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Articles

Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes†

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ABSTRACT: Lipid vesicles with a positive surface charge have been prepared by using mixtures of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) together with low mole fractions of a cationic lipid analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP). We have used measurements of vesicle aggregation, lipid mixing, contents mixing, and contents leakage to examine the interactions between these vesicles and similar vesicles that carry a negative surface charge. Mixtures of vesicles with opposite surface charges aggregate readily at physiological or lower ionic strengths, and the extent of this aggregation is enhanced for vesicles that contain high proportions of PE relative to PC. Mixing of lipids and aqueous contents can also be observed between such vesicles, particularly when the vesicles contain substantial proportions of PE. Surprisingly, these latter processes are strongly promoted by monovalent salts and do not proceed at very low ionic strengths. PE/DOTAP vesicles show substantial lipid mixing with negatively charged vesicles containing high proportions of phosphatidylcholine, as well as with human erythrocyte ghosts, on a time scale of a few minutes. These interactions are strongly promoted both by the presence of high levels of PE in the cationic vesicles and by the presence of complementary surface charges on the two membrane populations.

A number of studies in recent years have examined the fusion of lipid vesicles to model some aspects of the fusion of biological membranes (Liao & Prestegard, 1979; Papahadjopoulos et al., 1980; Wilschut et al., 1980, 1985; Uster & Deamer, 1981; Düzgünes et al., 1981a, 1985; Sundler & Papahadjopoulos, 1981; Sundler et al., 1981; Morris et al., 1985; Gagnê et al., 1985; Ellens et al., 1986, 1987a; Parente & Lentz, 1986; Leventis et al., 1986). Most studies of this sort have examined the interactions of charged lipid vesicles of like composition, which can be induced to aggregate and in some cases to fuse when electrostatic repulsions between vesicles are reduced through binding of counterions to the lipid surface (Lansmann & Haynes, 1975; Bentz & Nir, 1981; Düzgünes et al., 1981b; Rydhag et al., 1982; Ohki et al., 1982, 1984; Bentz & Düzgünes, 1985; Rupert et al., 1985; Carmona-Ribeiro et al., 1985). These systems are popular objects for study because the tendency of such vesicles to associate with one another can be readily controlled by varying the ionic composition of the medium.

A second possible means to promote interactions between lipid vesicles would be to mix two populations of vesicles with opposite surface charges, an approach that has not been widely employed to date. In some applications, this approach may offer significant advantages over the approach described above. Mixtures of vesicles with opposite surface charges may be useful, for example, to examine interactions between vesicles with different lipid compositions while suppressing interactions between vesicles of like composition, or to provide systems in which interactions between vesicles are not strongly dependent upon the binding of ions to the lipid surface, which may substantially alter the hydration and other properties of the surfaces involved.

In light of the considerations just noted, we have examined in this study the interactions between negatively and positively charged lipid vesicles, composed of neutral phospholipids as a majority component together with small amounts of anionic lipids or of a cationic lipid analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP).[1] Our results indicate

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[1] Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; 12-CPS-18-PC, 1-hexadecanoyl-2-[12-[[*N*-[4-[7-(dimethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]methyl]thio]-octadecanoyl]-*sn*-glycero-3-phosphocholine; 12-DABS-18-PC, 1-hexadecanoyl-2-[12-[*p*-[[*p*-(dimethylamino)phenyl]azo]-*N*-methylbenzenesulfonamido]octadecanoyl]-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane; DPX, *N,N'-p*-xylylenebis(pyridinium bromide); EDTA, ethylenediaminetetraacetic acid trisodium salt; LUV, large unilamellar vesicle(s); Mes, 2-(*N*-morpholino)ethanesulfonic acid sodium salt; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine (from egg yolk); PE, phosphatidylethanolamine (prepared by transphosphatidylation of egg yolk PC); PS, phosphatidylserine (dioleoyl); Rho-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt; HPLC, high-performance liquid chromatography.

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that the neutral lipid compositions of these vesicles influence significantly both their initial aggregation and the interactions that follow this initial association. Rather surprisingly, we also find that these further interactions, which can include mixing of lipids and aqueous contents between vesicles, are strongly promoted rather than hindered by increasing ionic strength. Finally, we demonstrate that cationic lipid vesicles that are rich in phosphatidylethanolamine (PE), a lipid with relatively weak surface hydration, can associate and intermix lipids with natural membranes or lipid vesicles that are rich in choline phospholipids, structures which are usually found to be rather refractory to processes of bilayer association and coalescence.

Materials and Methods

Materials. Phosphatidylcholine (PC) from egg yolk was obtained from Avanti Polar Lipids, Inc. Phosphatidylethanolamine (PE) was prepared from egg PC by transphosphatidylation with phospholipase D in the presence of ethanolamine as described previously (Comfurius & Zwaal, 1977; Silvius & Gagné, 1984a). Dioleoylphosphatidylmethanol was prepared from dioleoyl-PC by enzymic transphosphatidylation as described by Comfurius and Zwaal (1977), and dioleoylphosphatidylserine was synthesized chemically as described previously (Silvius & Gagné, 1984a).

1,2-Bis(oleoyloxy)-3-(trimethylammonio)propane was synthesized as follows: 1 mmol of 3-bromo-1,2-propanediol (Aldrich) was acylated for 48 h at 20 °C with 3 mmol of oleoyl chloride (freshly prepared from oleic acid and oxaloyl chloride) in dry, alcohol-free diethyl ether (20 mL) containing 5 mmol of dry pyridine. The precipitate of pyridinium hydrochloride was filtered off, and the filtrate was concentrated under nitrogen and redissolved in 10 mL of hexane. The hexane solution was washed 3 times with an equal volume of 1:1 methanol/0.1 N aqueous HCOONa, pH 3.0, 3 times with 1:1 methanol/0.1 N aqueous NaOH, and 1 time with 1% aqueous NaCl. The crude 3-bromo-1,2-bis(oleoyloxy)propane was then stirred for 72 h in a sealed tube with a solution of 15% trimethylamine in dry dimethyl sulfoxide (30 mL) at 25 °C. The products of this reaction were dissolved in chloroform (200 mL), which was repeatedly washed with 1:1 methanol/100 mM aqueous HCOONa, pH 3.0, and then evaporated in vacuo to yield a light yellow oil. This material was purified on a column of silicic acid (Bio-Sil A, Bio-Rad Laboratories), eluting with a 0–15% gradient of methanol in chloroform to give the desired product in pure form at 9–10% methanol. The purified product was a colorless, viscous oil that migrated with an R_f of 0.4 on thin-layer chromatographic plates (silica gel G) that were developed with 50:15:5:5:2 $CHCl_3$/acetone/CH_3OH/CH_3COOH/H_2O.

All other chemicals used in this study were of reagent grade or better. All solvents used were of reagent or HPLC grade and were redistilled before use. Diethyl ether used to prepare reverse-phase evaporation vesicles was distilled from P_2O_5, stabilized with 1% water, and stored in the dark at 4 °C.

Methods. Reverse-phase evaporation vesicles were prepared essentially as described by Wilschut et al. (1980) and were then passed through a 0.1-μm Nucleopore filter. Bath-sonicated vesicles were prepared by vortexing dried lipid samples in buffered sucrose solution and then sonicating for 2 min at room temperature. Trapped-volume measurements, using either carboxyfluorescein or ANTS as an aqueous-phase marker (Wilschut et al., 1980), indicated average diameters of 1100–1300 Å for LUV and ca. 400 Å for bath-sonicated vesicles. Such measurements could be carried out for DOTAP-containing vesicles only when these also contained at least 25 mol % PC; vesicles composed purely of DOTAP and PE adsorbed strongly to the gel filtration columns. Vesicle aggregation was monitored by measuring the turbidity of vesicle suspensions at 350 nm on a Beckmann DU-7 spectrophotometer. Lipid mixing between vesicles was measured by monitoring the relief of energy transfer between fluorescent donor and acceptor phospholipid probes, using the general procedure described by Struck et al. (1981). In these assays, vesicles labeled with 1 mol % donor and 0.4 mol % acceptor species were incubated with a 9-fold excess of unlabeled vesicles, at a total lipid concentration of 30 μM. In our initial experiments, we used NBD-PE as the energy-transfer donor and Rho-PE as the acceptor species. In later experiments, we employed a new combination of lipid probes, 12-CPS-18-PC as the donor and 12-DABS-18-PC as the acceptor, which have previously been shown to report faithfully the rates of lipid mixing between lipid vesicles in several systems (Silvius et al., 1987). While the two combinations of probes gave qualitatively similar results in most experiments, we observed in a few cases that the NBD-PE/Rho-PE combination appeared to transfer faster from anionic to cationic vesicles, and more slowly in the opposite direction, than did the 12-CPS-18-PC/12-DABS-18-PC combination. Therefore, in this paper, we report lipid mixing results obtained by using the latter probe combination except where otherwise indicated.

Mixing of contents between vesicles was examined by using the ANTS/DPX assay described by Ellens et al. (1985), using vesicles loaded with either 50 mM ANTS or 90 mM DPX. Leakage of vesicle contents was assayed by the procedure of Ellens et al. (1984), using vesicles loaded with 25 mM ANTS and 45 mM DPX. Contents mixing and leakage assays were carried out with a total lipid concentration of 30 μM, with a 1:4 ratio of ANTS- to DPX-containing vesicles in the former case, except where otherwise indicated. All experiments were carried out at 37 °C, and all solutions used were adjusted with sucrose to a total osmolarity of 290 mOsm and were buffered with 2 mM each of histidine and Tes to pH 7.4.

Resealed human erythrocyte ghosts were prepared from freshly collected blood by the method of Steck and Kant (1974), with the modification that the initial lysis of the cells was carried out by dilution into 100 volumes of distilled water. Binding of fluorescent-labeled vesicles to erythrocyte ghosts was assayed by incubating vesicles, containing 154 mM NaCl, 2 mM histidine, and 2 mM Tes, pH 7.4, with resealed ghosts loaded with a buffer containing the above components plus 100 mM sucrose. After incubation in the latter solution for 10 min, the incubation mixtures were centrifuged at 13000g for 5 min at room temperature, and the fluorescence in the pellet and supernatant fractions was measured after addition of 1% Triton X-100. Protein was assayed by the method of Markwell et al. (1981), and phospholipid was assayed by the method of Lowry and Tinsley (1974), with the modification that the digestion period was extended to 4 h.

Results

In preliminary experiments, we investigated the preparation and stability of vesicles combining DOTAP with neutral phospholipids. Stable vesicles, which did not discernibly aggregate over periods of hours in monovalent salt solutions, could readily by prepared by bath sonication or reverse-phase evaporation from mixtures of DOTAP and PC in any proportions. By contrast, mixtures of PE and DOTAP formed stable vesicles consistently using the reverse-phase evaporation procedure only when the vesicles contained at least 15–20 mol % DOTAP. Dispersions of PE/DOTAP vesicles were rapidly destabilized in the presence of millimolar concentrations of polyanions such as EDTA, phosphate, or calcein (not shown),

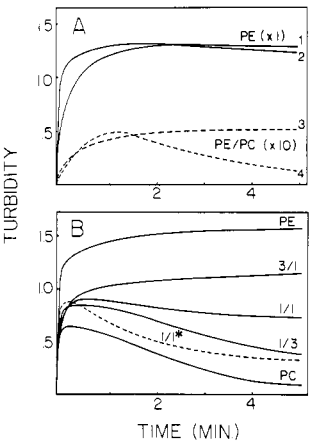

FIGURE 1: Time courses of turbidity (measured as absorbance at 350 nm) after anionic and cationic lipid vesicles are mixed at time zero. (A) Cationic and anionic LUV were mixed at concentrations of 30 μM each. (Curves 1 and 2) 80:20 (mol/mol) PE/DOTAP vesicles and 85:15 PE/PS vesicles were mixed in buffered solutions containing sucrose or 150 mM NaCl, respectively. (Curves 3 and 4) 40:40:20 PE/PC/DOTAP vesicles were mixed with 42.5:42.5:15 PE/PC/PS vesicles in buffered sucrose or buffered NaCl solutions, respectively. Curves 3 and 4 have been scaled up by a factor of 10 for clarity. (B) Bath-sonicated vesicles (200 μM each), prepared from 20 mol % DOTAP or 15 mol % PS plus PE and PC in the indicated molar proportions, were mixed at time zero in buffered sucrose solution (solid curves) or in buffered 150 mM NaCl (dashed curve).

a behavior observed previously for cationic vesicles prepared from dialkyldimethylammonium bromides (Carmona-Ribeiro et al., 1984, 1985; Rupert et al., 1985). DOTAP itself could readily be dispersed in buffers of physiological ionic strength by vortexing or bath sonication, giving stable, somewhat turbid suspensions that flocculated upon addition of millimolar concentrations of multivalent anions. We did not further investigate the properties of aqueous dispersions of pure DOTAP.

When two populations of phospholipid vesicles with opposite surface charges are mixed, rapid aggregation is observed, the extent of which depends on both the neutral lipid compositions of the vesicles and, in some cases, the ionic strength of the medium. These points are illustrated by the turbidity measurements shown in Figure 1. When PE/DOTAP (80:20) LUV are mixed with PE/PS (85:15) LUV in a medium of low ionic strength, a very rapid and dramatic increase in turbidity is observed (Figure 1A, curve 1). The time course of this turbidity increase appears similar at physiological ionic strengths (Figure 1A, curve 2). However, samples that are incubated at physiological ionic strength are visibly more heavily flocculated after several minutes incubation than are samples incubated at low ionic strength. When the above experiment is repeated using LUV in which the PE component is replaced by equimolar PE and PC, less extensive vesicle aggregation is observed (Figure 1A, curves 3 and 4). At higher ionic strengths, the initial rise in turbidity after mixing these latter vesicles is followed by a slow decrease, which is not a consequence of vesicle precipitation.

The results just presented suggest that LUV with opposite surface charges aggregate more extensively, with less sensitivity to the ionic strength, when they contain high proportions of PE relative to PC. Qualitatively similar results are also obtained in parallel experiments using smaller bath-sonicated vesicles prepared from PE/PC/DOTAP and PE/PC/PS mixtures, as shown in Figure 1B. Using these vesicles at a relatively high lipid concentration of 200 μM (compared to 30 μM for the LUV above), we observe rapid aggregation between oppositely charged vesicles even when the vesicles have

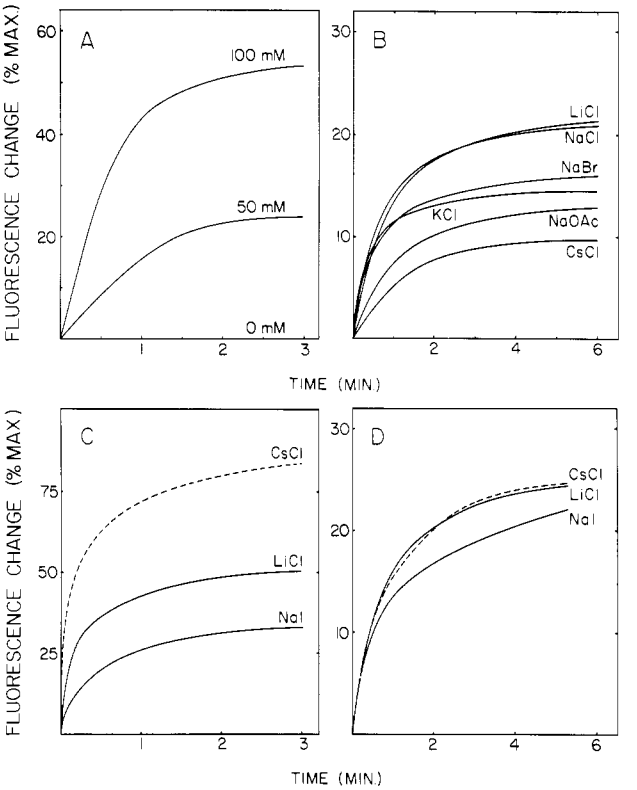

FIGURE 2: Time courses of lipid mixing, assayed as described under Materials and Methods, between lipid vesicles prepared from 80:20:1:0.4 (molar proportions) PE/DOTAP/12-CPS-18-PC/12-DABS-18-PC and vesicles prepared from 85:15 PE/PS. (a) LUV were mixed at time zero in buffered solutions containing the indicated concentrations of NaCl. (B) As in (A), using solutions containing the indicated monovalent salts at 50 mM. (C and D) Bath-sonicated vesicles were mixed at time zero in buffered solutions containing the indicated monovalent salts at 100 mM (C) or 5 mM (D). The time course for sonicated vesicles treated with CsCl is shown for clarity as a dashed line in (D) and, for consistency, in (C) as well. Time courses obtained using NaCl in the experiments shown in panels C and D were essentially superimposable on those shown for LiCl.

very high PC contents. However, as was observed for LUV, suspensions of bath-sonicated vesicles with opposite surface charges show lower maximum turbidities upon mixing, and a more pronounced decline in the turbidity signal after the initial rise, when the vesicles contain high levels of PC.

The aggregation of vesicles with opposite surface charges can be followed by significant intermixing of vesicle components, as we demonstrate below. Like the initial aggregation process, the mixing of vesicle lipids and contents is strongly influenced by the lipid compositions of the participating vesicles. However, in contrast to the aggregation process, these latter interactions are strongly promoted by increasing ionic strength. An example of this behavior is shown in Figure 2A, which shows time courses of lipid mixing between 80:20 PE/DOTAP LUV and 85:15 PE/PS LUV at varying ionic strengths. At NaCl concentrations up to roughly 20 mM, very little lipid mixing is observed. At higher concentrations of NaCl, substantial lipid mixing is observed, with the initial mixing rate increasing steadily with increasing salt concentration (Figure 3A). Bath-sonicated vesicles of the same compositions also show negligible lipid mixing at very low ionic strengths, although as little as 5 mM NaCl can promote significant lipid mixing in this case (not shown).

Further experiments were carried out in an effort to explain the somewhat surprising observation that lipid mixing between PE/DOTAP and PE/PS vesicles is salt dependent. We first verified that results identical with those shown in Figure 2A

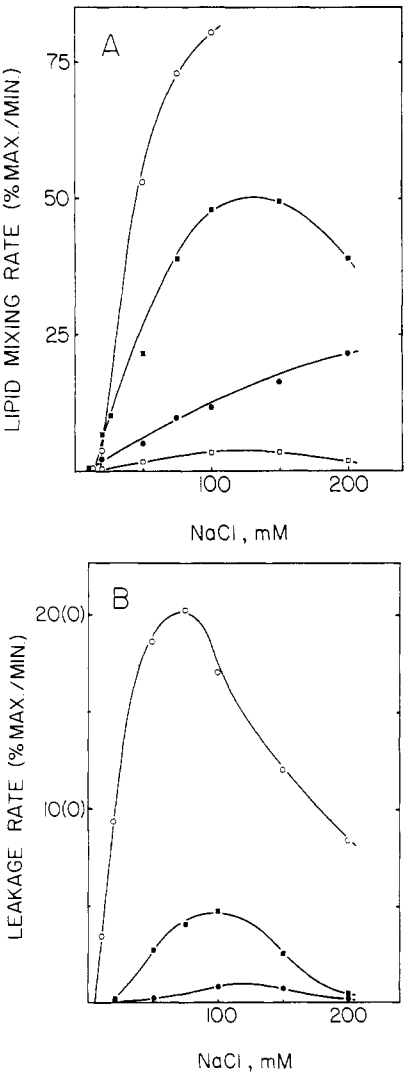

FIGURE 3: (A) Initial rates of lipid mixing, determined as in Figure 2, between DOTAP- and PS-containing LUV in buffered solutions containing the indicated concentrations of NaCl. (Open circles) 80:20 (molar proportions) PE/DOTAP vesicles plus 85:15 PE/PS vesicles. (Closed squares) 40:40:20 PE/PC/DOTAP vesicles plus 42.5:42.5:15 PE/PC/PS vesicles. (Closed circles) 80:20 PE/DOTAP vesicles plus 85:15 PC:PS vesicles. (Open squares) 80/20 PC/DOTAP vesicles plus 85:15 PC/PS vesicles. In all cases shown, the DOTAP-containing vesicles were labeled with 12-CPS-18-PC and 12-DABS-18-PC, as in Figure 2. Results very similar to those shown in this figure were obtained in parallel experiments in which the PS- rather than the DOTAP-containing vesicles were labeled (not shown). (B) Initial rates of leakage of contents, measured as described by Ellens et al. (1984), when PS-containing vesicles (6 μM lipid) loaded with ANTS and DPX were added at time zero to DOTAP-containing vesicles (24 μM lipid). The conditions of these experiments, and the symbols used to represent different vesicle compositions, are the same as in (A). For the upper curve, the maximum rate indicated on the y-axis scale is 200%/min, while for the lower two curves it is 20%/min. To compare the rates shown in (A) and (B) of this figure, it should be noted that a single round of interactions between vesicles can produce only ~45% of the maximum possible fluorescence change in the lipid mixing assay but can in principle lead to 100% leakage of vesicle contents.

were obtained with these vesicles when mannitol rather than sucrose was used to maintain a constant osmolarity in buffers of low ionic strength (results not shown). It thus appears that the mixing of lipids between PE/DOTAP and PE/PS vesicles is genuinely promoted by NaCl rather than being simply inhibited by sucrose. We next examined the salt dependence of lipid mixing between 80:20 PE/DOTAP vesicles and a series of PE/PS vesicles containing varying proportions of PS. The time courses of lipid mixing observed in these experiments were essentially indistinguishable, at any given concentration of NaCl from 0 to 150 mM, whether the latter population of vesicles contained 15, 20, 25, or 30 mol % PS (not shown). It thus does not appear that the salt requirement for lipid mixing in this system is in some way a consequence of disparities in the magnitudes of the surface charges of the two populations of vesicles.

In a final effort to clarify the origin of the salt requirement for lipid mixing between PE/PS and PE/DOTAP vesicles, we compared the abilities of different monovalent salts to promote this process. Representative results from these experiments, using either LUV or bath-sonicated vesicles, are shown in Figure 2B–D. At higher concentrations (50 or 100 mM), different alkali metal chlorides, and the sodium salts of different monovalent anions, support lipid mixing between vesicles with modestly differing efficiencies (Figure 2B,C). At lower ionic strengths, different monovalent salts support lipid mixing with very similar efficiencies, as is illustrated in Figure 2D. The different monovalent ions used in the experiments summarized in Figure 2 have been reported to bind to PS and to quaternary ammonium amphiphiles with widely varying affinities (Eisenberg et al., 1979; Abuin et al., 1983; Lissi et al., 1985). The salt requirement for lipid mixing between these vesicles thus appears to be fairly nonspecific, particularly when comparing different monovalent salts at low concentrations (<50 mM). We did not test the effects of multivalent ions on lipid mixing, since suspensions of PE/DOTAP or PE/PS vesicles self-aggregate in the presence of even low millimolar concentrations of multivalent anions (sulfate, phosphate, or EDTA) or cations (Ca^{2+} or Mg^{2+}), respectively.

Lipid mixing results qualitatively similar to those presented above are also obtained using vesicles that contain choline phospholipids as a major component. The 85:15 PC/PS LUV show essentially no lipid mixing with 85:15 PC/DOTAP vesicles at NaCl concentrations below ~25 mM, but at higher salt concentrations, a slow mixing of lipids is observed (Figure 3A). The 42.5:42.5:15 PE/PC/PS vesicles show lipid mixing with 40:40:20 PE/PC/DOTAP vesicles at NaCl concentrations above ~15 mM, and the rate of this process reaches a maximum at 100 mM NaCl, falling off again as the ionic strength is further increased (Figure 3A). The rates of lipid mixing between these PC-containing vesicles are considerably slower, at any given salt concentration, than are observed between PE/DOTAP and PE/PS vesicles at the same ionic strength. By contrast, the aggregation of vesicles with different surface charges is quite rapid, even when limited in amplitude, regardless of the vesicles' PC content (Figure 1).

As noted above, most studies of the interactions between lipid vesicles employ manipulations of the ionic composition of the aqueous medium to promote interactions between vesicles with like surface charges. We therefore examined the proton- and divalent cation-promoted mixing of lipids between PE/PC vesicles to compare with the lipid mixing observed between PE/PC/DOTAP vesicles and PE/PC/PS vesicles. LUV were prepared from 1:1 PE/PC at pH 9.5, and interactions between vesicles were then initiated either by reducing the pH or by adding divalent cations (Ellens et al., 1986, 1987a). When the PE/PC vesicles were abruptly exposed to a pH of 4.5 in 200 mM NaCl, an initial lipid mixing rate of 1.6%/min was measured at a lipid concentration of 30 μM. At pH 9.5, these vesicles showed initial lipid mixing rates of 6.8% or 5.1%, respectively, in the presence of 10 mM Ca^{2+} or Mg^{2+} (not shown). These rates are substantially slower than the observed rate of lipid mixing between PE/PC/DOTAP

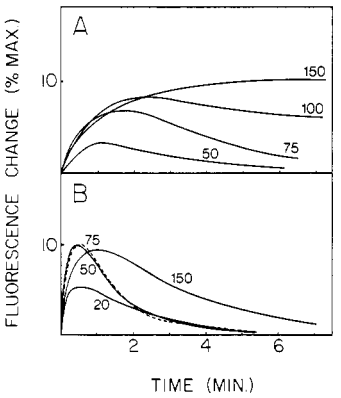

FIGURE 4: Time courses of contents mixing, assayed as described by Ellens et al. (1985), when DOTAP- and PS-containing LUV are mixed in buffered solutions containing the indicated concentrations of NaCl. (A) 40:40:20 (molar proportions) PE/PC/DOTAP vesicles loaded with DPX were mixed with 42.5:42.5:15 PE/PC/PS vesicles loaded with ANTS. (B) 40:40:20 PE/PC/DOTAP vesicles loaded with DPX were mixed with 42.5:42.5:15 PE/PS vesicles loaded with ANTS. Other details of the assay are described under Materials and Methods.

vesicles and PE/PC/PS vesicles at the same total lipid concentration (49%/min). It thus appears that the presence of fixed and complementary surface charges on two phospholipid vesicles can promote interactions between the vesicles considerably more efficiently than can a simple neutralization of charge repulsion between vesicles, or the possible cross-bridging of negatively charged lipid surfaces by divalent cations.

In parallel with the above lipid mixing experiments, we used the ANTS/DPX assays described by Ellens et al. (1984, 1985) to examine the mixing and release of vesicle contents during interaction of vesicles with opposite surface charges. As shown in Figure 4A, 42.5:42.5:15 PE/PC/PS LUV show detectable mixing of contents with 40:40:20 PE/PC/DOTAP vesicles at NaCl concentrations above roughly 20 mM. The initial rate of contents mixing is maximal at 75–100 mM NaCl and is significantly lower at higher salt concentrations. The leakage of contents from PE/PC/PS vesicles in the presence of PE/PC/DOTAP vesicles shows a very similar pattern of dependence on the salt concentration, as shown in Figure 3B.

PE/PS vesicles show a relatively rapid leakage of contents in the presence of PE/DOTAP vesicles (Figure 3B), and again, the leakage rate is strongly dependent on the salt concentration. Unfortunately, we could not examine the mixing of contents between PE/PS and PE/DOTAP vesicles, as the latter were difficult to prepare with high internal concentrations of ANTS or DPX, particularly at the stage of gel filtration. However, we were able to examine the mixing of contents between 80:20 PE/PS vesicles and 40:40:20 PE/PC/DOTAP vesicles, obtaining the results summarized in Figure 4B. Comparing these data to those shown in Figure 4A, it is apparent that the absence of PC in the former vesicles enhances significantly the rate at which they mix contents with PE/PC/DOTAP vesicles.

Interactions of PE-Rich Vesicles with PC-Rich Vesicles and Membranes. As noted above, the use of positively and negatively charged vesicles allows us to examine the interactions between two different types of surfaces, one highly fusion competent and one more refractory to fusion, while suppressing interactions between surfaces of the same type. We therefore examined the interactions between vesicles that are rich in PE, a lipid that is generally found to promote vesicle fusion, and vesicles and natural membranes that are rich in choline phospholipids, which are usually found to be inhibitory to vesicle fusion (Liao & Prestegard, 1979; Uster & Deamer, 1981; Düzgünes, 1981a,b; Sundler et al., 1981; Silvius & Gagné, 1984b).

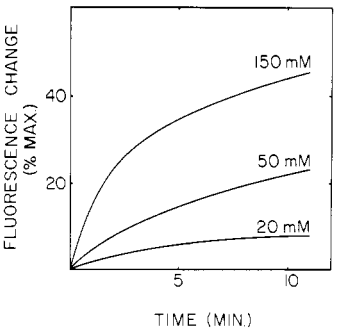

FIGURE 5: Time courses of lipid mixing when 80:20:1:0.4 (molar proportions) PE/DOTAP/12-CPS-18-PC/12-DABS-18-PC LUV are mixed with 85:15 PC/PS vesicles in solutions containing the indicated concentrations of NaCl. Other details are as for Figure 2.

In Figure 5 are shown time courses of lipid mixing observed when 80:20 PE/DOTAP vesicles are incubated with 85:15 PC/PS vesicles at various ionic strengths. As in the experiments described above, lipid mixing is strongly promoted by increasing ionic strength and is quite significant in both rate and extent at physiological salt concentrations. The time courses of lipid mixing in these experiments were unchanged when the ratio of labeled (PE/DOTAP) to unlabeled (PC/PS) vesicles was varied from 1:2 to 1:20 at a fixed concentration of the former (2.5 μM), suggesting that the process is not aggregation limited (Bentz et al., 1983). Similar results were obtained in experiments in which 85:15 PE/PS vesicles or 85:15 PE/DOPA-Me vesicles were mixed with 85:15 PC/DOTAP vesicles (not shown). By contrast, no significant lipid mixing was observed when 80:20 PE/DOTAP vesicles or 85:15 PE/PS vesicles were incubated with LUV prepared from PC alone. Likewise, unilamellar PE vesicles prepared at pH 9.5 (Ellens et al., 1986) showed negligible lipid mixing with a large excess of PC vesicles when a mixture of the two types of vesicles was abruptly exposed to pH 4.5 or 6.0 to eliminate the negative surface charge on the PE vesicles (P. Brown, unpublished observations). The presence of complementary surface charges on the two vesicle populations thus appears to be an important determinant of the ability of PE-rich vesicles to exhibit lipid mixing with PC-rich vesicles.

When 80:20 PE/PS vesicles loaded with ANTS were incubated with a 4-fold excess of 85:15 PC/DOTAP vesicles containing DPX, no significant mixing of vesicle contents could be detected on the time scale of the lipid mixing experiments described above (not shown). Parallel measurements of leakage of vesicle contents (Figure 4B) showed only a very slow release of contents from the PE/PS vesicles (<1%/min at 100 or 150 mM NaCl) in the presence of PC/DOTAP vesicles. This leakage required the presence of the PC/DOTAP vesicles but was only very weakly dependent on their concentration above a 1:1 ratio of cationic to anionic vesicles. We can thus conclude that the mixing of lipids between vesicles in this system is a much faster process than is subsequent destabilization of the PE-containing vesicles. The initial interactions of the PE-containing vesicles with PC-rich vesicles appear largely to constitute a process of "hemifusion", in which the outer monolayers of two apposed bilayers can intermix without simultaneous coalescence of their inner monolayers and internal aqueous compartments. We cannot determine from the present data whether the slower subsequent destabilization of the PE-containing vesicles in contact with PC-rich vesicles produces exclusively the leakage of vesicle contents or whether a slow mixing of contents also occurs but is followed by leakage. The steady-state signal that would be observed

Table I: Lipid Mixing between Cationic Lipid Vesicles and Human Erythrocyte Membranes or Phosphatidylcholine-Rich Vesicles[a]

"donor" vesicle composition	acceptor membrane	incubation time (min)	% lipid mixing
PE/DOTAP (85:15)	erythrocyte ghosts	10	68
		60	69
PC/DOTAP (85:15)	erythrocyte ghosts	10	1
		60	4
PE/DOTAP (85:15)	PC/PS (85:15) vesicles	10	58
		60	61
PE/DOTAP (85:15)	PC/ganglioside (95:5) vesicles	10	73
		60	75

[a] DOTAP-containing LUV, labeled with 1 mol % NBD-PE alone or with 1 mol % NBD-PE plus 0.4 mol % Rho-PE, were prepared in 25 mM NaCl, 240 mM sucrose, 5 mM histidine, and 5 mM Tes, pH 7.4. These vesicles (30 μM lipid) were incubated, at 37 °C and in a medium containing 100 mM NaCl, with acceptor membranes at levels of the latter equivalent to 300 μM phospholipid. At the end of the indicated incubation periods, the samples were diluted 10-fold with the buffer used for the incubations, and the sample fluorescence was immediately read before and after the addition of 1% Triton X-100. The extent of lipid mixing was determined from these readings as described in the Appendix.

in the contents mixing assay in either case would be too small to be detected reliably.

In view of the demonstrated ability of PE/DOTAP vesicles to aggregate and to mix lipids with anionic vesicles containing high levels of choline phospholipids, we also examined the interactions between cationic lipid vesicles and human erythrocyte ghosts. In preliminary experiments, we measured the extent of binding of the cationic vesicles to resealed ghosts by a centrifugation assay, as described under Materials and Methods. When 30 μM PE/DOTAP (85:15) vesicles are incubated with ghosts at a concentration corresponding to 300 μM erythrocyte membrane phospholipid, 85% of the vesicle lipid can be sedimented after 10 min of incubation in medium containing 150 mM NaCl. Under the same centrifugation conditions, <5% of the vesicle lipid is pelleted when the vesicles are incubated in the absence of ghosts. In a parallel experiment using PC/PE/DOTAP (42.5:42.5:15) vesicles in place of PE/DOTAP vesicles, only 8% of the vesicle lipid could be pelleted after 10-min incubation with ghosts. These results indicate that the addition of PC to PE/DOTAP vesicles can inhibit substantially the ability of such vesicles to associate with biological membranes.

In further experiments, we examined the ability of cationic lipid vesicles to intermix lipids with erythrocyte ghosts. Fluorescent-labeled vesicles containing 15 mol % DOTAP plus PE or PC were incubated with erythrocyte ghosts for various times at vesicle and ghost lipid concentrations of 30 and 300 μM, respectively. At the end of the incubation period, the samples were diluted 10-fold with buffer, and the extent of lipid mixing was determined by measuring the relief of quenching of the fluorescence of NBD-PE by Rho-PE. In parallel samples, ghosts were incubated with lipid vesicles that contained NBD-PE but no Rho-PE, and the fluorescence of these samples was also measured to allow correction for possible changes in the fluorescence of NBD-PE upon transfer from the vesicle to the ghost membranes. The extents of lipid mixing were then calculated by using the equations described in the Appendix, giving the results summarized in Table I. After 10-min incubation, PE/DOTAP vesicles show very substantial lipid mixing with the ghost membranes, while PC/DOTAP vesicles show very little lipid mixing under the same conditions. With either type of vesicle, the extent of lipid mixing increases only slightly when the time of incubation is increased up to 60 min. For purposes of comparison, the above experiments were also carried out using PE/DOTAP vesicles together with PC/PS or PC/ganglioside LUV in place of erythrocyte ghosts. As shown in Table I, the extent of mixing of lipids between PE/DOTAP vesicles and PC-rich LUV was similar to that observed with erythrocyte ghosts under the same incubation conditions.

Discussion

While numerous studies have examined the properties of vesicles formed from synthetic cationic amphiphiles (Kunitake et al., 1977; Kunitake & Sakamoto, 1978; Eibl & Wooley, 1979; Kano et al., 1979; Südholter et al., 1980; Rydhag et al., 1982; Carmona-Ribeiro et al., 1983, 1984, 1985; Rupert et al., 1985), fewer reports have examined the properties of liposomes that combine these species with natural phospholipids. Most of these latter studies have employed vesicles containing phosphatidylcholine together with stearylamine (Martin & MacDonald, 1976a; Deleers et al., 1982; Yoshihara et al., 1986). These vesicles have been shown to interact with negatively charged biological membranes (Martin & MacDonald, 1976a–c; Yoshihara et al., 1986), binding to the surfaces of erythrocytes and several cultured cell lines and eliciting cell agglutination under appropriate conditions. Stearylamine-containing liposomes have been shown to be appreciably cytotoxic to mammalian cells (Magee et al., 1974; Roozemond & Urli, 1982; Schwendener et al., 1984; Yoshihara & Nakae, 1986), although it is difficult to determine whether this cytotoxicity is attributable to direct interactions of the liposomes with the cells or to the exchange of stearylamine through the aqueous phase to bind to cellular membranes. None of these studies has defined in much detail the nature of the interactions between cationic liposomes and negatively charged membranes that follow the initial electrostatic binding of the liposomes to the membranes, although Martin and MacDonald (1976c) have shown that cationic liposomes that contain a substantial proportion of lysophosphatidylcholine can transfer a lipid probe to the surface membranes of cultured cells.

The results presented in this paper indicate that vesicles with opposite surface charges can interact with one another in a variety of ways, depending on the lipid compositions of the membrane surfaces and on the ionic strength of the medium. Vesicles that combine neutral phospholipids with small amounts of PS or DOTAP aggregate rapidly when mixed, as could be predicted from simple electrostatic considerations alone. However, the initial rate and extent of this aggregation show only a modest dependence on the ionic strength of the medium, particularly for vesicles that contain higher proportions of PE. Moreover, the extent of aggregation is significantly affected by the proportions of PE and PC in the vesicles, even under conditions where aggregation is not followed by vesicle coalescence (i.e., by intermixing of vesicle lipids and/or aqueous contents). These results are readily understandable in light of current models of the aggregation process (Verwey & Overbeek, 1948; Nir & Bentz, 1978; Ninham, 1981), which consider that the free energy of this process depends not only on the electrostatic interactions between vesicles but also on the van der Waals attractions and repulsive "hydration forces" between surfaces (Cowley et al., 1978; Lis et al., 1982; Loosely-Millman et al., 1982). The superior ability of PE vs PC to promote vesicle aggregation in the present systems may be attributable to a weaker surface hydration of PE-rich vesicles, a stronger van der Waals attraction between surfaces that are rich in PE, or a combination of these two factors.

Probably the most surprising result obtained in this study is our observation that the coalescence, as opposed to the

aggregation, of lipid vesicles with opposite surface charges shows a marked salt dependence. From our results to date, we cannot propose a unique explanation for this effect, but we can rule out certain possibilities. Most fundamentally, we suggest that the salt dependence of vesicle coalescence does not reflect the binding of counterions to vesicle surfaces, which could in principle modulate such properties as surface hydration. Using previously published analyses and binding constants (Eisenberg et al., 1979; Winiski et al., 1986), we can calculate that fewer than 25% of the charged lipid molecules in vesicles containing 15–20 mol % PS or DOTAP will be bound by counterions at NaCl concentrations below 50 mM [we assume that chloride binds to DOTAP with an affinity similar to that estimated previously for didodecyldimethylammonium (Winiski et al., 1986)]. Moreover, different monovalent salts show fairly similar abilities to promote lipid mixing between vesicles with opposite surface charges, particularly at lower ionic strengths, even though the different ions studied bind to PS or to quaternary ammonium amphiphiles with widely varying affinities (Eisenberg et al., 1979; Abuin et al., 1983; Lissi et al., 1985). These observations suggest that the salt requirement for mixing of components between anionic and cationic vesicles does not arise from a requirement for binding of counterions to the vesicle surfaces. Counterion binding to vesicle surfaces may be important at higher ionic strengths (>100 mM), where the rates of coalescence and destabilization of some types of vesicles decline markedly (Figures 3 and 4).

If counterion binding is not important in determining the salt requirement for coalescence of cationic and anionic lipid vesicles, it remains to account for this requirement through other mechanisms. As already noted, calculations based on macroscopic treatments of the electrostatic, van der Waals, and hydration forces between lipid bilayers (Verwey & Overbeek, 1948; Nir & Bentz, 1978; Bentz & Nir, 1981; Evans & Parsegian, 1986) can account satisfactorily for the aggregation behavior of these vesicles. However, such calculations, when applied to the systems examined here, shed little light on the reasons why these vesicles fail to coalesce, i.e., show no intermixing of lipids or aqueous contents, at low ionic strength (I. Graham and M. J. Zuckermann, personal communication). We would suggest two additional factors, which are not fully considered in the analyses just noted, that may be important in determining the salt dependence of the coalescence of vesicles with opposite surface charges. First, fluctuations of surface geometry may be very important in determining the rate at which apposed surfaces proceed to coalescence (Siegel, 1984, 1986), and the nature and amplitude of these fluctuations could depend significantly on the electrostatic properties of the surfaces and their associated double layers. Second, current theoretical analyses of the forces between bilayer surfaces assume that the different forces (electrostatic, van der Waals, and hydration) can be considered independently, an assumption that may not be justified (Bentz & Ellens, 1987). In the systems studied here, for example, it is possible that the distribution of fixed and mobile charges at and near the bilayer surfaces may have significant effects on the hydration as well as the electrostatic properties of the surfaces. Further study will be required to clarify these points.

It should be noted that some of the interactions between vesicles that we observe in this study may take place, at least in part, within aggregates of vesicles that are larger than simple "dimers". In our experiments, extensive "superaggregation" of vesicles was avoided by employing high ratios of anionic to cationic vesicles (or vice versa) in all assays except the turbidometric experiments shown in Figure 1. Smaller complexes of several associated vesicles may still form rapidly under these conditions, particularly in systems where the vesicles show strong tendencies to aggregate (e.g., PE/PS and PE/DOTAP vesicles). Nevertheless, we find that the major qualitative features of the interactions between anionic and cationic lipid vesicles, and particularly the remarkable salt dependence of these interactions, are similar for strongly aggregating systems (e.g., PE/PS and PE/DOTAP vesicles) and for more weakly aggregating systems (e.g., PE/PC/PS and PE/PC/DOTAP LUV). We thus feel that the results presented here provide at least a qualitatively valid reflection of the nature of the interactions between simple pairs of vesicles with opposite surface charges.

One of the more interesting properties of cationic vesicles that are rich in PE is their ability to interact with negatively charged surfaces even when the latter are rich in choline phospholipids and/or surface proteins. PE/DOTAP vesicles, but not PC/DOTAP vesicles, readily adsorb to erythrocyte ghosts in media of physiological ionic strength, and this adsorption is followed by substantial mixing of lipids between the vesicle and ghost membranes. We have also observed the efficient transfer of fluorescent phospholipids from PE/DOTAP vesicles into the surface membranes of cultured CV-1 cells by fluorescence microscopy (P. Brown and J. Silvius, unpublished results). Lipid mixing between phospholipid vesicles and normally nonfusogenic biological membranes has been reported previously in a few systems (Schneider et al., 1980; Chazotte et al., 1985; Driessen et al., 1985), but extremes of pH, or high concentrations of divalent cations or poly(ethylene glycol), are usually required to promote these processes. We cannot conclude from our present results that the mixing of lipids between PE/DOTAP vesicles and ghost membranes reflects an equally efficient fusion between the two structures. Several systems have been described in which lipid mixing takes place between the outer monolayers of two apposed membranes, in a process termed "hemifusion", without coalescence of the inner monolayers or of the aqueous compartments bounded by the membranes (Wojcieszyn et al., 1983; Ellens et al., 1985; Leventis et al., 1986). Our own results suggest that at least the initial interactions between PE/DOTAP and PC/PS vesicles are largely of this type, and the same may well be true of the interactions between PE/DOTAP vesicles and erythrocyte membranes. Further studies, using somewhat different approaches, will be required to determine the rates and efficiencies of true fusion between PE/DOTAP vesicles and biological membranes. It seems clear from the present results, however, that PE/DOTAP vesicles can readily establish points of continuity with the surfaces of biological membranes, in the process overcoming the "hydration barrier" that represents one of the major obstacles to intermembrane fusion (Cowley et al., 1978; Lis et al., 1982; Loosely-Millman et al., 1982). These results may be useful in guiding further efforts to design lipid vesicles that can fuse efficiently with biological membranes. The potential usefulness of this approach is suggested by the very recent report of Felgner et al. (1987) that liposomes prepared from PE (but not PC) and the diether analogue of DOTAP can be used to promote efficient DNA transfection of animal cells in tissue culture.

APPENDIX

Estimation of the Extent of Lipid Mixing between Lipid Vesicles and Erythrocyte Ghosts. We assume that the transfer of fluorescent phospholipids from lipid vesicles to erythrocyte membranes can lead to a change in the intrinsic fluorescence

of the donor as well as to a reduction in the efficiency of quenching of the donor fluorescence by the acceptor. To separate these effects, we perform parallel incubations of erythrocyte ghosts with vesicles labeled either with the donor only or with the donor and acceptor species. It we assume that the transfer of lipid probes from vesicles to the much larger erythrocyte ghosts essentially eliminates energy transfer between the probes, we can use the following equations for each pair of samples:

$$\frac{\text{fluorescence(donor only)}}{\text{fluorescence(donor/Triton)}} = f_{mix}F_{eryth} + (1 - f_{mix})F_{ves} \quad (1)$$

$$\frac{\text{fluorescence(colabeled vesicles)}}{\text{fluorescence(colabeled/Triton)}} = f_{mix}F_{eryth} + (1 - f_{mix})F_{ves}Q \quad (2)$$

The terms in these equations are defined as follows. The numerators of the terms on the left represent the fluorescence measured for samples of ghosts incubated with vesicles containing donor only (in eq 1) or donor plus acceptor (in eq 2), and the corresponding denominators represent the fluorescence measured for these samples after addition of 1% Triton. The term f_{mix} is the extent of lipid mixing between vesicles and ghosts (=1.0 for complete intermixing), which is assumed to be the same in the two samples since they are treated identically. F_{ves} and F_{eryth} represent the fluorescence of the donor in lipid vesicles containing no acceptor probe and in the ghost membrane, respectively, normalized to the donor fluorescence in 1% Triton. Finally, Q represents the normalized fluorescence intensity measured for the donor species in lipid vesicles containing the acceptor probe divided by the normalized fluorescence intensity measured for the donor in vesicles containing no acceptor.

All of the quantities in the above equations except f_{mix} and F_{eryth} can be measured directly. We can thus calculate these latter two parameters by simultaneous solution of eq 1 and 2, using fluorescence data obtained from pairs of samples prepared as described above. As a check on the consistency of the results obtained, we verified in each experiment that very similar estimates of F_{eryth} were obtained from all sample pairs that used the same donor species and the same preparation of ghosts.

Registry No. DOTAP, 113669-21-9; NaCl, 7647-14-5; CsCl, 7647-17-8; LiCl, 7447-41-8; NaBr, 7647-15-6; KCl, 7447-40-7; NaOAc, 127-09-3; NaI, 7681-82-5; 3-bromo-1,2-propanediol, 4704-77-2; oleoyl chloride, 112-77-6; oleic acid, 112-80-1; oxaloyl chloride, 79-37-8; 3-bromo-1,2-bis(oleoyloxy)propane, 14437-89-9; trimethylamine, 75-50-3.

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Phospholipid Transfer between Phosphatidylcholine–Taurocholate Mixed Micelles†

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ABSTRACT: The transfer of fluorescent-labeled *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) between phosphatidylcholine–taurocholate mixed micelles was measured by monitoring the increase in fluorescence as *N*-NBD-PE, initially contained in mixed micelles at self-quenching concentrations, was diluted into unlabeled mixed micelles. The half-times for transfer of a homologous series of *N*-NBD-PEs differing in saturated acyl chain length from 11 to 16 carbons increased with acyl chain length from 4 to 35 s. The half-times for transfer of the same *N*-NBD-PEs between phosphatidylcholine vesicles without taurocholate were 200–6000 times slower than those between the mixed micelles. A kinetic analysis of initial transfer rate data was used to determine the mechanistic model that best described the data. According to this analysis, the increased rate of intermicellar phospholipid transfer relative to that of intervesicular transfer is a result of (1) exchange between micelles during transient micelle collisions which is not observed between vesicles and (2) an increased rate of monomer diffusion due to a faster rate of phospholipid dissociation from mixed micelles into the water phase than from vesicles. The relative significance of collision-dependent versus monomer diffusion transfer increases with acyl chain length and hydrophobicity.

The release of mixed micelles containing phospholipids, cholesterol, and bile salts from the gall bladder following a fatty meal is essential for the efficient digestion and absorption of fats and other insoluble amphiphiles. Although the thermodynamics of mixed bile salt micelle formation and the factors that determine their size and structure have been studied in detail [for reviews, see Cary and Small (1972), Hofmann and Roda (1984), and Whiting (1986)], virtually nothing is known about the rates and mechanisms by which the water-insoluble components of these micelles are exchanged.

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