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Glycolipid Transfer Protein from Bovine Brain†

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ABSTRACT: Glycolipid transfer protein from bovine brain has been purified partially by ammonium sulfate precipitation, CM-52 ion-exchange, and Sephadex G-75 column chromatography. Both pyrene-labeled and tritium-labeled glucocerebrosides have been used to study the kinetics of protein-mediated transfer between donor and acceptor vesicles. Protein accelerates glucocerebroside transfer but does not accelerate phospholipid transfer. In colyophilized small sonicated vesicles (10% glucocerebroside, 90% 1-palmitoyl-2-oleoylphosphatidylcholine) about two-thirds of the glycolipid is transferred in 2 h and the remaining one-third does not transfer (up to 5 h). For donor and acceptor vesicles made of dipalmitoylphosphatidylcholine or 1-palmitoyl-2-oleoylphosphatidylcholine, glucocerebroside (10% in donors) is transferred rapidly only when both the donor and acceptor matrix phospholipids are in the liquid-crystalline state. If either donor or acceptor vesicles are in the gel state, transfer protein mediated transfer is much reduced. The amount of transfer protein bound specifically to glucocerebroside-containing vesicles is nearly equal above and below the matrix phospholipid phase transition temperature. Bound protein transfers glucocerebroside upon addition of acceptor vesicles.

Glycolipids are components of biological membranes located primarily on the extracellular surface (Steck & Dawson, 1974; Gamberg & Hakomori, 1975; Moss et al., 1977; Critchley et al., 1981). They are thought to be receptors for lectins, hormones, and toxins (Fishman & Brady, 1976; Mullin et al., 1976; Hanson et al., 1977).

Small amounts of glycolipids have a relatively large effect on the physical properties of phospholipid membranes (Correa-Freire et al., 1979; Barenholz et al., 1983). The lateral organization of glycolipids in phospholipid membranes has been studied by using freeze–etch electron microscopy. Glycolipids have been localized in phospholipid bilayers and in biological membranes by attaching electron dense ferritin or colloidal gold particles to an affinity label (Tillack et al., 1982, 1983). These studies suggest that glycolipids are laterally organized in domains that change in size and geometry with temperature. Further evidence comes from measurements in which the spontaneous intervesicular transfer rates of glycosphingolipids in several systems have been shown to be very

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slow with half-times in excess of 30 days (Correa-Freire et al., 1982).

Proteins that accelerate the transfer of glycolipids between membranes have been reported in bovine spleen, pig brain, and rat brain (Metz & Radin, 1982; Abe et al., 1982; Yamada & Sasaki, 1982). We have found a similiar glycolipid transfer protein in bovine brain. We herein report studies of its transfer properties using pyrene- and tritium-labeled glucocerebroside in phosphatidylcholine unilamellar vesicles. Experiments performed above and below the matrix phospholipid phase transition temperature show that the protein-mediated glucocerebroside transfer occurs rapidly only when both donor and acceptor matrix phospholipids are in the liquid-crystalline state. If either donor or acceptor vesicles are in the gel state, protein-mediated transfer is much reduced. A preliminary report of portions of this work has been published previously (Wong et al., 1983).

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC),[1] 1-palmitoyl-2-oleoylphosphatidylcholine (POPC),[1] dipalmitoylphosphatidic acid, and (9,10-dibromostearoyl)phosphatidylcholine (BrPC)[1] were purchased from Avanti Biochemicals and gave a single spot on silicic acid thin-layer chromatographs. Glucocerebroside (GlcCer),[1] extracted from a spleen biopsy of a patient with Gaucher disease, was a gift from the Hadassah Medical School of the Hebrew University, Jerusalem. The fatty acid composition was previously reported by Correa-Freire et al. (1979). The fluorescent derivative, pyrenyl glucocerebroside (PyrGlcCer)[1] was prepared by the method described by Correa-Freire et al. (1982). [^{3}H]Glucocerebroside ([^{3}H]GlcCer), labeled at the 4,5-positions of the sphingosine base, was prepared by the method of Schwarzmann (1978). To ensure purity, cerebrosides and their labeled derivatives were chromatographed on silica gel G plates by using a 85:15:1.5 chloroform–methanol–water solvent system. Pyrenylphosphatidylcholine (PyrPC)[1] was prepared from palmitoyllysophosphatidylcholine and pyrenedecanoic acid by the method of Mason et al. (1981). A single spot was obtained by thin-layer chromatography ($CHCl_3$/MeOH/H_2O, 65:25:4).

Glycolipid Transfer Protein. Glycolipid transfer protein was prepared from bovine brain following a modification of the procedure reported by Abe et al. (1982) for pig brain. All steps in the purification were performed at 4 °C. A 30% homogenate of bovine brain (1 kg) was prepared by disruption with a Waring Blendor (45 s, medium speed) in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride, and 0.02% NaN_3. Cellular debris was removed by centrifugation for 30 min at 5500*g*. Solid ammonium sulfate was slowly dissolved into the supernatant (35% saturation), and after gentle stirring for 15 min, the precipitate was removed by centrifuging for 30 min at 5500*g*. Next, the supernatant was adjusted to 70% $(NH_4)_2SO_4$ saturation and stirred gently for 15 min. The precipitate was collected by centrifuging for 45 min at 5500g and resuspended in 10 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT and 0.02% NaN_3 (buffer A). After exhaustive dialysis against buffer A and removal of precipitate by centrifugation for 40 min at 5000*g*, the protein solution was loaded onto a Whatman CM-52 column (5 × 30 cm), washed with 3 L of buffer A, and fractionated (12.5 mL/tube) with a linear gradient (0–0.3 M NaCl; 0.5 L) of buffer A. The transfer activity peak, which eluted at about 70 mM NaCl, was precipitated with $(NH_4)_2SO_4$ (70% saturation), dialyzed exhaustively against 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT and 0.02% NaN_3 (buffer B), and concentrated to about 20 mL by ultrafiltration (Amicon YM 10 filter). Approximately 10 mL of the concentrated solution (125–135 mg of protein) was loaded onto a Sephadex G-75 column (2.5 × 130 cm) and fractionated (13.5 mL/tube) by elution with buffer B. The colorless peak of transfer activity (Figure 2) was pooled and stored at 4 °C. The specific activity, determined by using the DEAE ion-exchange minicolumn assay (see Measurements of [^{3}H]GlcCer Transfer), was 164.5 nmol of GlcCer min^{-1} (mg of protein)$^{-1}$. This corresponded to a 410-fold increase in specific activity relative to the original 30% homogenate [0.40 nmmol of GlcCer min^{-1} (mg of protein)$^{-1}$] and a 125-fold increase relative to the first 5500*g* supernatant [1.30 nmol of GlcCer min^{-1} (mg of protein)$^{-1}$].

A vesicle-to-RBC ghost transfer assay was used to determine the transfer elution profile from the columns in the purification procedure. Donors were POPC small sonicated vesicles containing 2 mol % [^{3}H]GlcCer, a trace amount of [^{14}C]-cholesteryl oleate nonexchangeable marker, and 10 mol % dipalmitoylphosphatidic acid to minimize aggregation. Acceptors were RBC ghosts, prepared acording to Hanahan & Ekholm (1974). For each assay, donor vesicles (4 μmol total phospholipid) and 0.75 mL of packed RBC ghosts plus protein (10–200 μg) were incubated in a total volume of 1.5 mL for 2 h at 37 °C with shaking. Additional buffer (4 mL) was added, the sample was centrifuged for 10 min at 60000*g*, and the supernatant was aspirated off. After two additional washings, the pellet was counted in a scintillation counter. The nonexchangeable marker counts were used to correct for fusion and aggregation between the donors and acceptors. In later protein purifications, BrPC/POPC (1:1) sonicated vesicles were substituted for RBC ghosts (see below).

Protein concentration was determined with a fluorescence assay (Bohlen et al., 1973) in which the sample aliquot (10–100 μL) was pH adjusted by addition of 1 mL of 0.15 M borate buffer at pH 8.2, and then 0.5 mL of fluorescamine (15 mg/mL acetone) was added while vortexing. Excitation was at 390 nm. Emitted light was monitored at 475 nm through a 430-nm cutoff filter. Bovine serum albumin was used as a standard (0–100 μg).

The specific phospholipid transfer protein from bovine liver was prepared according the the procedure of Kamp et al. (1973).

Preparation of Vesicles. Small unilamellar vesicles were prepared from spectral grade chloroform solutions of phospholipids or mixtures of phospholipids and glycolipids dried in vacuo overnight, suspended in 10 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT and 0.02% NaN_3 (buffer B) above the gel–liquid-crystalline phase transition temperature, sonicated under nitrogen (Huang, 1969), and fractionated by centrifugation (Barenholz et al., 1977). Large unilamellar DPPC vesicles, about 700 Å in diameter, were prepared by using the following modification of a previously published procedure (Wong et al., 1982). Small unilamellar DPPC vesicles were prepared in buffer B at 50 °C, stored at room temperature for 1 day, and then stored at 4 °C for 7–8

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; GlcCer, monoglucosylceramide; T_m, gel–liquid-crystalline phase transition temperature; PyrGlcCer, pyrenyl glucocerebroside, 1-*O*-(β-D-glucopyranosyl)-*N*-[10-(1-pyrenyl)decanoyl]-D-*erythro*-sphingosine; [^{3}H]GlcCer, [^{3}H]glucocerebroside; BrPC, (9,10-dibromostearoyl)phosphatidylcholine; PyrPC, 1-palmitoyl-2-(pyrenyldecanoyl)phosphatidylcholine.

days. The vesicles were centrifuged in a clinical centrifuge for 10 min at 1500*g* to remove small amounts of aggregated material and then stored at room temperature. Sepharose CL-2B column chromatography was used to characterize the size distribution of small and large vesicles before and after incubation with transfer protein. Vesicle elution profiles were determined with diphenylhexatriene (London & Feigenson, 1978). Phospholipid concentrations were measured by the method of Bartlett (1959).

Measurements of [^{3}H]GlcCer Transfer. To measure the transfer of radiolabeled [^{3}H]GlcCer, donor DPPC or POPC vesicles containing 5–10 mol % [^{3}H]GlcCer, a trace amount of [^{14}C]cholesteryl oleate, and 10 mol % dipalmitoylphosphatidic acid were incubated with acceptor vesicles at an acceptor/donor phospholipid ratio of 25. At appropriate times donor vesicles were separated from acceptors on small columns of 2 mL bed volume containing DEAE-Sephacel (Pharmacia) equilibrated with buffer and presaturated with small unilamellar POPC vesicles. Incubation mixes with and without transfer protein were run in parallel. The difference between the two curves was taken to be the protein-mediated transfer of glycolipid.

Control experiments were performed to make sure that the ^{3}H counts, which transferred to the acceptor vesicles, were due to [^{3}H]GlcCer and not some other enzymatic breakdown product. Thin-layer chromatographic analysis ($CHCl_3$/MeOH/H_2O, 85:15:1.5) indicated that over 95% of the ^{3}H counts recovered from acceptor vesicles comigrated with glucosylceramide. This experiment ruled out the possibility of "lipase-like" activity in the Sephadex G-75 purified transfer protein.

Fluorescence Measurements of Transfer. Fluorescence experiments were performed with an SLM 4800S in the ratio mode. The sample intensity was measured against a reference solution of rhodamine B (3 g/L in ethylene glycol) in a triangular cell. PyrGlcCer- or PyrPC-containing samples were excited at 345 nm, and the emission spectra were monitored from 360 to 600 nm. A control sample prepared without fluorophor gave a less than 1% scattering signal. The sample cuvette was temperature regulated to ±0.1 °C and stirred continuously. The emission spectra of pure donors (7 mol % PyrGlcCer, 93 mol % phospholipid) was first recorded. Next, acceptors were added at an acceptor/donor lipid ratio of 25, and the emission spectrum was monitored to allow completion of rapid spontaneous transfer of any trace amounts of pyrene-labeled free fatty acid generated by sonication (Correa-Freire et al., 1982). Transfer protein was then added, and the excimer-to-monomer intensity ratio was recorded continuously with emission monochromators set at 378 and 470 nm.

Analysis of Excimer-to-Monomer Transfer Data. When the donor and acceptor vesicles contained the same matix phospholipid, the concentration of fluorescent probe remaining in the donor vesicles was calculated from the excimer-to-monomer intensity ratio by using a mathematical expression (Roseman & Thompson, 1980; Correa-Freire et al., 1982), which was modified for the case of exchange only between the outer monolayers of the donor and acceptor vesicles (eq 1).

$$E/M = \{[[\tfrac{1}{3}C_0^2/(C_0 + C_h)] + [\tfrac{2}{3}C^2/(C + C_h)] + [\tfrac{2}{3}(C_0 - C)^2/(C_0 - C + RC_h)]]/[[\tfrac{1}{3}C_0C_h/(C_0 + C_h)] + [\tfrac{2}{3}CC_h/(C + C_h)] + [\tfrac{2}{3}(C_0 - C_h)RC_h/(C_0 - C + RC_h)]]\}(K_1/K_2)\,(\eta_{E_{max}}/\eta_{M_{max}}) \quad (1)$$

Here, C is the concentration of the pyrene probe remaining in the outer monolayer of the donor vesicles, C_0 is the initial value of C before addition of transfer protein, and R is the molar ratio of lipid in the outer monolayer of the acceptors to the lipid in the outer monolayer of the donors. The constant C_h is the half-maximal concentration and is defined by eq 2a,b

$$\eta_E = [C/(C + C_h)]\eta_{E_{max}} \quad (2a)$$

$$\eta_M = [C_h/(C + C_h)]\eta_{M_{max}} \quad (2b)$$

where η_E is the quantum yield of excimer and $\eta_{E_{max}}$ is the maximum value ($C \rightarrow \infty$); η_M is the quantum yield of monomer and $\eta_{M_{max}}$ is the maximum value ($C \rightarrow 0$). C_h was evaluated following the procedure of Forster, developed for pyrene in solution (Forster, 1969; Forster & Kasper, 1955), and adapted for PyrGlcCer in phospholipid bilayers. A series of small sonicated vesicles containing 0.0357 μmol of PyrGlcCer and varying amounts of phospholipid were prepared as described above. The samples were excited at 346 nm, and emission spectra were recorded. The curves were normalized to equal integrated areas, and the relative monomer (370–428 nm) and the relative excimer areas (428–600 nm) were plotted as a function of the mole percent PyrGlcCer, as shown in Figure 1A for PyrGlcCer in POPC small sonicated vesicles at 50 °C. The two curves intersect at a concentration equal to C_h. To evaluate the constant $[(K_1/K_2)(\eta_{E_{max}}/\eta_{M_{max}})]$, the ratio of the excimer peak height to the monomer peak height was plotted as a function of concentration. This gave a straight line (correlation coefficient greater than 0.998 for POPC and 0.994 for DPPC), as shown in Figure 1B, for the case of PyrGlcCer in POPC small sonicated vesicles at 37, 45, and 50 °C. Multiplication of the slope of the line by C_h determined the constant $[(K_1/K_2)(\eta_{E_{max}}/\eta_{M_{max}})]$ as shown in eq 12 from Roseman & Thompson (1980). For PyrGlcCer in POPC small sonicated vesicles, C_h = 0.14 (37 °C), 0.12 (45 °C), and 0.11 (50 °C), and $(K_1/K_2)(\eta_{E_{max}}/\eta_{M_{max}})$ = 0.51 (37 °C), 0.54 (45 °C), and 0.58 (50 °C). For PyrGlcCer in DPPC small unilamellar vesicles, C_h = 0.059 (37 °C), 0.064 (45 °C), and 0.063 (50 °C), and $(K_1/K_2)(\eta_{E_{max}}/\eta_{M_{max}})$ = 0.46 (37 °C), 0.48 (45 °C), and 0.51 (50 °C). Linear plots of the excimer to

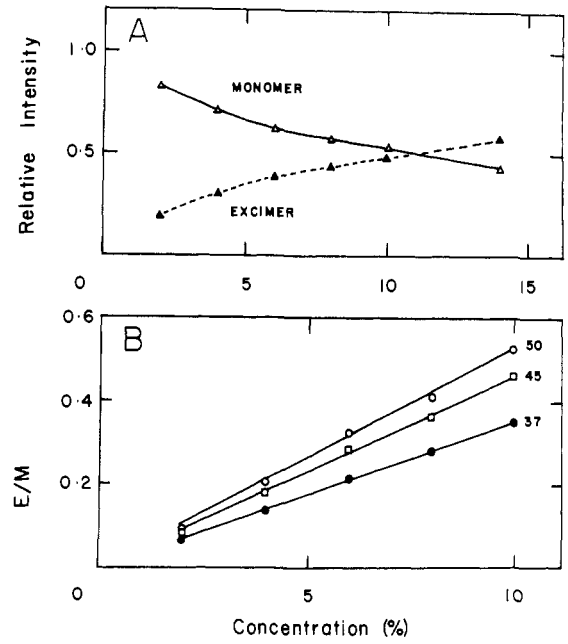

FIGURE 1: (A) Relative intensity (integrated areas) of PyrGlcCer monomer (370–428 nm) (△) and excimer (428–600 nm) (▲) as a function of the mole percent PyrGlcCer in POPC small unilamellar vesicles at 50 °C. (B) The ratio of the excimer peak height at 470 nm to the monomer peak height at 378 nm as a function of the mole percent of PyrGlcCer in POPC small unilamellar vesicles [(●) 37, (□) 45, and (○) 50 °C].

monomer intensity ratio vs. concentration were observed for PyrGlcCer in DPPC small sonicated vesicles at 37, 45, and 50 °C (data not shown). Qualitatively similar results have been reported previously for free pyrene in DPPC vesicles above the phase transition temperature (Galla & Sackman, 1974) and for pyrene derivatives of phosphatidylcholine, glucosylceramide, and sphingomyelin in DMPC (36 °C), DPPC (45 °C), and POPC (50 °C), respectively (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Frank et al., 1983).

When the donor and acceptor vesicles contained different matrix phospholipids, the concentration of fluorescent probe remaining in the donor vesicles was estimated from eq 1 by using the constants C_h and $(K_1/K_2)(\eta_{E_{max}}/\eta_{M_{max}})$ evaluated for the matrix phospholipid of the donor vesicles. Estimates obtained by this procedure were in good agreement with measurements made with [^{3}H]GlcCer.

Vesicle-to-BrPC Vesicle Transfer Assay. Donors were POPC-sonicated unilamellar vesicles containing 2 mol % [^{3}H]GlcCer, 10 mol % DPPA, and a trace of [^{14}C]cholesteryl oleate. Acceptors were prepared by sonicating colyophilized mixtures of BrPC/POPC (1:1) under nitrogen at 20 °C at lipid concentrations between 40 and 100 mM with a sapphire-tipped probe (Heat Systems-Ultrasonics, Plainview, NY). The use of this probe minimized the amount of titanium that was generated by the extensive sonication required to obtain good yields of small BrPC/POPC vesicles. Acceptors were recovered from the upper two-thirds of the supernatant after centrifugation for 20 min at 60000*g*. Donors and acceptors were mixed together at a ratio of 1/12.5 or 1/25 along with transfer protein (0.1–2.0 μg of protein) in a total volume of 0.175 mL and incubated in a Beckman airfuge tube (5 × 20 mm) for 10 min at 37 °C. The acceptors were recovered by centrifuging at 125000*g* for 30 min. These sedimentation conditions yielded 80–90% recovery of control BrPC/POPC acceptors containing [^{14}C]POPC. The supernatant in region 3 (Barenholz et al., 1977) was carefully removed immediately after centrifugation. The acceptor vesicle pellet was washed once by recentrifuging and then collected by solubilizing with 20 μL of 10% Triton X-100. Correction for the small amount of donors, which remained associated with the pelleted acceptors, was made by including [^{14}C]cholesteryl oleate, a nonexchangeable marker, in the donors. The amount of transfer was quantitated by liquid scintillation counting.

Results

The elution profile of glycolipid transfer protein, chromatographed on a Sephadex G-75 column, is shown in Figure 2. The transfer activity peak, measured by using the vesicle-to-RBC ghost assay, eluted at a position which corresponds to an apparent molecular weight of 18 200. This is in good agreement with the size of the glycolipid transfer protein from pig and rat brain reported by Abe et al. (1982) and Yamada & Sasaki (1982), respectively, and is smaller than the transfer protein from bovine spleen (M_r 21 000) reported by Metz & Radin (1980).

The PyrGlcCer transfer assay was used to investigate the effect of the physical state of donor and acceptor vesicle matrix lipid on the protein-mediated transfer of glucosylceramide. Representative data are shown in Figure 3 in which excimer-to-monomer intensity ratio as a function of time is given for a number of donor–acceptor systems at 37 and 50 °C. Two phosphatidylcholines are used as matrix lipids: POPC and DPPC. POPC, with a phase transition temperature of −5 °C, is in the liquid-crystalline state at both 37 and 50 °C. DPPC, which has a gel–liquid-crystalline phase transition at about

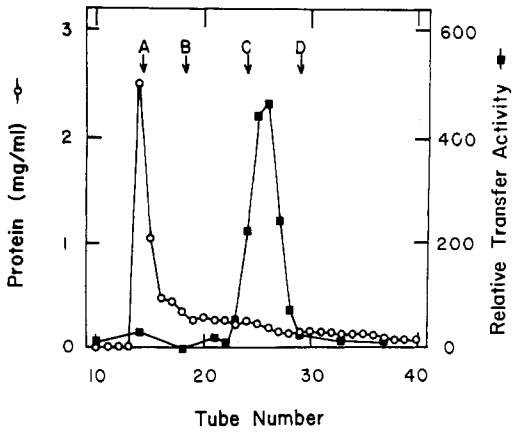

FIGURE 2: Sephadex G-75 column (2.5 × 130 cm), fraction size = 13.5 mL/tube. Column calibrated with (A) bovine serum albumin (M_r 68 000), (B) ovalbumin (M_r 43 000), (C) trypsin (M_r 23 300), and (D) cytochrome *c* (M_r 11 700). The protein (○) and glucocerebroside transfer activity (■) profiles are shown.

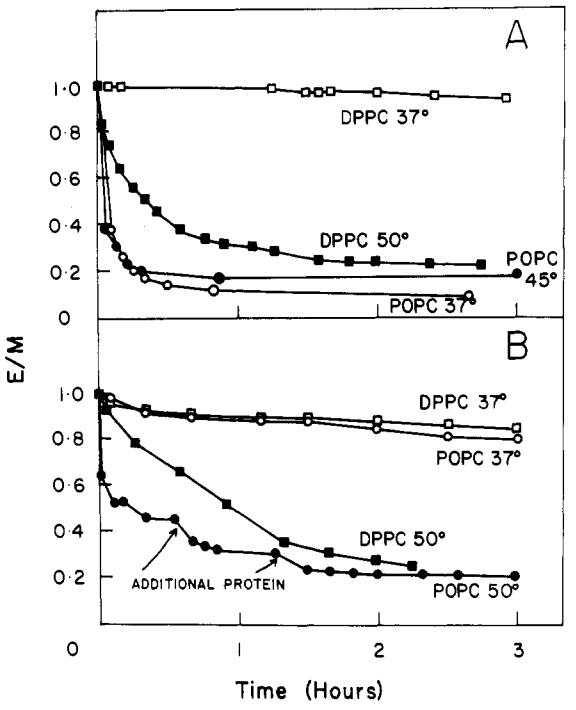

FIGURE 3: (A) Excimer-to-monomer ratio as a function of time in the presence of transfer protein for POPC/PyrGlcCer (93:7) small unilamellar vesicle donors with POPC small unilamellar acceptors at 37 (○) and 50 °C (●) and DPPC large unilamellar vesicle acceptors at 37 (□) and 50 °C (■). POPC T_m = −5 °C, and DPPC T_m = 41 °C. Each cuvette contaned 0.8 μmol of donors, 20 μmol of acceptors, and 0.4 mg of glycolipid transfer protein. The designation on each curve is the matrix lipid of the acceptor followed by the temperature of the experiment. (B) Same as in (A) but using DPPC small unilamellar donor vesicles with POPC small unilamellar vesicles as acceptors at 37 (○) and 50 °C (●) and DPPC large unilamellar vesicles as acceptors at 37 (□) and 50 °C (■).

41 °C, is in the gel state at 37 °C and is liquid crystalline at 50 °C. The donors used to obtain the data in Figure 3A were small unilamellar vesicles comprised of POPC and were thus liquid crystalline at both temperatures. The upper curve shows that when the acceptors were large unilamellar vesicles comprised of DPPC at 37 °C, and hence in the gel state, the transfer rate was very slow. However, when the acceptors were DPPC large unilamellar vesicles at 50 °C or POPC small unilamellar vesicles at either 37 or 50 °C, protein-mediated transfer was rapid. Under the conditions of the assay, $t_{1/2}$ for transfer (calculated after converting E/M data to percent

transfer) to POPC small unilamellar vesicles at 37 °C was 17 min and at 45 °C was about 6 min. The $t_{1/2}$ for transfer to large unilamellar vesicles at 50 °C was about 27 min. The data in Figure 3A clearly show that protein-mediated transfer of glucosylceramide is rapid if both donor and acceptor matrix lipids are in the liquid-crystalline state. However, if the acceptor lipid is gel state, the transfer rate is slow.

The donors used to obtain the data in Figure 3B were small unilamellar vesicles made of DPPC, and the acceptors were either small unilamellar vesicles comprised of POPC or large unilamellar DPPC vesicles. The two upper curves in Figure 3B show that very little transfer occurred at 37 °C when the donor vesicles were in the gel state. The estimated half-times for protein-mediated transfer under the assay conditions are greater than 15 h. The two lower curves in Figure 3B show that rapid transfer occurred at 50 °C when both donor and acceptor matrix lipids were liquid crystalline. The $t_{1/2}$ is about 17 min when the acceptors were small unilamellar POPC vesicles and about 68 min when the acceptors were DPPC large unilamellar vesicles. The lowest curve in Figure 3B shows a small change in the transfer time course when fresh transfer protein was added after 30 and 70 min. This suggests that the transfer protein added initially may have lost some activity with time during incubation at 50 °C. The data in both A and B of Figure 3 show the transfer half-times may be larger when the acceptors are large unilamellar vesicles than when the acceptors are small unilamellar vesicles. This difference could be due to an effect of the vesicle radius of curvature on transfer rate which would be in agreement with observations reported for the specific phospholipid transfer protein (DiCorleto & Zilversmit, 1977; Wirtz et al., 1979; Machida & Ohnishi, 1980).

The incubation mixtures from the experiments in Figure 3 were fractionated on Sepharose CL-2B to ascertain whether or not fusion between the donor and acceptor vesicles had occurred (data now shown). Negligible (less than 2%) phospholipid and glycolipid eluted in the void volume, indicating that changes in the excimer-to-monomer intensity ratio could not be due to vesicle fusion. In addition, after transfer from small unilamellar donors to large unilamellar acceptors (acceptor/donors = 25), the relative amount of PyrGlcCer associated with the fractionated donor and acceptor vesicles was determined. By solubilization of fraction aliquots in tetrahydrofuran and measurement of the monomer emission intensity at 378 nm, it was determined that approximately two-thirds of the PyrGlcCer coeluted with the large vesicle peak fractions, and one-third remained with the small unilamellar vesicles (data not shown). Therefore, this control experiment demonstrated that the excimer-to-monomer intensity changes, noted in Figure 3, were due to transfer to PyrGlcCer from donor to acceptor vesicles and were not simply the result of lateral rearrangement of pyrene probe within the plane of the donor membranes.

The results obtained with PyrGlcCer were augmented by transfer experiments performed with [^{3}H]GlcCer. The data shown in Figure 4 were obtained by using a different experimental assay system (compared to Figure 3 assay system) and by employing [^{3}H]GlcCer, which is nearly identical with the natural biological molecule. Both the donors and the acceptors were small sonicated vesicles. The donors contained 5 mol % [^{3}H]GlcCer in either POPC (Figure 4A) or DPPC (Figure 4B). The upper curves show that when the acceptors were POPC vesicles in the liquid-crystalline state, approximately 62% or roughly two-thirds of the [^{3}H]GlcCer was transferred in 1.5–2 h and no further transfer occurred for up to 5 h. This

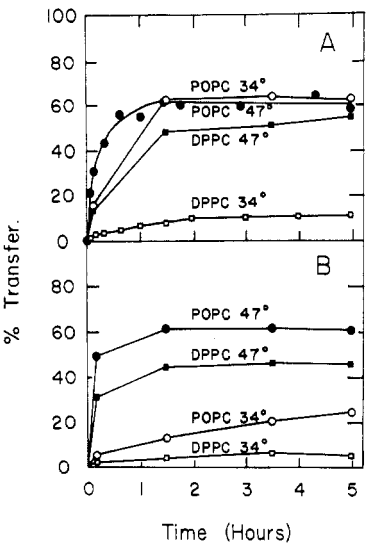

FIGURE 4: (A) Time course of protein-mediated transfer of [^{3}H]GlcCer from POPC small unilamellar donor vesicles containing 10 mol % dipalmitoylphosphatidic acid. The acceptors were small unilamellar vesicles either comprised of POPC at 34 (○) and 47 °C (●) or comprised of DPPC at 34 (□) and 47 °C (■). Mole ratio of [^{3}H]-GlcCer/POPC = 5/95. At each time point, incubation aliquots containing 1 μmol of donors, 25 μmol of acceptors, and 0.5 mg of glycolipid transfer protein were separated on DEAE-Sephacel minicolumns as described under Materials and Methods. The designation on each curve is the matrix lipid of the acceptor followed by the temperature of the experiment. (B) Same as in (A) except that the donor matrix lipid was DPPC in small unilamellar vesicles containing 10 mol % dipalmitoylphosphatidic acid. The acceptors were small unilamellar vesicles either comprised of POPC at 34 (○) and 47 °C (●) or comprised of DPPC at 34 (□) and 47 °C (■).

result is consistent with nearly equal relative concentrations of glucocerebroside in the inner and outer monolayers of the bilayers of small unilamellar vesicles, rapid transfer from the outer monolayer of the donor vesicles to acceptor vesicles, and no measureable glucocerebroside flip-flop from the inner to the outer monolayer of the donor vesicles over a 5-h period. Similiar results were obtained in an electron spin resonance experiment reported by Sharom & Grant (1977), who incorporated a TEMPO derivative of the glycolipid, GM_1, into small sonicated egg phosphatidylcholine vesicles and measured the accessibility of probe to ascorbate reduction.

Glucocerebroside transferred rapidly into DPPC acceptors at 47 °C (Figure 4A,B). The total amount transferred after 5 h was slightly less (50–55%) than with POPC acceptors. However, when either the donor or acceptor matrix phospholipid was in the gel state, glucocerebroside transfer was markedly decreased (5–20% after 5 h).

Phospholipid transfer activity has been reported in bovine brain (Helmkamp et al., 1974, 1976, 1980; Demel et al., 1977, 1982; Zborowski & Demel, 1982; Harvey et al., 1973) and might be present in the glycolipid transfer protein preparation. A test for phospholipid transfer activity was made by using small unilamellar donor vesicles containing PyrPC in POPC (10:90) and is shown in Figure 5. Small unilamellar acceptor vesicles were made of POPC. The glycolipid transfer protein did not significantly accelerate the transfer of phospholipid. After 3 h, approximately 5% PyrPC had departed from the donors. The estimated half-time for this departure was 30 h, which is close to the spontaneous transfer half-time (27 h) for PyrPC between DMPC vesicles (Roseman & Thompson, 1980).

One possible explanation for the observation that glycolipid transfer occurs more readily above the gel–liquid-crystalline phase transition temperature of the donor vesicle matrix

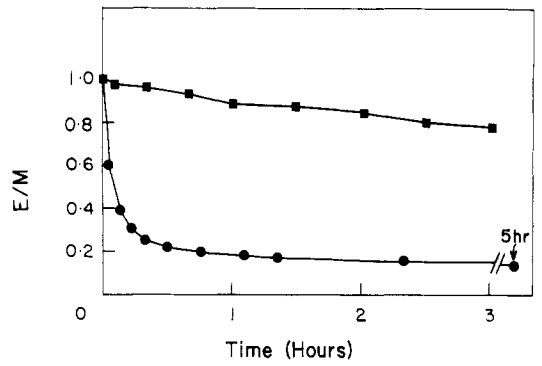

FIGURE 5: Excimer-to-monomer intensity ratio as a function of time for POPC/PyrPC (90:10) small unilamellar vesicle donors and POPC small unilamellar vesicle acceptors upon addition of either the glycolipid transfer protein (0.4 mg) from bovine brain (■) or the specific phospholipid transfer protein (0.4 mg) from bovine liver (●). Incubation at 37 °C. Acceptor/donor = 25.

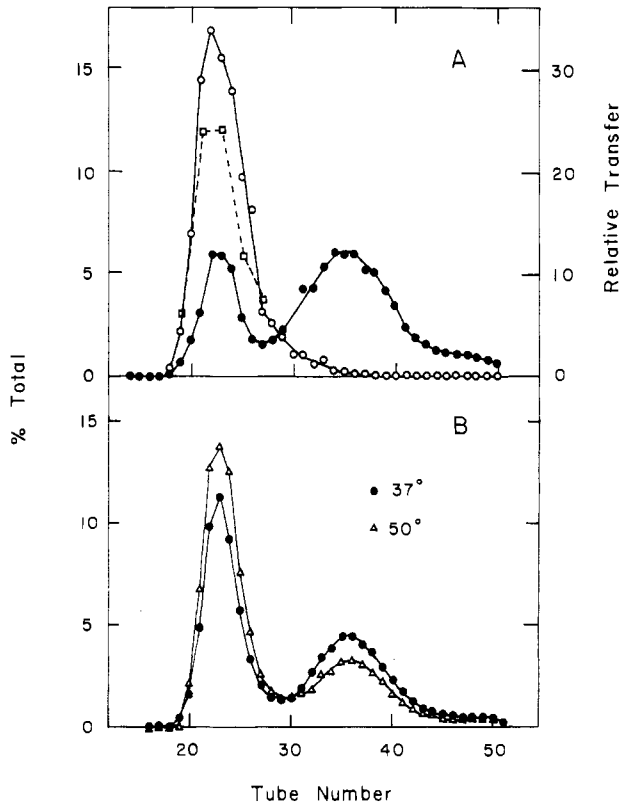

FIGURE 6: (A) Sephadex G-75 column (0.7 × 29 cm) profile of glycolipid transfer protein incubated with POPC/[^{3}H]GlcCer (90:10) small unilamellar vesicles for 2 h at 37 °C (fraction size = 0.25 mL). The [^{3}H]GlcCer profile (○) and the protein profile (●) are shown. The ratio of glycolipid to protein was approximately 20/1 (using the protein molecular weight = 18 200). The glycolipid transfer activity (□) was determined by use of the BrPC centrifugation assay described under Materials and Methods. The activity was normalized to 100% acceptor vesicle recovery. (B) Sephadex G-75 column profile of glycolipid transfer protein after incubation with DPPC/GlcCer (90:10) small unilamellar vesicles for 2 h at 37 (●) or 50 °C (▵). Fraction size = 0.25 mL. The ratio of glycolipid to protein was 30:1.

phospholipid is that protein association with vesicles containing glycolipid is phase state dependent. Figure 6A shows a Sephadex G-75 column elution profile of POPC/[^{3}H]GlcCer (90:10) vesicles following incubation with glycolipid transfer protein for 2 h at 37 °C. All of the [^{3}H]GlcCer eluted with the vesicles in the void volume peak. No appreciable amount was associated with the free protein peak. Of the total protein applied to the column, 30–40% coeluted with the POPC/[^{3}H]GlcCer vesicles in the void volume, and the remainder

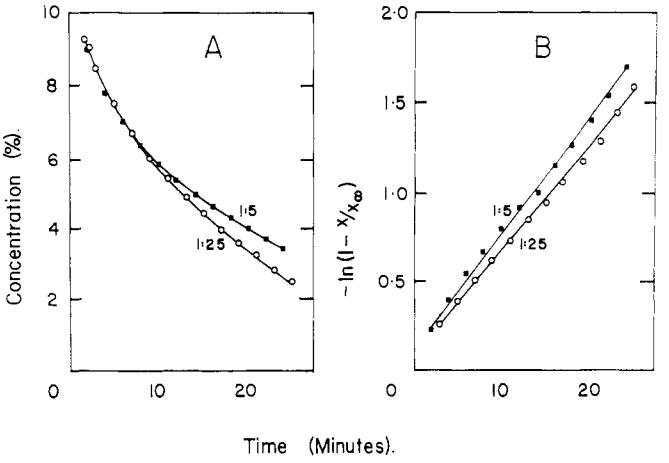

FIGURE 7: (A) Concentration of PyrGlcCer remaining in POPC small unilamellar donor vesicles as a function of time of incubation at 37 °C with glycolipid transfer protein and POPC small unilamellar acceptor vesicles at a donor-to-acceptor ratio of 1/5 (■) or 1/25 (○). Approximately 2.3 μg of protein and 5 nmol of PyrGlcCer were used. (B) Logarithmic plot of data in (A). X is the fractional transfer of label at time t, and X_∞ is the fractional transfer at infinite time.

of the protein was included in the column. In control incubations with vesicles containing no glycolipid, less than 10% of the total protein eluted with the vesicles peak (data not shown). Nearly identical elution profiles were obtained for vesicles, [^{3}H]GlcCer, and protein when similar experiments were performed at 50 °C rather than 37 °C (data not shown). When the fractions (Figure 6A) with the POPC/[^{3}H]GlcCer vesicles and the bound protein were assayed for activity by incubation with BrPC/POPC acceptor vesicles at 37 °C, rapid glycolipid transfer occurred. The activity profile in Figure 6A (□) shows that the levels of transfer are proportional to the amounts of bound protein and [^{3}H]GlcCer found in each fraction.

In order to test whether binding to vesicles containing glucocerebroside was diminished in the presence of a gel-state matrix, the transfer protein was incubated for 2 with DPPC/GlcCer (90:10) small sonicated vesicles at either 37 or 50 °C and then fractionated by Sephadex G-75 column chromatography. Figure 6B shows the amount of protein that is bound to the DPPC/[^{3}H]GlcCer vesicles (void volume) as well as the amount of protein that is free (included volume). Only 10–15% more protein coelutes with the liquid-crystalline state vesicles (▵) than with the gel-state vesicles (●). Therefore, a change in the phase state of the matrix phospholipid only slightly alters the amount of protein binding to donor vesicles and cannot explain the marked acceleration in the rate of [^{3}H]GlcCer transfer observed when the matrix is in the liquid-crystalline state rather than the gel state (Figures 3 and 4).

Figure 7A shows the concentration of PyrGlcCer remaining in POPC donor vesicles as a function of time of incubation with glycolipid transfer protein when the donor-to-acceptor vesicle ratio was either 1/5 (■) or 1/25 (○). The kinetic data were linearized by using eq 3 (McKay, 1938; McLean & Phillips, 1981). Here k is the pseudo-first-order rate constant, a is the acceptor concentration, b is the donor concentration, x is the fractional transfer at time t, and x_∞ is the fractional transfer at infinite time. The linearized plots are shown in Figure 7B.

$$-kt = [a/(a + b)] \ln (1 - x/x_\infty) \quad (3)$$

True first-order and second-order kinetic processes can be differentiated by varying the concentration of acceptors and

calculating the rate constant k by using eq 3. For a second-order process, k is directly proportional to the acceptor vesicle concentration, while for a true first-order process, k is independent of acceptor vesicle concentration. When the donor-to-acceptor ratio is 1/5, k equals 0.0538 min^{-1}; when the donor-to acceptor ratio is 1/25, k equals 0.0553 min^{-1}. Therefore, when the donor and acceptor matrix phospholipids are POPC in the liquid-crystalline state, the transfer of glucocerebroside by the transfer protein is a true first-order process.

Discussion

Bovine brain contains a protein with a molecular weight of about 18 200 which rapidly transfers glucocerebroside between phospholipid vesicles in the liquid-crystalline state. In contrast, the spontaneous transfer of this glucosphingolipid between similar phospholipid systems under the same conditions is a very slow process; Correa-Freire and co-workers (1982) have shown the half-time of transfer at 37 °C between dimyristoylphosphatidylcholine vesicles to be in excess of 30 days. Data presented in Figure 7 show the transfer protein mediated process to be kinetically first order and thus to be limited by the off-rate of the glycolipid. This result rules out the possibility that transfer occurs when donor and acceptor vesicles collide.

The data in Figures 3 and 4 show that rapid intervesicular transfer of glucosylceramide by the transfer protein occurs only when both donor and acceptor matrix lipids are in the liquid-crystalline state. An analogous dependence of the rate of transfer on the phase state of the donor and acceptor vesicles has also been noted for the phosphatidylcholine-specific transfer proteins from bovine liver (Jackson et al., 1979, 1980; Kasper & Helmkamp, 1981; Welti & Helmkamp, 1984). In the case of the glycosphingolipid transfer protein, the data in Figure 6A show that the binding of the protein to vesicles occurs to essentially the same extent both above and below the gel–liquid-crystalline phase transition temperature, T_m. Decreased transfer observed when either donor or acceptor vesicles are in the gel state cannot, in this case, be due to a decrease in the binding constant of transfer protein to gel-state matrix lipid.

Several mechanisms are possible by which the glycolipid transfer protein can enhance the rate of intervesicular glycolipid transfer. Each mechanism requires that the transfer protein bind transiently to the donor bilayer. Once bound the protein might (i) increase the off-rate of the glycolipid molecule by perturbing locally the structure of the bilayer or (ii) bind glycolipid and leave the bilayer as a glycolipid–protein complex. The apparent off-rate of the glycolipid would then be that of the complex. Once in the aqueous phase, the complex could either dissociate and the glycolipid travel as a free molecule to the acceptor bilayer or the complex could persist and deliver the glycolipid to the acceptor by interaction with it. With either mechanism (i) or mechanism (ii), the low rate of transfer observed with the acceptor bilayer in the gel state can only be explained by a low on-rate for glycolipid at the acceptor bilayer.

The dependence of transfer rate on the phase state of the donor is more complex. Work from this laboratory has suggested that neutral glycosphingolipids, and in particular glucosylceramide, are present in liquid-crystalline phospholipid bilayers and some biological membranes as small dispersed gel-like domains (Correa-Freire et al., 1979, 1982; Tillack et al., 1982, 1983). The gel-like state of these small domains has been used to explain the very slow rate of spontaneous interbilayer transfer of glucosylceramide (Correa-Freire et al., 1982). Assuming this to be correct, then either mechanism (i) or mechanism (ii) requires that the transfer protein interact with the vesicle in such a way as to disrupt this phase structure locally. If this disruption is taken to be roughly equivalent to melting the small gel-like domain containing glucosylceramide, then each small domain would be expected to increase in surface area by about 30% (Lichtenberg et al., 1982; Tardieu et al., 1973). With the surrounding matrix lipid in the liquid-crystalline state this increase in area of the small domains could be accommodated because of the fluid character of the matrix. However, if the matrix surrounding the small domains of glucosylceramide is a rigid gel phase, minimal accommodation would be possible. In effect the gel-state matrix would then act as a constant area container for each isolated domain of glycolipid. The net effect would be that, although the transfer protein could bind to the glucosylceramide domains, it could not increase the rate of transfer.

The actual physiological role of this particular glycosphingolipid transfer protein remains to be established. At the present time, the intracellular localization is not known. If its localization is in the cytoplasm, it is difficult to understand how the transfer protein functions since the site of glycosphingolipid biosynthesis is within the cisternal space of the Golgi–endoplasmic reticulum complex while the ultimate destination of newly synthesized glycosphingolipids is the external surface of the plasma membrane. On the other hand, if the glycolipid transfer protein localization is within the lumen of the Golgi–endoplasmic reticulum complex, then its transfer function within this space is reasonable. Alternatively, these transfer proteins may be activator proteins of the type found within lysosomes (Berent & Radin, 1982; Li & Li, 1982). The function of intralysosomal activators is to facilitate the interaction between soluble glycosidases and their insoluble lipid substrates (Sandhoff & Conzelmann, 1979; Conzelmann et al., 1982a). It is believed that this facilitation is accomplished by the formation of a lipid–activator complex which is the true substrate for the glycosidase. In fact, recent studies by Sandhoff and co-workers (Conzelmann et al., 1982b) indicate that a 20 000-dalton protein, which activates lysosomal hexosaminidase A, also can function as a glycolipid transfer protein under certain conditions.

The use of the glycolipid transfer protein as a tool for preparing small unilamellar vesicles containing glycosphingolipids only in the outer bilayer surface will be reported in a subsequent communication.

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Registry No. DPPC, 2644-64-6; POPC, 6753-55-5; dipalmitoylphosphatidic acid, 19698-29-4.

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