Interbilayer Transfer of Phospholipid-Anchored Macromolecules via Monomer Diffusion†

John R. Silvius*,‡ and Martin J. Zuckermann§

Departments of Biochemistry and Physics, McGill University, Montréal, Québec, Canada H3G 1Y6

Received September 11, 1992; Revised Manuscript Received November 17, 1992

ABSTRACT: A series of conjugates has been prepared by linking various hydrophilic macromolecules [poly(ethylene glycols), polylysine, aminodextrans, or apotransferrin] to synthetic phosphatidylethanolamines via linker moieties incorporating a fluorescent bimane group. Using a fluorescence energy transfer-based assay, the rate of transfer of these species between phospholipid vesicles has been monitored as a function of the nature and size of the coupled macromolecule and of the acyl chain composition of the lipid anchor. Conjugates in which the phospholipid anchor is linked to a small hydrophilic terminal residue (e.g., ethanolamine or ethylenediamine) transfer between large unilamellar vesicles of egg phosphatidylcholine with half-times ranging from tens of minutes (for dimyristoyl lipid conjugates) to a few tens of hours (for dipalmitoyl and 1-palmitoyl-2-oleoyl lipid conjugates), in agreement with previous results for unlabeled phospholipids. Conjugation of these same lipid anchors to larger hydrophilic molecules markedly accelerates their rates of intermembrane transfer, by factors ranging from 5–7-fold (for conjugates with apotransferrin and aminodextrans of molecular weight 10,000–70 000) to over 25-fold [for conjugates with poly(ethylene glycol)-5000]. In all cases the observed transfer appears to reflect the diffusion of lipid monomers through the aqueous phase. Our results suggest that substantial intermembrane transfer can occur, on a time scale of several hours or less, for hydrophilic macromolecules conjugated to diacyl(/alkyl) lipids with 14- to 18-carbon chains unless portions of the conjugate other than the lipid anchor also interact strongly with the membrane.

"Anchored" conjugates, linking phospho- and sphingolipids to soluble macromolecules such as proteins, oligosaccharides or simple hydrophilic polymers occur widely in nature (Ledeen & Yu, 1982; Ferguson & Williams, 1988; Gaulton et al., 1988; Low, 1989; Stadler et al., 1989; Cross, 1990; Thomas et al., 1990; Takeda & Maizel, 1990; Conzelmann et al., 1992) and as well are increasingly used in biotechnological applications [e.g., for "targeting" or stabilization of liposomes (Heath & Martin, 1986; Afzelius et al., 1989; Klibanov et al., 1990; Blume & Cevc, 1990; Allen et al., 1991; Senior et al., 1991; Woodle & Lasic, 1992)]. An important question for understanding the functions and behavior of such conjugates is the strength of the membrane anchorage that the coupled lipid moiety confers on the tethered macromolecule. Most available evidence suggests that species such as phosphatidylinositol(PI)[1]-glycan-anchored proteins are "firmly" membrane-anchored in the sense that they are preponderantly membrane-associated and cannot be extracted into the aqueous phase without grossly disrupting the membrane (Ferguson & Williams, 1988; Hooper & Turner, 1988; Low, 1989; Thomas et al., 1990; Cross, 1990). However, it is less clear that phospho- or sphingolipid-anchored conjugates of hydrophilic macromolecules are so firmly associated with a given membrane that they cannot transfer between distinct membranes through the aqueous phase on biologically meaningful time scales. Evidence has in fact been reported that at least one PI-glycan-anchored protein, the dimyristoylglycerol-anchored variant surface glycoprotein of *Trypanosoma brucei*, can transfer to a detectable extent between the surfaces of different cells on a time scale of a few hours (Rifkin & Landsberger, 1990).

In the present study we have used fluorescence resonance energy transfer to examine the transfer of defined phospholipid–macromolecule conjugates, varying both in acyl chain composition and in the size and nature of the coupled macromolecule, between the surfaces of phospholipid vesicles. Our results suggest that hydrophilic macromolecules anchored to lipid bilayers by diacyl(/alkyl) phospholipid residues with C_{14}–C_{18} hydrocarbon chains may generally be able to transfer between membranes via monomer diffusion, with half-times of a few tens of hours or less, unless this potential is suppressed by significant additional interactions between the soluble portion of the anchored conjugate and the membrane.

MATERIALS AND METHODS

Materials

Phosphatidylcholines (PCs), obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) or from Sigma (St. Louis, MO),

† This work was supported by grants from the Medical Research Council of Canada (J.S.), the Natural Sciences and Engineering Research Council of Canada (M.Z.), and les Fonds FCAR du Québec. J.S. gratefully acknowledges additional support in the form of an MRC of Canada Scientist award.

‡ Department of Biochemistry.

§ Department of Physics.

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DABS-PC, 1-palmitoyl-2-[12-[[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]octadecanoyl]-*sn*-glycero-3-phosphocholine; DMCA-NHS, succinimidyl 7-dimethylaminocoumarin-4-acetate; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; DTNB, 5,5′-dithio(bis(2-nitrobenzoic acid)); EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA, ethylenediaminetetraacetic acid trisodium salt; LUV, large unilamellar vesicles; NBD-fluoride, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PEG, poly(ethylene glycol); PG, phosphatidylglycerol prepared from egg PC by transphosphatidylation; PI, phosphatidylinositol; SAMSA, *S*-acetylmercaptosuccinic anhydride; SUV, small unilamellar vesicles; Tes, sodium tris(hydroxymethyl)methanesulfonate; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; TPE, PE prepared from egg PC by transphosphatidylation.

0006-2960/93/0432-3153$04.00/0

were converted to phosphatidylethanolamines (PEs) as described elsewhere (Silvius et al., 1986). DABS-PC was prepared as described previously (Silvius et al., 1987). Monomethoxy-PEGs (mePEGs), polylysine, the *N*-hydroxysuccinimidyl ester of succinyl mePEG-5000, EDC, SAMSA, and CHAPS were obtained from Sigma (St. Louis, MO). PEGs and mePEGs were purified by ether precipitation (Honda et al., 1981). Monobromobimane and aminodextrans were obtained from Molecular Probes (Eugene, OR). Cholic acid (Anachemia, Montréal, PQ) was treated with activated charcoal and then recrystallized three times from 85% aqueous ethanol. All common chemicals and solvents were of at least reagent grade. Chloroform for coupling reactions was redistilled from P_2O_5.

α-Amino-ω-methoxyPEGs were synthesized as follows: MePEGs (50 mg/mL in dry CH_2Cl_2) were reacted for 3 h at −10 °C and then for 1 h at 25 °C with a 5-fold molar excess of methanesulfonyl chloride and a 2-fold excess of triethylamine. The reaction mixtures were concentrated under nitrogen, cautiously mixed with concentrated NH_4OH (1 mL/100 mg of mePEG), stirred for 30 min at 25 °C and finally heated to 70 °C for 40 h after adding an equal volume of ethanol. The solvents were then evaporated under nitrogen, with repeated additions of chloroform and methanol, until essentially all water was removed. The residue was partitioned between chloroform and 1:1 methanol/0.1 M aqueous NaCl, recovering the chloroform phase to yield material that in each case migrated as a single ninhydrin-positive spot on TLC in 85:15:0.3 $CHCl_3$/CH_3OH/concentrated NH_4OH. α-Amino-ω-methoxy-PEG-5000 prepared in this manner comigrated with one of the two major ninhydrin-positive spots detected in a commercial preparation of this compound (Sigma).

Small samples of the products from the above reactions were labeled with a substoichiometric amount of NBD-fluoride or DMCA-NHS in chloroform solution in the presence of triethylamine, and the fluorescent products were recovered and analyzed by gel filtration on Bio-Gel P-4 and P-10. Each labeled PEG gave a single peak, and the relative elution volumes measured for the different PEG derivatives varied essentially as predicted from the mean molecular weights specified.

Synthetic Methods

General. Lipid derivatives were extracted from reaction mixtures by partitioning in 2:1:1 $CHCl_3$/CH_3OH/aqueous mixtures, washing the chloroform phase once with fresh upper phase, and then concentrating under nitrogen. Lipid derivatives were normally purified by preparative TLC on silica gel 60 plates (Brinkmann, Montréal, PQ). After visualization of the developed plates by fluorescence or by opacity, the desired bands were scraped off and eluted with 1:2:0.4 $CHCl_3$/CH_3OH/0.1 M aqueous EDTA, pH 7.4. The proportions of the above solvents were then adjusted to 2:1:1 for extraction of the products as described above.

Lipid derivatives and intermediates expected to contain free amino or sulfhydryl groups were tested for stoichiometric reaction with TNBS or DTNB, respectively (Nordlund et al., 1981; Ganong & Bell, 1984).

Synthesis of N-(S-Bimanylmercapto)succinyl-PE (Species I and II). In a typical preparation, 50 μmol of PE was reacted for 45 min at 25 °C with a 4-fold excess of SAMSA in 2 mL of $CHCl_3$ containing 20 μL of triethylamine. The reaction products were extracted as described above, using 0.1 M EDTA, pH 7.4, as the aqueous component, and then purified by preparative TLC using solvent system A (50:15:7.5:7.5:3

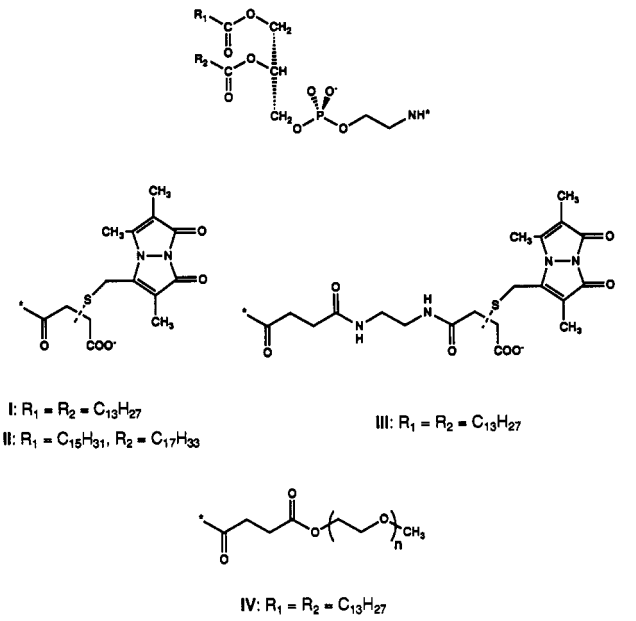

FIGURE 1: "Parent" compounds for the types of lipid conjugates utilized in this study. Species I, III, and IV are derived from dimyristoyl-PE and species II from 1-palmitoyl-2-oleoyl-PE. As indicated, the structure of the bimane-labeled linker in species I–III was not determined unambiguously. However, these compounds as isolated represent in each case only one of two chromatographically resolved fractions and probably comprise a mixture of two stereoisomers with the same site of attachment of the bimanylmercapto group.

$CHCl_3$/acetone/CH_3OH/CH_3COOH/H_2O). Two major phosphate-containing bands were visualized with molybdate reagent (Dittmer & Lester, 1964); the larger upper band (R_f 0.75) was recovered, deprotected by incubation for 45 min at 25 °C in 5 mL of 1:2:0.4 $CHCl_3$/CH_3OH/0.5 M aqueous hydroxylamine, pH 7.0, and then extracted as above, using 0.2 M NaCl as the aqueous component. The product was immediately reacted for 2 h at 25 °C with a 1.3-fold molar excess of monobromobimane in 1:2:0.4 $CHCl_3$/CH_3OH/0.1 M sodium phosphate, pH 8.0. The labeled products were extracted as above and purified by preparative TLC in solvent system B (50:20:10:10:5 $CHCl_3$/acetone/CH_3OH/CH_3COOH/H_2O), which gave a single major fluorescent yellow band (R_f 0.7) in a 30–40% overall yield.

Synthesis of Simple Amine Conjugates of Species I and II. A 10 mM solution of species I in $CHCl_3$ was reacted for 6 h at 25 °C with 20 molar equiv of the appropriate amine and 10 molar equivalents of EDC. The products were extracted as above using 0.1 M HCOONa, pH 3.0, as the aqueous component and then purified by preparative TLC using solvent system B; in all cases a single major product was obtained, in yields of 70–90% based on recovered fluorescence.

The ethylenediamine conjugate of species I (1 mM in dry $CHCl_3$) was reacted for 1 h at 25 °C with 1 and 20 molar equiv of triethylamine and acetic or succinic anhydride, respectively, and then extracted and purified as for the other conjugates just described. Acylation was quantitative in both reactions. The ethylenediamine conjugate of I, but not its acylated derivatives, gave a ninhydrin-positive reaction on TLC.

Species III in Figure 1 was prepared by first derivatizing PE with succinic anhydride (Nayar & Schroit, 1985) in essentially quantitative yield and then coupling to ethylenediamine in the presence of EDC as described above. The major reaction product was purified by TLC in solvent system A and then coupled to SAMSA, deprotected with neutral

hydroxylamine, and finally labeled with monobromobimane, all as described above.

Synthesis of mePEG Conjugates of Species I and II. A 10 mM solution of species I or II in dry chloroform containing 30 mM triethylamine was reacted for 16 h at 25 °C with a 10-fold molar excess of α-amino-ω-methoxy-PEG-350 or -750, or with a 5-fold molar excess of α-amino-ω-methoxy-PEG-2000 or -5000, together with a 5-fold molar excess of EDC. The products were extracted as above and then purified by two successive preparative TLC steps. Initial TLC separation in solvent system B gave three major bands, the slowest migrating of which (R_f 0.6–0.4, decreasing with increasing PEG mass) was recovered and rerun by TLC in 75:25:1 $CHCl_3/CH_3OH$/concentrated NH_4OH. The predominant, slower migrating band from the latter TLC step was recovered as described above, with final yields ranging from 45–55% for mePEG-350 derivatives to 15–22% for mePEG-2000 derivatives.

Preparation of the mePEG-5000 conjugate by the above approach gave relatively low yields (≤10%). As an alternative, the ethylenediamine conjugate of species I was reacted, as a 10 mM solution in dry chloroform, with 1 molar equiv of triethylamine and 3 equiv of the *N*-hydroxysuccinimide ester of α-succinyl-ω-methoxyPEG-5000 for 6 h at 25 °C. The products were isolated as above and purified by preparative TLC in solvent B, recovering the major fluorescent band (yield = 40%). Species IV in Figure 1 was synthesized in the same manner from DMPE and the *N*-hydroxysuccinimide ester of α-succinyl-ω-methoxyPEG-5000.

Synthesis of Polylysine and Aminodextran Conjugates. Fifty nanomoles of species I or II was dispersed in 200 μL of water containing 3 mg of CHAPS and a 5-fold molar excess of polylysine or aminodextran. A 10-fold molar excess of EDC (relative to lipid) was added, and the solution was adjusted to pH 8.0 and then stirred for 6 h in the dark at room temperature. Acetone (1.8 mL) was then slowly added, and after extensive vortexing the mixture was centrifuged at 1000*g* for 10 min and the supernatant discarded. The pellet was washed with a small volume of 90% aqueous acetone and then redissolved in 200 μL of 0.1 M NaCl and reprecipitated with 1.8 mL of acetone. The above precipitation/washing was repeated three times more, removing all traces of uncoupled lipid and detergent detectable by extraction and TLC and fluorescence analysis. The final precipitate was redissolved in 200 μL of 150 mM KCl, 10 mM Tes, and 0.1 mM EDTA, pH 7.4, and dialyzed for 72 h at 4 °C against two changes of the same buffer (1 L each). Conjugate yields under these (nonoptimized) conditions ranged from 4% (for aminodextran-70 000) to 35% (for polylysine) based on recovery of fluorescence.

Coupling of the Conjugate I-ED to Apotransferrin. Conjugate I-ED (100 nmol) was reacted with a 2- and 4-fold molar excess of triethylamine and of bis(succinimidylsuccinyl)-ethylene glycol (Pierce), respectively, for 4 h in dry chloroform at room temperature. The reaction mixture was immediately dried down under nitrogen, and the residue was redissolved in 100 μL of phosphate-buffered saline containing 3.5 mg of CHAPS. After brief sonication, 20 mg of apotransferrin (previously dialyzed against phosphate-buffered saline, pH 7.4) in 250 μL of saline was added, and the mixture was stirred overnight at room temperature. The reaction mixture was chromatographed on a 30-cm column of Sephadex G-75 equilibrated with 150 mM KCl, 10 mM Tris, and 15 mM sodium cholate, pH 8.1, and the fluorescent void-volume fractions were pooled and rechromatographed on a similar column without cholate. The void-volume fraction from the latter column (estimated to contain 1.4 nmol of conjugate by fluorescence in 1% Triton X-100) was mixed with 700 nmol of sonicated/freeze-thawed 85:15:1.5 egg PC/TPE/DABS-PC vesicles (see below) and dialyzed for 72 h at 4 °C against two changes of 150 mM KCl, 10 mM Tes, and 0.1 mM EDTA, pH 7.4. In the final dialysis, sonicated egg PC vesicles (10 μmol of lipid) were included in the external solution as an additional "sink" for possible residual detergent. The dialyzed preparation was incubated for 3 h at 37 °C and then used directly as "donor" vesicles for conjugate-transfer measurements as described below. TLC showed no evidence of lipid degradation. Afzelius et al. (1989) have showed that lipid-conjugated, liposome-bound transferrin prepared by a procedure very similar to the above retains its ability to bind specifically to cellular receptors.

Vesicle Preparation and Probe Transfer Measurements. Donor lipid vesicles were prepared as follows: Well-dried lipid samples including 1–1.5 mol % DABS-PC were first bath-sonicated to virtual clarity at ca. 2 mM lipid in distilled water (0.1 mM NaOH for PC/PE vesicles) and then resonicated for 3 min after adjustment of the aqueous phase to 150 mM KCl, 10 mM Tes, and 0.1 mM EDTA, pH 7.4. The sonicated lipid dispersions were finally freeze/thawed three times in dry ice/ethanol to yield vesicles with an average diameter of approximately 350 nm as estimated by carboxyfluorescein trapping (Wilschut et al., 1980). Vesicles prepared in this manner and "symmetrically" labeled with exchangeable lipid probes (by including the probe in the lipid mixture used to prepare the vesicles) typically showed an exchangeable fraction of probe almost exactly half that observed for similar vesicles labeled "asymmetrically" as described below, suggesting that the vesicles were predominantly unilamellar. Acceptor vesicles were prepared by sonication as described above, without DABS-PC and omitting the freeze–thaw cycles.

Donor vesicles were asymmetrically loaded with fluorescent probes (other than the apotransferrin conjugate) by bath-sonicating the probe (usually at 3 μM) for 3 min in 150 mM KCl, 10 mM Tes, and 0.1 mM EDTA, pH 7.4, and then adding lipid vesicles to 0.6 mM and incubating at 37 °C (or, for dipalmitoyl probes, at 45 °C) for 3–6 h. For probe-transfer measurements, 200 or 400 nmol of acceptor vesicles was normally added, at 37 °C and with stirring, to 30 or 60 nmol of probe-labeled donor vesicles in 3 mL of the above buffer, while continuously monitoring the fluorescence at 468 nm in a Perkin-Elmer LS-5 spectrofluorimeter with excitation at 393 nm (Gardam et al., 1988). A parallel sample was incubated for 3–12 h at 37 °C to permit complete equilibration of probes between the outer surfaces of donor and acceptor vesicles. The probe content of each sample was determined by measuring its fluorescence in the presence of 1% Triton X-100, which relieved quenching effects (Struck et al., 1981). Initial rates of probe transfer from donor to acceptor vesicles were determined using the equations

$$\text{(initial transfer rate)} = [(dF(t)/dt)_{t=0}]/\Delta F_{max} \quad (1)$$

with

$$\Delta F_{max} = [(F[\text{preinc}])(F_{Tx}/F_{Tx}[\text{preinc}])] - F_o \quad (2)$$

where F_o, $F(t)$, and F_{Tx} denote the sample fluorescence before and after acceptor vesicle addition and after final addition of Triton, respectively, and $F[\text{preinc}]$ and $F_{Tx}[\text{preinc}]$ represent the fluorescence of the preincubated control sample before and after Triton addition. All fluorescence readings were corrected to the appropriate blank values.

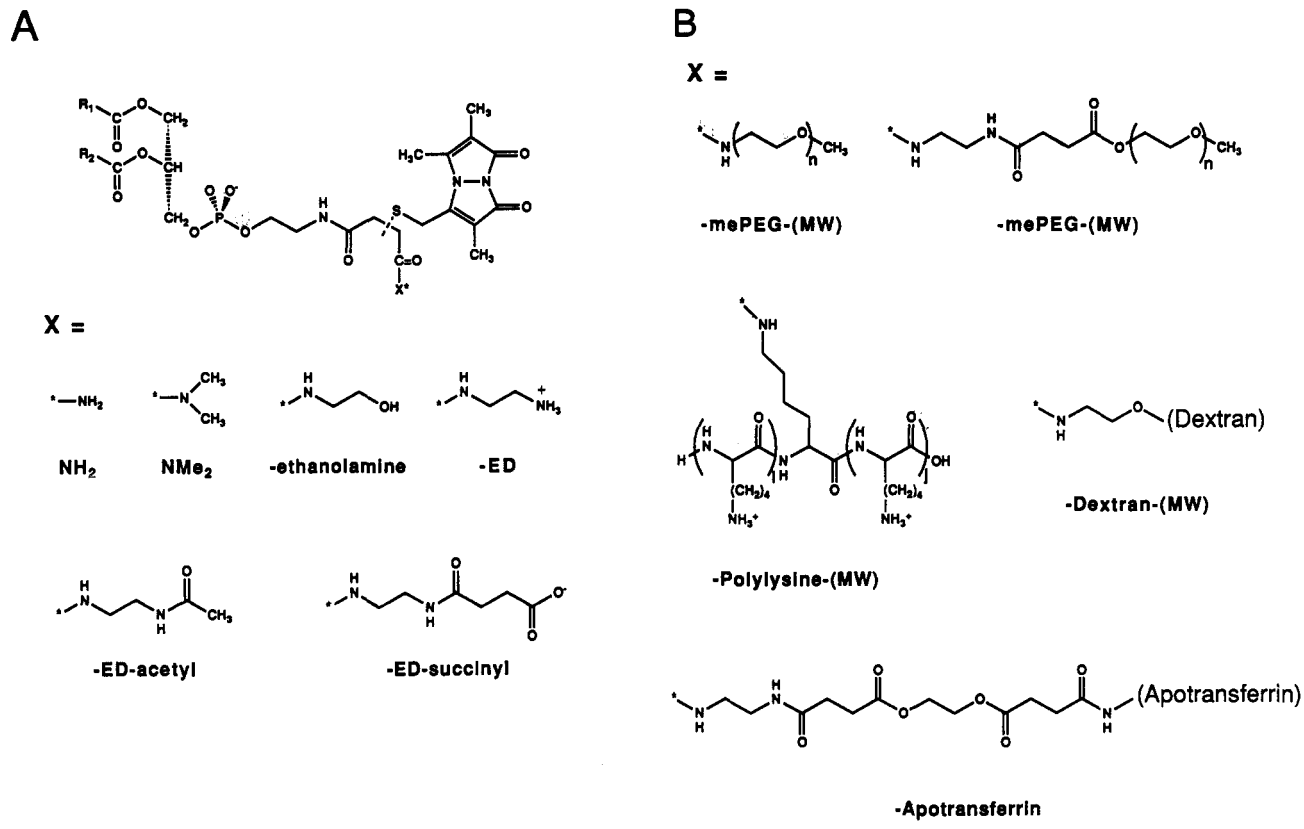

FIGURE 2: Structures of the conjugates of species I and II examined in this study. (Panel A) Conjugates with low molecular weight substituents. (Panel B) Conjugates with hydrophilic polymers, indicating the denotation for each type of conjugate in the text.

RESULTS

The structures and abbreviations of the fluorescent lipid conjugates examined in this study are given in Figures 1 and 2. Species I and II and their conjugates with simple amines and amino alcohols gave turbid dispersions when vortexed in aqueous buffers. By contrast, conjugates with larger polylysine or mePEG headgroups (particularly for molecular weights 750 and higher) formed clear dispersions which foamed extensively under the same conditions, suggesting that these species form micellar rather than bilayer dispersions in aqueous media.

When large unilamellar phospholipid vesicles were added to sonicated dispersions of species I or its conjugates, the fluorescence rapidly increased (over a few minutes for simple conjugates, a few seconds for higher molecular weight species) to a new value that was stable over long times (not shown). A similarly rapid decrease in fluorescence was observed when the phospholipid vesicles contained the nonexchangeable quencher DABS-PC (see below). In both cases subsequent transfer of the probes between vesicles, assessed as discussed below, was much slower than the fluorescence changes just described. These observations are consistent with the assumption that the rapid fluorescence changes noted above reflect insertion of the fluorescent lipid conjugates into the vesicle bilayers upon mixing sonicated suspensions of the conjugates with phospholipid vesicles. Except where otherwise noted, the experiments described below were carried out using vesicles "asymmetrically" labeled with probe molecules in the manner just described, in order to maximize the exchangeable fraction of the vesicle-associated probes (Pagano et al., 1981; Gardam et al., 1988) and to allow comparison of the transfer of multiple types of probe molecules from the same population of donor vesicles.

As noted above, the fluorescence of species I and of its derivatives is efficiently quenched by low mole fractions of the nonexchangeable energy-transfer acceptor DABS-PC when the two probes are present in the same vesicle (Silvius et al., 1987). Interbilayer transfer of the bimane-labeled probes can thus be monitored as a time-dependent dequenching of fluorescence when donor vesicles, incorporating both a bimane-labeled probe and DABS-PC, are mixed with unlabeled acceptor vesicles (Nichols & Pagano, 1982; Gardam et al., 1988). This approach is illustrated in Figure 3, where 85:15:1.5 egg PC/TPE/DABS-PC donor LUV, asymmetrically labeled with 0.5 mol % of species I, are mixed at time zero with a 6-fold excess of egg PC/TPE SUV. (For simplicity, in the remainder of this paper the term "donor" vesicles will denote large unilamellar vesicles containing, in addition to the major lipid components specified, 1.5 mol % of DABS-PC). As described under Materials and Methods, by measuring the initial rate of fluorescence dequenching and by measuring the plateau level of fluorescence in an identical sample after prolonged incubation, the effective rate constant for intervesicle probe transfer can be determined directly (Nichols & Pagano, 1982).

In Table I are listed the rate constants measured for the transfer of a series of low-molecular weight derivatives of species I and III between 85:15 egg PC/TPE donor vesicles and sonicated 85:15 egg PC/TPE acceptor vesicles at 37 °C and pH 7.4. The absolute rates measured for intervesicle transfer of a given conjugate could vary significantly (by up to 40%) between experiments using different preparations of donor vesicles or when symmetrically vs asymmetrically labeled donor vesicles (which also constitute distinct donor preparations) were compared. However, the *relative* rates of transfer of different probes varied considerably less (typically by <10–15%) between different donor preparations. Therefore, in

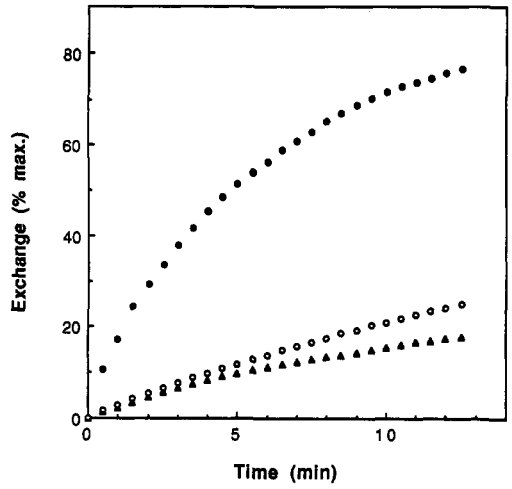

FIGURE 3: Time courses of transfer of conjugates (●) I–mePEG-2000, (○) I–ethanolamine, and (▲) II–mePEG–2000 between 85:15 egg PC/TPE large unilamellar donor vesicles (incorporating also 1.5 mol % DABS-PC) and an excess of small unilamellar acceptor vesicles of like composition (without quencher) at 37 °C. Data points shown are taken from continuous fluorescence traces and scaled in each case to present the fluorescence change as a percentage of the maximal fluorescence change measured for parallel samples equilibrated for 9 h. Other experimental details are given under Materials and Methods.

Table I: First-Order Rate Constants for Desorption of Low Molecular Weight Conjugates of Species I from 85:15:1.5 Egg PC/Egg PG/DABS-PC LUV at 37 °C

probe	k_d/k_d (I–ethanolamine)[a]
I–$NHCH_2CH_2NH_3$ (I-ED)	0.21 ± 0.08
I–$NHCH_2CH_2OH$	(1.00)
I–NH_2	0.74 ± 0.06
I–$N(CH_3)_2$	0.72 ± 0.06
I-ED-acetyl	1.81 ± 0.17
I	2.58 ± 0.27
III	3.62 ± 0.06
I-ED-succinyl	3.39 ± 0.04

[a] k_d values determined as described in the text were normalized to the mean transfer rate measured for species I–ethanolamine in the same experiment and then averaged over a total of 12 runs in four independent experiments. Values are given as mean ± SD. The average value of k_d measured for species I–ethanolamine in these experiments was 0.0204 ± 0.0035 min^{-1}.

Tables I and II the conjugate-transfer rates presented are normalized to the value measured for the conjugate I–ethanolamine, allowing more meaningful averaging of transfer rates between experiments, and the range of absolute transfer rates measured for this reference species is indicated in the legend.

As indicated in Table I, the rate constants measured for intervesicle transfer of simple zwitterionic and monoanionic derivatives of species I are comparable in magnitude to those reported previously for transfer of dimyristoyl-PC between liquid-crystalline bilayers under similar conditions (Ferrell et al., 1985; Wimley & Thompson, 1990, 1991). It is noteworthy that the transfer kinetics for the isomeric dianionic species III and I-ED-succinyl are very similar, suggesting that the specific location of the bimanyl moiety on the probe headgroup is not a major determinant of the transfer rate. Very similar relative transfer rates were measured for the various conjugates of species I when egg PC donor and acceptor vesicles were used in place of 85:15 egg PC/TPE vesicles or when the buffer pH was reduced to 6.5 to ensure complete protonation of PE (not shown), indicating that vesicle surface charge is not a factor in the above pattern of results.

Table II: First-Order Rate Constants for Desorption of High Molecular Weight Conjugates of Species I from LUV at 37 °C

conjugate	vesicle composition[a]	k_d/k_d (I–ethanolamine)
I–aminodextran-10K	egg PC[b]	6.1 ± 1.3[c]
I–aminodextran-40K	egg PC[b]	7.6 ± 0.1[c]
I–aminodextran-70K	egg PC[b]	4.2 ± 0.6[c]
I–polylysine-12.6K	egg PC[b]	14.4 ± 1.6[c]
I–apotransferrin	egg PC/TPE (85:15)	5.7 ± 1.2[d]

[a] All donor vesicles contained 1.5 mol % DABS-PC in addition to the components shown. [b] Similar ratios were measured in parallel experiments using 85:15 egg PC/TPE donor vesicles. PC vesicles were used here to rule out any possible effect of vesicle surface charge on the measured rates. [c] Values were normalized to the mean transfer rate measured for species I–ethanolamine in the same experiment and then averaged over a total of six determinations for two independent preparations of the conjugates. The average value of k_d measured for species I–ethanolamine in these experiments was 0.0135 ± 0.0034 min^{-1}. [d] Parallel determination of k_d for species I–ethanolamine using the same donor vesicles was not possible because of the method of donor preparation. The value indicated was estimated from average k_d values of 0.118 ± 0.010 min^{-1}, measured for the I–apotransferrin conjugate, and of 0.0208 ± 0.0035 min^{-1} measured for species I–ethanolamine using five different preparations of donor vesicles of the same composition.

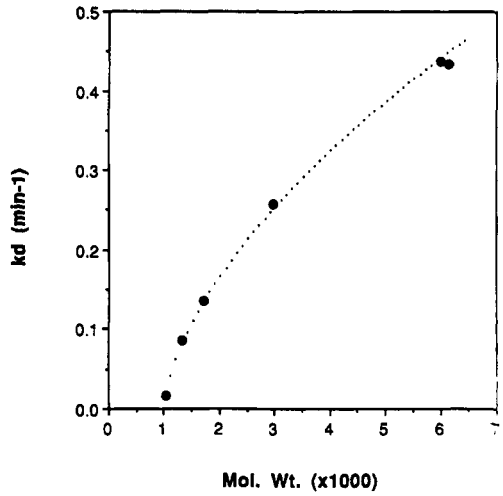

FIGURE 4: Initial rate constants determined for transfer of conjugates of species I with mePEGs of varying molecular weights between 85:15 egg PC/TPE large unilamellar donor vesicles (incorporating also 1.5 mol % DABS-PC) and an excess of small unilamellar acceptor vesicles at 37 °C. The left-hand data point shown for the mePEG-5000 conjugate represents the conjugate of species I with α-amino-ω-methoxyPEG-5000, while the right-hand point represents the conjugate between species I-ED and succinyl-mePEG-5000 (see Figure 2B). Other experimental details are as given in the legend to Figure 3. The dashed curve is fit to the data using the functional form described in the text.

In Figure 4 are shown the intervesicle-transfer rate constants measured, again using 85:15 egg PC/TPE donor and acceptor vesicles, for conjugates of species I with ethanolamine and with α-amino-ω-methoxyPEGs of various molecular weights. The transfer rate increases steadily and dramatically with increasing molecular weight of the attached mePEG component, such that conjugates of species I with mePEG-5000 transfer over 25-fold faster than does the conjugate I-ethanolamine under the same conditions. It is noteworthy that conjugates containing species I coupled in two different ways to mePEG-5000 show very similar rates of intervesicle transfer. The data in Figure 4 can be well-described by a function of the form $k_d = k_d^\circ N^{0.60}$ (dashed curve), where k_d° is the transfer rate for the conjugate I–ethanolamine and N is the number of ethyleneoxy units in the coupled PEG (or ethanolamine) moiety. The data shown can also be fit reasonably well using functions of the form $k_d = k_d^\circ M^\alpha N^\beta$ (M = total molecular

weight), although in this case unique values for the exponents α and β cannot be assigned individually.

The results of several control experiments help to clarify the mechanism of the intervesicle transfer process observed in the above experiments. First, the rate of transfer of several conjugates tested, including the ethanolamine and mePEG-2000 and -5000 conjugates of species I, from 85:15 egg PC/TPE or egg PC vesicles showed negligible variation (<5%) when the total vesicle lipid concentration was varied over a 10-fold range (20–200 μM) at a constant ratio of donor/acceptor vesicles (not shown). Similar results were obtained for a representative set of homologues of the above conjugates, as well as for the other classes of conjugates discussed below. This result suggests that intervesicle collisions play no significant role in the mechanism of intervesicle conjugate transfer at the relatively low lipid concentrations employed here, in agreement with previous observations for various phospholipids (Pagano et al., 1981; Nichols & Pagano, 1982; Jones & Thompson, 1989; Wimley & Thompson, 1991). Under these conditions the measured transfer rate provides a direct estimate of k_d, the first-order rate constant for dissociation of conjugate molecules for the donor vesicles. Second, the rate of transfer of conjugates I-mePEG-2000 and I-mePEG-5000 from PC donor vesicles was unaffected by varying the level of incorporated probe from 0.2 mol % up to at least 1 mol %, or by incorporating up to 2 mol % of the unlabeled DMPE-mePEG-5000 conjugate IV (see Figure 1) into the donor vesicles. These results suggest that under the normal experimental conditions probes with large polar headgroups transfer between bilayers as monomers rather than as aggregates of like or structurally similar molecules (e.g., micelles). By similar criteria the conjugate I–ethanolamine also appears to transfer between vesicles primarily via collision-independent monomer diffusion, as has been reported previously for various phospholipids under conditions like those employed here (Nichols & Pagano, 1982; McLean & Phillips, 1984; Nichols, 1985, 1986; Ferell et al., 1985; Gardam et al., 1988; Jones & Thompson, 1989). Interestingly, however, inclusion of 4 mol % of species IV in the donor vesicles significantly accelerated the transfer of the conjugate I–mePEG-5000 but again had no effect on the transfer of the conjugate I–ethanolamine. This result suggests that conjugates with large polar headgroups may be able to transfer between bilayers as micelles as well as monomers when present in the bilayers at high mole fractions.

The interbilayer transfer of three other classes of lipid–polymer conjugates was also investigated in experiments similar to those described above, giving the results summarized in Table II. Conjugates of species I with aminodextran-10 000, -40 000 and -70 000 transfer between egg PC vesicles, or between 85:15 egg PC/TPE vesicles, at rates several-fold faster than do simple monoanionic conjugates of species I under the same conditions. However, for these conjugates the transfer rate shows a different (and apparently weaker) dependence on the molecular weight of the coupled polymer than is observed for the mePEG conjugates discussed above. The transfer rate constant for the conjugate I–polylysine–12 600 is substantially greater than those for the dextran conjugates but is somewhat smaller than that which would be predicted for a mePEG conjugate of comparable mass by extrapolation from Figure 4.

The transfer rate for the polylysine-12 600 conjugate of species I increased almost 2-fold when the donor vesicles included 10 mol % of DOTAP, a cationic lipid analogue, and decreased by a similar factor when the donor vesicles included 5 mol % PG (not shown). Interestingly, in the presence of DOTAP the transfer rate for aminodextran conjugates of species I *decreased* by roughly 2-fold, suggesting a net attractive electrostatic interaction between these conjugates and the cationic vesicle surface. This latter result may reflect the possibility that for the sparsely amino-substituted dextran conjugates the positively charged amino groups lie well away from the charged surface, in a region of weak electrostatic potential [see Winiski et al. (1988) and Cevc (1990)], so that the interfacially disposed phosphodiester group dominates the electrostatic interactions between the conjugate and the charged surface. By contrast, in the highly charged polylysine conjugates some positively charged groups must be disposed near the membrane surface, and as expected their electrostatic interactions with the surface charge collectively appear to exceed (and oppose) that of the phosphodiester group.

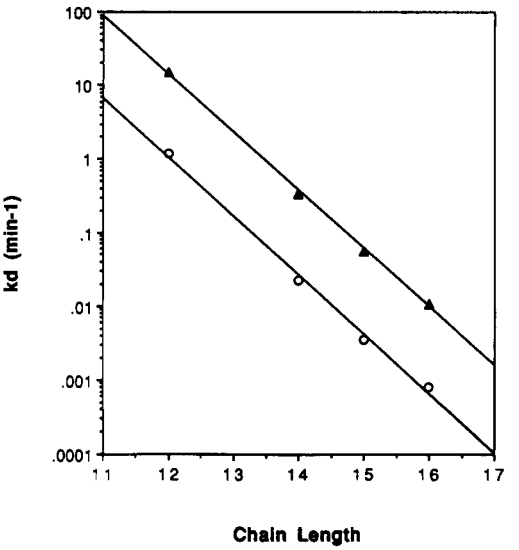

FIGURE 5: Initial rate constants for the transfer of various saturated di-*n*-acyl homologues of conjugates (○) I–ethanolamine and (▲) I–mePEG-2000 between 85:15 egg PC/TPE large unilamellar donor vesicles and an excess of small unilamellar acceptor vesicles at 37 °C. Other experimental details are as given in the legend to Figure 3.

In a final experiment in this series, we examined the rate of intervesicle transfer of a conjugate of species I coupled to apotransferrin through an ethylenediamine–(bis-succinylethylene glycol) linker. As discussed under Materials and Methods, the conjugate was first gel-filtered in the presence of cholate-containing buffer (to remove uncoupled lipid) and then rechromatographed in the absence of cholate, combined with preformed 85:15 egg PC/TPE donor vesicles, and finally dialyzed to remove possible residual detergent (Afzelius et al., 1989). The transfer rate for the donor vesicle-associated conjugate was found to be essentially the same before and after the dialysis step (not shown), suggesting that residual detergent, if present in the final preparation, did not substantially affect the measured transfer rate (Nichols, 1986). However, some 28% of the fluorescent lipid associated with the final preparation appeared to be uncoupled to protein, as judged by its ready extractability into chloroform and its migration on TLC. The true rate of intervesicle transfer for the apotransferrin conjugate is therefore probably underestimated, possibly by as much 40%, by the value given in Table II. It is nonetheless clear that the apotransferrin conjugate of species I transfers between vesicles several-fold faster than do simple conjugates of species I under the same conditions.

Effects of Conjugate Acyl Chain Length. In Figure 5 are plotted the rate constants measured for transfer of various

homologues of the ethanolamine and mePEG-2000 conjugates of species I between 85:15 egg PC/TPE vesicles. For both classes of conjugates the transfer rate decreases exponentially with increasing acyl chain length. The slopes of the lines fit to the semilog plot shown in Figure 5 are proportional to the increment in the apparent free energy of probe desorption, $\Delta G^{\ddagger}_{dis}$, that results from the addition of one methylene group to each acyl chain of the lipid conjugate. The value of this increment is essentially identical (1.13 and 1.14 kcal mol^{-1}, respectively) for the homologous series of ethanolamine and mePEG-2000 conjugates, corresponding to a decrease of roughly 6.3-fold in the rate of intervesicle transfer when each acyl chain is extended by one methylene unit. Very similar values for this increment can be estimated from the data of Nichols (1985) and of Homan and Pownall (1988), obtained using quite different types of fluorescent-labeled phospholipids, if we compare the behavior of different probes with reference to their "effective carbon numbers" as discussed by Nichols (1985) and by Tanford (1972, 1980). A similar dependence of lipid transfer rates on acyl chain length has been estimated by Ferrell et al. (1985) for the transfer of saturated phosphatidylcholines from vesicles of these species into erythrocyte membranes.

In parallel experiments, we also examined the intervesicle transfer of certain conjugates of the 1-palmitoyl-2-oleoyl phospholipid II with large hydrophilic headgroups. Interestingly, significant transfer of the polylysine-12 600 and mePEG-2000 and -5000 conjugates of species II between egg PC or 85:15 egg PC/TPE vesicles was observed on a time scale of tens of minutes (see, for example, Figure 1), and virtually complete redistribution of these conjugates was observed after a 6-h incubation at 37 °C. Considerably slower transfer was observed for conjugates with simpler headgroups, such as species II and its ethanolamine conjugate. In general, 1-palmitoyl-2-oleoyl phospholipid conjugates transferred between vesicles 30–40-fold more slowly than did the analogous dimyristoyl lipid conjugates (e.g., 32-fold slower for II- vs I-mePEG-2000, 34-fold slower for II- vs I-mePEG-5000, and 34-fold slower for species II vs I), in agreement with previous estimates of the relative rates of intervesicle transfer for 1-palmitoyl-2-oleoyl-PC vs dimyristoyl-PC (Jones & Thompson, 1989; Wimley & Thompson, 1990, 1991). However, unlike the other species examined in the present study, some conjugates of species II appeared to show nonexponential time courses of intervesicle transfer. The origin of this behavior was not established, although certain simple explanations (e.g., heterogeneity of the probe) could be ruled out. We are currently investigating this point in more detail.

DISCUSSION

The fluorescent lipid probes from which the conjugates examined here were derived are relatively simple to prepare and possess an unsubstituted diacylglycerophospho- anchor moiety. This latter feature is important, as it reduces the risk that the packing of the lipid anchor within the bilayer, and hence the dynamics and energetics of probe-bilayer dissociation, will be markedly different from those of natural phospholipids. In agreement with this expectation, the rates of interbilayer transfer measured here for simple zwitterionic and monoanionic derivatives of the dimyristoyl phospholipid derivative I are comparable to those reported by Jones and Thompson (1990) and by Ferrell et al. (1985) for desorption of dimyristoyl-PC from small unilamellar PC vesicles. In addition, as already noted, the pattern of dependence of the bilayer desorption rate on the acyl chain length for various homologues of probes I–ethanolamine and I–mePEG-2000 (Figure 5) agrees well with that reported for several other homologous series of fluorescent-labeled and unlabeled phospholipids (Nichols, 1985; Ferrell et al., 1985; Homan & Pownall, 1988).

Nichols (1985) has noted that the addition of successive methylene units to a diacylphospholipid produces very similar incremental changes in $\Delta G^{\ddagger}_{dis}$ and in the equilibrium free energy of transfer of the lipid from the micellar (or vesicular) to the monomeric state (Tanford, 1980). This and other results (McLean & Phillips, 1984; Nichols, 1985; Wimley & Thompson, 1990) suggest that formation of the transition state for desorption of lipid (conjugate) monomers from a bilayer entails a significant displacement of the lipid hydrocarbon chains into the aqueous phase and probably a similar displacement of the lipid headgroup away from the bilayer surface. In this case we can propose at least three ways in which the hydrophilic portion of a lipid–macromolecule conjugate can modulate the free energy of activation (and hence the observed rate) for the desorption of the conjugate from a lipid bilayer.

Two mechanisms by which the hydrophilic portion of a lipid–macromolecule conjugate may influence the rate of conjugate desorption from a membrane are quite general in nature. First, the transfer of any molecule from a supramolecular complex or aggregate to a monomeric state entails an inherent gain of translational and rotational entropy (Janin & Chothia, 1978; Finkelstein & Janin, 1989). While the magnitude of this entropy gain is difficult to calculate precisely except in idealized cases, analyses suggest that this factor contributes significantly to the net free energy of dissociation (Finkelstein & Janin, 1989). Second, a flexible polymer that is anchored at a bilayer/water interface and that cannot readily penetrate the hydrocarbon region will exhibit a significantly lower conformational entropy than would the free polymer. The entropy gain resulting upon dissociation of a lipid-anchored polymer from the bilayer will be particularly substantial for highly flexible polymers but may be significant in any case where the anchored hydrophilic moiety (including its tether to the lipid residue) is not completely rigid. The contributions of the above two factors to the *equilibrium* free energy of desorption for a membrane-bound molecule are predicted to scale in the first case as the logarithm of the molecular weight and in the second (for long polymer chains) as the logarithm of the number of flexibly linked repeating units (Janin & Chothia, 1978; Finkelstein & Janin, 1989; Whittington, 1975). The contributions of these factors to the free energy of activation for conjugate desorption ($\Delta G^{\ddagger}_{dis}$) are probably somewhat smaller, as the conjugate is not completely released from the bilayer in the transition state, but may nonetheless be significant. The desorption rates for various mePEG conjugates of species I (Figure 4) can in fact be well replicated by assuming that $\Delta G^{\ddagger}_{dis}$ includes contributions of logarithmic form from both of these entropic factors (i.e., that $k_d = k_d^{\circ} M^{\alpha} N^{\beta}$, where M is the molecular weight and N is the polymerization number of the mePEG headgroup), although the relative contributions of the two factors cannot be uniquely assigned from these data. It is noteworthy that we find that a lipid-anchored conjugate of a folded globular protein, apotransferrin, transfers between bilayers markedly faster than does the lipid anchor alone but that conjugates linking the same anchor to large highly flexible polymers (polylysine or particularly mePEGs) exhibit still faster interbilayer transfer. These observations suggest that both of the above entropic

factors contribute significantly to the net free energy of activation for the desorption process.

The above effects of a tethered macromolecule on the free energy of lipid–macromolecule conjugate desorption are fairly general ones, to which may be added other contributions more specific to the nature of the anchored moiety. Favorable hydrophobic or other interactions of the anchored macromolecule with the surface or with the polar portions of specific membrane components (e.g., other membrane proteins or even other molecules of the same species) may decrease the rate of transfer of the anchored species. Our observation that lipid–aminodextran conjugates show slower interbilayer transfer than do lipid–polylysine or lipid–mePEG conjugates of similar molecular weight, for example, may reflect in part the known tendency of dextrans to adsorb weakly to lipid bilayers (Minetti et al., 1979; Evans & Metcalfe, 1984). In principle, electrostatic interactions between the anchored species and the membrane surface could significantly influence the rate of conjugate desorption. However, our observations on the desorption of lipid–polylysine and lipid–aminodextran conjugates from charged lipid vesicles suggest that such interactions will only modestly influence the desorption rate at the moderate surface charge densities (equivalent to $\leq$7 mol % charged lipid) typical of biological membranes (Cevc, 1990). A final way in which the "soluble" domain could influence the kinetics of desorption of a lipid–macromolecule conjugate is through a direct interaction of this domain with the lipid anchor (e.g., interactions of the hydrocarbon chains with hydrophobic sites on the soluble domain). For certain species (e.g., lipid-anchored globular proteins possessing hydrophobic "crevices" or surface binding sites), such interactions could significantly decrease the unfavorable hydrophobic free energy of the transition state and thereby accelerate interbilayer transfer.[2]

Naturally occurring phospholipids transfer between membranes at rates that are slow but not necessarily negligible on biologically relevant time scales. 1-Palmitoyl-2-oleoylphosphatidylcholine, a representative membrane phospholipid, has been estimated to transfer between phospholipid vesicles, via a monomer-transfer mechanism, with a half-time of the order of 50 h at 37 °C (McLean & Phillips, 1984; Jones & Thompson, 1989). Extrapolating from this value using results from this and other studies (Ferrell et al., 1985; Nichols, 1985; Homan & Pownall, 1988; J. Silvius, unpublished results), we predict that various mono- and polyunsaturated di-C_{18}-phosphatidylcholines will transfer between phospholipid bilayers with half-times from ca. 20 to 300 h. Our present results indicate that coupling a given phospholipid anchor to a variety of soluble macromolecules can accelerate the rate of interbilayer transfer via monomer diffusion by 5- to >25-fold over that found for the unmodified anchor moiety. Additional mechanisms of conjugate transfer, such as contact-dependent intermembrane transfer (Jones & Thompson, 1989; Wimley & Thompson, 1991) or possible transfer of conjugates in the form of micelles as well as monomers, could potentially further enhance the rate of transfer of certain conjugates between bilayers under particular conditions.

The above observations, taken together, raise the very real possibility that significant intermembrane transfer may occur, on a biologically relevant time scale of a few tens of hours or less, for a variety of naturally occurring lipid–macromolecule conjugates, including not only the dimyristoyl-PI-glycan-anchored variant surface glycoproteins of certain parasites (Ferguson et al., 1985; Rifkin & Landsberger, 1990) but also those PI-glycan membrane proteins and glycolipids of higher eukaryotes whose anchors carry two acyl (or alkyl) chains of 14–18 carbons (Ferguson & Williams, 1988; Ogata et al., 1988, 1990; Gaulton et al., 1988). A similar potential for intermembrane transfer must be considered in assessing the behavior of artificial lipid–macromolecule conjugates with similar anchors, whether these are produced chemically (Heath & Martin, 1986; Afzelius et al., 1989; Woodle & Lasic, 1992) or by genetically engineered anchorage of an otherwise soluble protein residue to a diacyl(/alkyl)-PI-glycan moiety (Orchansky et al., 1988; Paborsky et al., 1991; Duval et al., 1992). Some unusual features of the membrane anchors of certain glycolipids and PI-glycan proteins, such as the presence of very long acyl or alkyl groups (Orlandi & Turco, 1987; Rosen et al., 1989; Luhrs & Slomiany, 1989; McConville & Bacic, 1989; Schneider et al., 1990) or the coupling of a third acyl group to the inositol ring (Roberts et al., 1988; Luhrs & Slomiany, 1989; Walter et al., 1990; Mayor et al., 1990), may have evolved to suppress the potential for intermembrane redistribution (at least by monomer transfer) in cases where such redistribution would be biologically disadvantageous.

The sample of lipid–macromolecule conjugates examined here of course may not model all aspects of the behavior of particular lipid–protein conjugates. As already noted, favorable interactions between the "soluble" portions of an anchored conjugate and other membrane components (Hooper & Turner, 1988; Rademacher et al., 1991) may substantially modulate the rates of intermembrane transfer of individual anchored species. Nonetheless, the generality of the trends observed here for diverse types of phospholipid–macromolecule conjugates suggests that the possibility of intermembrane transfer of other lipid-anchored macromolecules should be addressed in all cases where the anchor moiety carries two acyl or alkyl moieties of 18 carbons or less, and where such intermembrane redistribution could entail functionally significant consequences.

[2] We have considered the possibility that the accelerated interbilayer transfer rates observed for mePEG-containing conjugates could reflect a significant (albeit partial) shielding of the lipid acyl chains from the aqueous environment by the mePEG headgroup. In our opinion at least two points argue against this suggestion. First, polylysine conjugates also show a large acceleration of interbilayer transfer rates; polylysine-12 600 conjugates transfer only about 2-fold more slowly than do the corresponding mePEG-5000 conjugates. Given that polylysine-12 600 has *fewer* backbone bonds with appreciable rotational freedom than does mePEG-5000 (roughly 200 vs 340) and moreover that the allowable dihedral angles for these bonds are likely to be more restricted for polylysine than for mePEG, the intervesicle transfer of the mePEG conjugates does not appear to be anomalously rapid. Second, the data of Figure 5 indicate that the presence of a mePEG-2000 headgroup has no effect on the decrement in the intervesicle transfer rate per pair of methylene units added to the acyl chains of a lipid probe. This result indicates that any possible shielding of conjugate acyl chains by the PEG headgroup would have to be confined entirely to the portions of the acyl chains near the glycerol backbone—at best an unlikely degree of discrimination given the large size of the PEG-2000 headgroup. It is also not clear that a "chain-shielding" effect of the mePEG headgroup would lead to a relationship between conjugate headgroup size and transfer rate of the form found in Figure 4, particularly if this effect is entirely confined to portions of the acyl chain near the headgroup.

REFERENCES

Afzelius, P., Demant, E. J. F., Hansen, G. H., & Jensen, P. B. (1989) *Biochim. Biophys. Acta 979*, 231–238.

Allen, T. M., Hansen, C., Martin, F., Redemann, C., & Yau-Young, A. (1991) *Biochim. Biophys. Acta 1066*, 29–36.

Blume, G., & Cevc, G. (1990) *Biochim. Biophys. Acta 1029*, 91–97.

Cevc, G. (1990) *Biochim. Biophys. Acta 1031*, 311–382.

Conzelmann, A., Puoti, A., Lester, R. L., & Desponds, C. (1992) *EMBO J. 11*, 457–466.
Cross, G. A. M. (1990) *Annu. Rev. Cell Biol. 6*, 1–39.
Dittmer, J. C., & Lester, R. L. (1964) *J. Lipid Res. 5*, 126–127.
Duval, N., Krejci, E., Grassi, J., Coussen, F., Massoulié, J., & Bon, S. (1992) *EMBO J. 11*, 3255–3261.
Evans, E., & Metcalfe, M. (1984) *Biophys. J. 45*, 715–720.
Ferguson, M. A. J., & Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285–320.
Ferguson, M. A. J., Haldar, K., & Cross, G. M. (1985) *J. Biol. Chem. 260*, 4963–4968.
Ferrell, J. E., Jr., Lee, K.-L., & Huestis, W. (1985) *Biochemistry 24*, 2857–2864.
Finkelstein, A. V., & Janin, J. (1989) *Protein Eng. 3*, 1–3.
Ganong, B. R., & Bell, R. M. (1984) *Biochemistry 23*, 4977–4983.
Gardam, M. A., Itovich, J. J., & Silvius, J. R. (1988) *Biochemistry 27*, 884–893.
Gaulton, G. N., Kelly, K. L., Pawlowski, J., Mato, J. M., & Jarett, L. (1988) *Cell 53*, 963–970.
Heath, T. D., & Martin, F. J. (1986) *Chem. Phys. Lipids 40*, 347–358.
Homan, R., & Pownall, H. J. (1988) *Biochim. Biophys. Acta 938*, 155–166.
Honda, K., Maeda, Y., Sasakawa, S., Ohna, H., & Tsuchida, E. (1981) *Biochem. Biophys. Res. Commun. 100*, 442–448.
Hooper, N. M., & Turner, A. J. (1988) *Biochem. J. 250*, 865–869.
Jones, J. D., & Thompson, T. E. (1989) Biochemistry *28*, 129–134.
Klibanov, A. L., Maruyama, K., Torchilin, V. P., & Huang, L. (1990) *FEBS Lett. 268*, 235–237.
Ledeen, R. W., & Yu, R. K. (1982) *Methods Enzymol. 83*, 139–190.
Low, M. G. (1989) *Biochim. Biophys. Acta 988*, 427–454.
Luhrs, C. A., & Slomiany, B. L. (1989) *J. Biol. Chem. 264*, 21446–21449.
Mayor, S., Menon, A. K., & Cross, G. A. M. (1990) *J. Biol. Chem. 265*, 6174–6181.
McConville, M. J., & Bacic, A. (1989) *J. Biol. Chem. 264*, 757–766.
McLean, L. R., & Phillips, M. C. (1984) *Biochemistry 23*, 4624–4630.
Minetti, M., Aducci, P., & Viti, V. (1979) *Biochemistry 18*, 2541–2548.
Nayar, R., & Schroit, A. J. (1985) *Biochemistry 24*, 5967–5971.
Nichols, J. W. (1985) *Biochemistry 24*, 6390–6398.
Nichols, J. W. (1986) *Biochemistry 25*, 4596–4601.
Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720–1726.
Nordlund, J. R., Schmidt, C. F., Dicken, S. N., & Thompson, T. E. (1981) *Biochemistry 20*, 3237–3241.
Ogata, S., Hayashi, Y., Takami, N., & Ikehara, Y. (1988) *J. Biol. Chem. 263*, 10489–10494.
Ogata, S., Hayashi, Y., Misumi, Y., & Ikehara, Y. (1990) *Biochemistry 29*, 7923–7927.
Orchanksy, P. L., Escobedo, J. A., & Williams, L. T. (1988) *J. Biol. Chem. 263*, 15159–15165.
Orlandi, P. A., & Turco, S. J. (1987) *J. Biol. Chem. 262*, 10384–10391.
Paborsky, L. R., Caras, I. W., Fisher, K. L., & Corman, C. M. (1991) *J. Biol. Chem. 266*, 21911–21916.
Pagano, R. E., Martin, O. C., Schroit, A. J., & Struck, D. K. (1981) *Biochemistry 20*, 4920–4927.
Rademacher, T. W., Edge, C. J., & Dwek, R. A. (1991) *Curr. Biol. 1*, 41–42.
Rifkin, M. R., & Landsberger, F. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 801–805.
Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G., & Rosenberry, T. L. (1988) *J. Biol. Chem. 263*, 18766–18775.
Rosen, G., Påhlsson, P., Londner, M. V., Westerman, M. E., & Nilsson, B. (1989) *J. Biol. Chem. 264*, 10457–19463.
Schneider, P., Ferguson, M. A. J., McConville, M. J., Mehlert, A., Homans, S. W., & Bordier, C. (1990) *J. Biol. Chem. 265*, 16955–16964.
Senior, J., Delgado, C., Fisher, D., Tilcock, C., & Gregoriadis, G. (1991) *Biochim. Biophys. Acta 1062*, 77–82.
Silvius, J. R., Brown, P. M., & O'Leary, T. J. (1986) *Biochemistry 25*, 4242–4249.
Silvius, J. R., Leventis, R., Brown, P. M., & Zuckerman, M. J. (1987) *Biochemistry 26*, 4279–4287.
Stadler, J., Keenan, T. W., Bauer, G., & Gerisch, G. (1989) *EMBO J. 8*, 371–377.
Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4102.
Takeda, A., & Maizel, A. L. (1990) *Science 250*, 676–679.
Tanford, C. (1972) *J. Mol. Biol. 67*, 59–74.
Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed., John Wiley and Sons, New York.
Thomas, J. R., Dwek, R. A., & Rademacher, T. W. (1990) *Biochemistry 29*, 5413–5422.
Walter, E. I., Roberts, W. L., Rosenberry, T. L., Ratnoff, W. D., & Medof, M. E. (1990) *J. Immunol. 144*, 1030–1036.
Whittington, S. G. (1975) *J. Chem. Phys. 63*, 779–785.
Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.
Wimley, W. C., & Thompson, T. E. (1990) *Biochemistry 29*, 1296–1303.
Wimley, W. C., & Thompson, T. E. (1991) *Biochemistry 30*, 4200–4204.
Winiski, A. P., Eisenberg, M., Langner, M. K., & McLaughlin, S. (1988) *Biochemistry 27*, 386–392.
Woodle, M. C., & Lasic, D. D. (1992) *Biochim. Biophys. Acta 1113*, 171–200.