molecules. Using the data shown for the 8/(11-carbazole)-phospholipid probes in Table II, we can then estimate the net free energy of interlipid hydrogen bonding in a hydrated lipid-crystalline phospholipid bilayer as roughly −300 cal mol^{-1}, −300 cal mol^{-1}, −200 cal mol^{-1}, and −170 cal mol^{-1} for the interactions of PE, phosphatidyl-2-amino-1-propanol, *N*-methyl-PE, and *N,N*-dimethyl-PE, respectively, with surrounding phospholipids. These free-energy estimates represent the *net* change in free energy of hydrogen bonding of the lipid probe headgroups when they are transfered from vesicles where all hydrogen bonds must be made to interfacial water (HODMA vesicles) to vesicles where probe–lipid hydrogen bonds are also possible (PC-rich vesicles).

While the results reported here are strictly relevant to PC-rich bilayers, the very modest differences that we have observed previously in the partitioning of PE vs PC probes between PC-rich, PE-rich, and PC/cholesterol-rich bilayers (Gardam et al., 1989) suggest that very similar results would be expected for phospholipid bilayers of other compositions as well. It is also important to note that, in the experiments described here, the surface charge per se does not appear to influence significantly the relative strength of interactions of PE vs PC probes with the surrounding lipid environment. This conclusion is based on comparisons of desorption rates for these two types of probes from differently charged PC-rich vesicles and on comparisons of the equilibrium partitioning of the probes between such vesicles (this study and Gardam et al., 1989). This point is important, as variations in bilayer surface charge have been reported to influence the orientation of phospholipid headgroups (Seelig et al., 1987; Scherer & Seelig, 1989), which in principle could affect the relative strength of lipid–lipid vs lipid–water hydrogen bonding.

The present data, which suggest that hydrogen-bonding can make a measurable but modest energetic contribution to the interactions between neutral aminophospholipids and neighboring phospholipid molecules in liquid-crystalline bilayers, are broadly consistent with some previous theoretical and experimental findings for related phospholipid systems. Theoretical analyses suggest that the higher gel-to-liquid-crystalline transition temperatures observed for "hydrogen-bonding" phospholipids compared to "non-hydrogen-bonding" species can be explained by invoking rather modest changes in interlipid hydrogen-bonding energies at the chain-melting transition (Eibl & Wooley, 1979) or a small contribution of hydrogen bonding to the overall energy of interlipid interactions above and below the transition (Nagle, 1976). The critical aggregation concentration measured for dihexanoyl-PE (Plückthun et al., 1985) is roughly 1.6-fold smaller than that for the corresponding PC (Johnson et al., 1981), and a spin-labeled PE with one shortened acyl chain has been reported to show a critical micelle concentration 2-fold smaller than that for the corresponding spin-labeled PC (King & Marsh, 1987). Thermodynamic data more rigorously comparable (and complementary) to those reported here could be obtained by measuring the partitioning of small amounts of short-chain phospholipids, with different headgroups but the same acyl chains, between the aqueous phase and phospholipid vesicles of a fixed composition. To the best of our knowledge, such measurements have not been reported to date.

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Registry No. POPC, 26853-31-6; DOTAP, 113669-21-9; HODMA, 134311-09-4; 10/(3-DPH)-PC, 134311-10-7; 10/(3-DPH)-*N,N*-dimethyl PE, 134311-11-8; 10/(3-DPH)-*N*-methyl PE, 134311-12-9; 10/(3-DPH)-PE, 134311-13-0; 10/(3-DPH)-phosphatidyl-2-amino-1-propanol, 134311-14-1; 8/(11-carbazole)-PC, 134311-15-2; 8/(11-carbazole)-*N,N*-dimethyl PE, 134311-16-3; 8/(11-carbazole)-*N*-methyl PE, 134311-17-4; 8/(11-carbazole)-PE, 134332-52-8; 8/(11-carbazole)-phosphatidyl-2-amino-1-propanol, 134311-18-5; 10/(8-indoyl)-PE, 134311-19-6; indole, 120-72-9; 8-bromooctanoic acid, 17696-11-6; 8-inddyloctanoic acid, 134311-20-9; hexadecyl mesylate, 20779-14-0; dimethylamine, 124-40-3; *cis*-9′-octadecenyl mesylate, 134311-21-0.

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