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PREPARATION OF ASYMMETRIC, CEREBROSIDE SULFATE-CONTAINING PHOSPHOLIPID VESICLES

BENVENUTO CESTARO [a], ELVIRA PISTOLESI [a], NORBERT HERSHKOWITZ [b] and SHIMON GÁTT [c]

[a] *Department of Biological Chemistry, Faculty of Medicine, University of Milan (Italy),* [b] *Neurochemistry Division, Childrens Hospital, University of Bern (Switzerland), and* [c] *Laboratory of Neurochemistry, Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem (Israel)*

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A procedure is described which inserts asymmetrically cerebroside sulfate ('sulfatide') into the outer leaflet of bilayered phospholipid vesicles. Cerebroside sulfate is adsorbed onto a cellulose, filter-paper support and, when incubated with phosphatidylcholine vesicles is transferred to and inserted into the outer leaflet of these vesicles. This transfer occurs at, or above the transition temperature of the phospholipid and follows a similar pattern with small or larger ('fused') unilamellar vesicles. The transfer is linear with time for 1–2 h and is maximal after about 6 h, when the sulfatide content reaches about 6 mol% of the total quantity of phospholipid, corresponding to about 10 mol% of the phospholipids present in the outer layer. Initial rates of sulfatide transfer were somewhat increased when the vesicles contained a positively charged lipid (e.g. stearylamine) and decreased when this lipid was negatively charged (e.g. dicetyl phosphate) or hydrophobic (e.g. cholesterol). Divalent ions markedly inhibited sulfatide transfer and monovalent ions did so to a lesser degree. Once incorporated into the outer leaflet of the vesicle, the sulfatide could not be removed by washing with buffer, 1 M NaCl or 1 M urea.

Introduction

Cell surface glycolipids are embedded in the biomembrane architecture formed by proteins, phospholipids and cholesterol. Though their precise biological role has not been fully clarified, it has been shown that they serve as receptors for various toxins, viruses, hormones, neurotransmitters [1–5]. It has also been suggested that their pattern is changed in neoplasia [4,6,7].

Ceramide galactose 3-sulfate ('sulfatide') is an acidic glycolipid which is present, in relative abundance in nerve membranes where it might bind to and influence the conformation of myelin basic protein [8]. Because of its pronounced acidic nature the sulfatide can combine with inorganic cations or organic amines [9], thereby assisting in maintenance of the electrical neutrality of neuronal membranes. It may also play a function in the transport of Na^+ and K^+ [10]. It has been suggested though not fully proved that cerebroside sulfate is an integral component of an opiate and β-endorphin receptor in mammalian brain [11,12]. An important localization characteristic of myelin glycolipids (e.g. ceramide galactose and ceramide

Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

galactose 3-sulfate) is their asymmetric distribution in myelin lamellae [13,14], being present only on the external surface of the membrane, thereby mimicking the common features of membrane glycoconjugates [15,16].

In general, studies on the postulated role of membrane glycolipids were facilitated by modifying the existing glycocalix through incorporation of exogenous glycolipids.

Two complementary lines of investigation have been followed, one used biological membranes [17,18], while the other was based on studies of experimental systems which employed liposomal dispersions [19,20]. In this paper we report preparation of small or fused unilamellar vesicles of several phosphatidylcholines containing sulfatide incorporated into the outer surface only of the bilayered lipids. This was achieved by spontaneous transfer of the sulfatide from a cellulose-support to the preformed bilayered lipid vesicles. This compositionally asymmetric bilayer system is analogous to the bilayered plasmamembrane of the mammalian cell. We hope that the present study of the parameters governing this spontaneous glycolipid transfer from the cellulose-support to phosphatidylcholine vesicles will provide a guideline for future modification of the external surface of cell membranes by incorporation of exogenous sulfatide.

Materials and Methods

Analytical grade chemicals, distilled solvents and doubly-distilled water were used; dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), egg phosphatidylcholine (egg PC), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphatidylethanolamine (DMPE), egg phosphatidylethanolamine (egg PE) and DNA (type 1) were purchased from Sigma Chem. Co.; acridine orange from Merck; Sepharose 4B and Sepharose CL-2B from Pharmacia; cellulose support (filter paper No. 580) from Schleicher and Schuell; liquid scintillation counting mixture (Instagel) from Packard. Radioactivity measurement were done on a Packard 3385 scintillation spectrometer.

Preparation of substrates. Cerebroside sulfate (sulfatide) was obtained from Supelco (Lot. No. LA-00826). It was further purified on a Florisil column according to Radin [21], and converted to the sodium salt by the procedure of Abramson et al. [22]. The final purity was checked by high performance thin-layer chromatography in chloroform/methanol/water (70:30:4, v/v) [21]. The plates were developed with iodine vapors or by spraying with the lipid reagent of Rauser et al. [23], or with α-naphthol for detecting sugars [24]. All sulfatide preparations showed a single spot with no detectable lipid contaminants.

Radioactive sulfatide [^{14}C]stearoylsulfogalactosylsphingosine was prepared by coupling sphingosylgalactosylsulfate and [^{14}C]stearoyl chloride [25] and had a specific radioactivity of 51 μCi/μmol.

Preparation of small unilamellar vesicle. Chloroform-methanol solutions of DPPC, DMPC, egg PC, with or without sulfatide were evaporated under nitrogen and further dried in high vacuum for 24 h. The dried lipids were suspended in 50 mM Tris-HCl, pH 7.0 (lipid concentration 50 to 60 mM). This suspension was subjected to sonic irradiation in an MSE PG 1116 apparatus at 45–50°C under nitrogen using a microprobe. The dispersion was centrifuged according to Barenholz et al. [26]. The supernatant, which was carefully pipetted off the pelleted titanium and residual multilamellar vesicles, was diluted with the same buffer to a final lipid concentration of 2 μmol/ml and represented the small unilamellar vesicles. Negative-staining electron microscopy indicated that this preparation contained vesicles having a diameter of 200 ± 50 Å.

Preparation of dipalmitoylphosphatidylcholine fused unilamellar vesicles. Unilamellar vesicles ranging in size from 500 to 1000 Å in diameter can be prepared from saturated diacylphosphatidylcholine by spontaneous fusion of small sonicated vesicles below the gel-liquid crystalline phase -transition [27]. A solution of small unilamellar vesicles of dipalmitoylphosphatidylcholine (lipid concentration 50 mM) in Tris-HCl 50 mM, pH 7.0, prepared as described above was incubated at 25°C for 48 h. The dispersion (which became progressively milky white) was applied to a Sepharose CL-2B column (15 × 40 cm) and eluted with 50 mM Tris-HCl, pH 7.0. Two lipid peaks were obtained: the first corresponding approximately to

the void volume (as determined with DNA), the second was somewhat retarded. The fractions of the first peak (about 50% of the total lipid) were pooled, diluted with Tris buffer to a final lipid concentration of 2 μmol/ml, and represented the fused unilamellar vesicles. Negative-staining microscopy indicated that this preparation contained vesicles with a diameter of about 700 ± 150 Å.

Preparation of sulfatide adsorbed onto cellulose. 25 μl of a solution of [^{14}C]sulfatide (containing 0.2 μCi in 0.25 μmol sulfatide) in chloroform/methanol (2:1, v/v) were pipetted onto 15 mg of a dry cellulose support (filter paper No. 580 from Schleicher and Schuell); the solvent was evaporated under nitrogen and the cellulose, now containing the adsorbed sulfatides was further dried in high vacuum for 1 h. For incubation with vesicles (see below) the filter paper was cut to small pieces or, alternatively rolled and introduced into the test tube without cutting.

Preparation of micellar sulfatide. A solution of [^{14}C]sulfatide (250 nmol, 0.2 μCi) in chloroform/methanol 2:1, v/v) was evaporated in vacuo in a round bottom flask and further dried in high vacuum. The lipid was dispersed in 0.5 ml of Tris-HCl, 50 mM, pH 7.0 and this, somewhat opalescent dispersion was transferred to a test tube and subjected to sonic irradiation under nitrogen until clear. This dispersion was prepared immediately before use.

Incubation of vesicles with sulfatide, adsorbed onto cellulose. 0.5 ml of a suspension of vesicles, containing 1 μmol of phospholipid and 0.25 μmol (0.2 μCi) of cellulose-adsorbed sulfatide were incubated in a shaking incubator as described in the respective experiments (see Results). In parallel, cellulose-adsorbed sulfatide was incubated in the same buffer solution in the absence of vesicles. At the end of incubation the tube was centrifuged at 5000 rev./min for 10 min and the supernatant, containing the vesicle-associated sulfatide was applied to a 1.5 × 40 cm Sepharose 4B column, previously equilibrated with 50 mM Tris-HCl, pH 7.0; this buffer was also used as the eluting solvent. The elution rate was 0.5 ml/min and 1 ml fractions were collected. The columns were maintained at the constant temperature (identical with that used during the incubation). The phospholipid recoveries of fused or small unilamellar vesicles were always high (between 95% and 100% of the total lipids) and were independent of the amount of vesicle-associated sulfatide. The void volume was estimated using a solution of DNA (mol. wt. 10^7).

Trinitrobenzene sulfonic acid. For determining the distribution of lipid in the two respective layers of the vesicle a mixture of phosphatidylcholine and phosphatidylethanolamine, mol ratio 9:1 was used. The total phosphatidylethanolamine of these vesicles as well as that part which resides in the outer leaflet were estimated with the non-penetrating reagent trinitrobenzenesulfonic acid (TNBS) according to the method of Barenholz et al. [26].

Determination of the sulfatide content in the outer layer of vesicles using acridine orange. Massari et al. [28] showed that negatively-charged lipids quench the absorbance of acridine orange by forming dimers of this dye. This procedure was used to estimate the content of sulfatide in the outer leaflet relative to the total content of this lipid in the vesicle. For this purpose a dispersion of vesicles was added to a solution of acridine orange and the decrease in absorbance, caused by interaction of the dye with sulfatide of the outer leaflet was recorded. This dispersion was subjected to sonic irradiation, now permitting interaction of the dye with the total sulfatide content and the decrease in absorbance was again recorded.

Differential thermical analysis. All calorimetric scans were measured with a DTA Mettler TA-200 equipped with 500 μl high pressure cells. Calorimetric and thermometric calibrations were done using the transition of indium sulfate or *p*-nitrotoluene as standards.

Fused unilamellar vesicles (5μmol/500 μl) by themselves or having 6 mol% of sulfatide, in 50 mM Tris-HCl, pH 7.0, were scanned from 20 to 70°C at a rate of 30 K/h. The enthalpy (ΔH) for each transition was determined by calculating the area of the calorimetric peak with the help of a numeric integration computer program.

Other methods. The concentration of vesicle phospholipids was determined according to the method of Bartlett [29].

The sulfatide concentration of each vesicle preparation was monitored by radioactive determination and/or by the azur A method of Kean [30].

Results

Transfer of sulfatide from cellulose support to phosphatidylcholine vesicles

Fig. 1 shows gel filtration profiles of fused and small, unilamellar vesicles of dipalmitoylphosphatidylcholine, in the absence and presence of sulfatide. Sepharose 4B, which has an exclusion limit of $4 \cdot 10^6$ daltons was used for this purpose. Fused unilamellar vesicles were eluted in the void volume while the small unilamellar vesicles were somewhat retarded. In contrast, an aqueous dispersion of sulfatide was considerably retarded on the column and was eluted in two asymmetric peaks, suggesting that these lipids are present as aggregates having a smaller diameter than that of fused or small unilamellar vesicles. This evidence confirms previous data on the behaviour of sulfatide in aqueous solutions, already reported as containing micelles [31,32] whose molecular weight of about 180 000 corresponds to an aggregation number of about 180 [33]; the double peak of the elution profile suggests that micellar aggregates were also present as reported by Jeffrey et al. [33]. When sulfatide (250 nmol) was adsorbed onto cellulose filter paper and incubated with DPPC vesicles, a portion dissociated from the solid cellulose support and was transferred to the vesicles. In the experiment shown in Fig. 1, 65 ± 5 and 60 ± 5 nmol of sulfatide co-eluted with fused (Fig. 1B) or small unilamellar vesicles (Fig. 1C), respectively, suggesting the presence of vesicles now containing 6.5 ± 0.5 and 6.0 ± 0.5 mol% sulfatide associated with these respective two types of DPPC vesicles. The data of Fig. 1 show that sulfatide micelles were entirely absent. Despite their different fatty acid composition, labelled and unlabelled sulfatides behave similarly in transfer from donor to acceptor: the amount of vesicle-associated sulfatide measured by ^{14}C-radioactive counts is always the same as that calculated with the azur A method used as control.

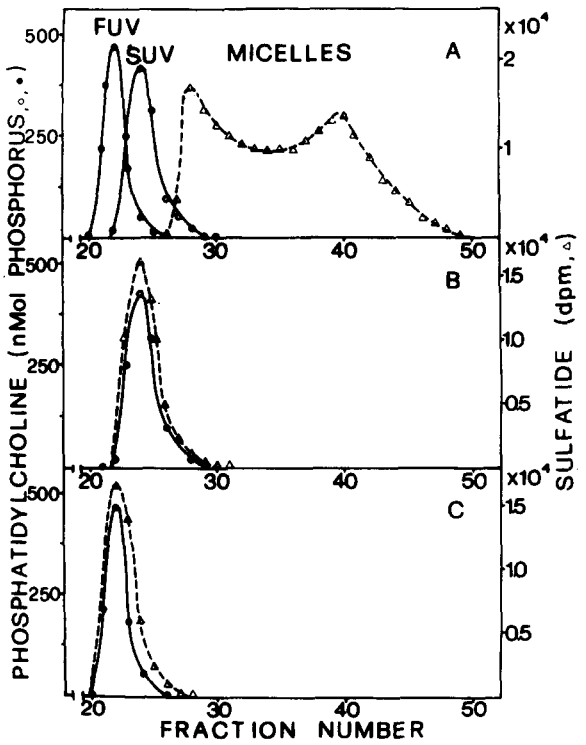

Fig. 1. Elution profiles of small and fused DPPC vesicles and of sulfatide micelles. (A) 0.5 ml, each of small, unilamellar DPPC vesicles (SUV), fused unilamellar DPPC vesicles (FUV) (1 μmol phosphorus) or a micellar dispersion of [^{14}C]cerebroside sulfate (0.250 μmol, 0.2 μCi in 50 mM Tris-HCl, pH 7.0) were applied to a column of Sepharose 4B (40×1.5 cm) and eluted with the same buffer. 1-ml fractions were collected and their phosphorus content and radioactivity estimated. (B) Sulfatide (0.250 μmol, 0.2 μCi) was adsorbed onto 15 mg of cellulose filter paper. The latter was incubated with 0.5 ml of small, unilamellar vesicles of DPPC (1 μmol phosphorus) for 2 h at 45°C. The tube was centrifuged for 5 min at 3000 rev./min and the supernatant applied to a column of Sepharose 4B and eluted as described in the legend to Fig. 1A. (C) The experiment of Fig. 1B was repeated now using fused unilamellar vesicles of DPPC.

The residual filter paper was washed with buffer and extracted with chloroform-methanol. Phospholipids were not detected in this extract, thereby suggesting a unidirectional transfer of sulfatide from the cellulose to the vesicles. Control experiments showed that cellulose-adsorbed sulfatide, incubated in the absence of phospholipid vesicles released but a negligible portion (5 ± 1 nmol) of sulfatide to the aqueous phase.

Association of sulfatide with PC vesicles did not affect their integrity. This was assessed by using vesicles composed of a mixture of phosphatidylethanolamine and phosphatidylcholine, at a mol ratio of 1:9. In these sealed vesicles only 60% of the phosphatidylethanolamine interacts with trinitrobenzene-sulfonic acid [34,35]. This value did not change after incubating with cellulose-adsorbed sulfatide for 2–24 h at 45°C. The

integrity of these vesicles was further verified by electron microscopy, using negative staining with 2% ammonium molybdate. Presence of the sulfatide did not reveal any change in the size or shape of the vesicles.

Asymmetric distribution of sulfatide in the phospholipid vesicles

Massari et al. [36] showed that the absorbance of acridine orange decreased upon association with negatively-charged lipids present in bilayer vesicles; this is caused by formation of dye dimers bound to paired negative lipids occupying nearby positions, not exceeding 9–10 Å.

Since acridine orange does not penetrate closed vesicles and the different curvature of the internal with respect to the external layer does not influence the pairing of negative lipids [28], the external/total ratio of negatively-charged lipids in vesicles can be calculated by dividing the amount of dye dimers before and after cosonication of acridine orange with vesicles. This procedure was utilized to ascertain that the sulfatide, transferred from the cellulose to the vesicles was indeed distributed asymmetrically.

When a mixture of sulfatide and DPPC (mol ratio 1:9) was subjected to sonic irradiation, resulting in a symmetric distribution of the sulfatide in the two respective leaflets, 30 ± 2% of the sulfatide did not react with dye as expected in vesicle whose diameter is about 200 Å; moreover this percent distribution compares well with previously reported data for phosphatidic acid, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol [28], phosphatidylethanolamine [34] and gangliosides [35].

In contrast, the sulfatide which was transferred from the cellulose support to the fused or small unilamellar vesicles was fully accessible to interaction with acridine orange. This provided further evidences that sulfatide was indeed asymmetrically distributed and associated only with the outer leaflet of the lipid bilayer. Even after further incubation of the sulfatide-associated vesicles for 24 h at 45°C, the entire sulfatide still interacted with the dye, suggesting the absence of a transbilayer migration (flip-flop) from the outer to the inner leaflet.

Thermotropic behavior of sulfatide-associated fused unilamellar vesicles

Fused unilamellar vesicles of dipalmitoylphosphatidylcholine showed a transition temperature (T_m) of 41.2 ± 0.2°C and an enthalpy (ΔH) of 8.4 ± 0.2 kcal/mol. This compares well with previously reported data for multilamellar DPPC-liposomes [37,38]. Asymmetric incorporation of 6.5 mol% sulfatide resulted in a negligible (0.2°C) decrease in the T_m but a 20% change of ΔH to 6.7 ± 0.2 kcal/mol.

Effect of temperature on the rate of transfer of sulfatide to DPPC vesicles

Unilamellar vesicles of egg PC, as well as DMPC and DPPC were prepared and incubated at varying temperature with sulfatide, adsorbed onto the cellulose filter paper.

Fig. 2 shows that the transfer of sulfatide to PC vesicles is related to the transition temperature of the respective lipid. Thus, sulfatide incorporation into DMPC and DPPC vesicles was negligible at temperatures less than about 23 and 37°C with

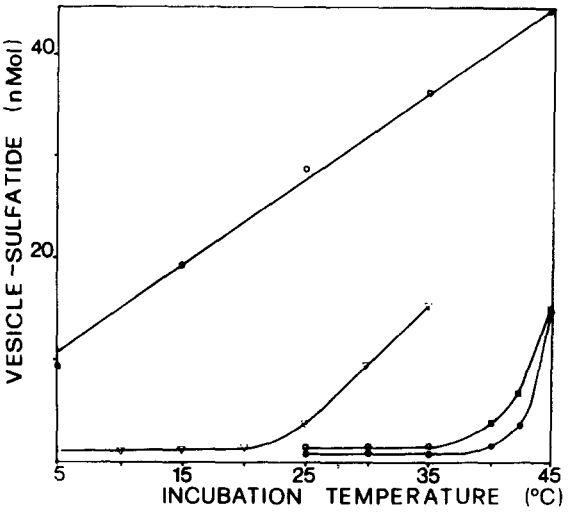

Fig. 2. Effect of incubation temperature on the transfer of cellulose-adsorbed sulfatide to PC vesicles. 0.5-ml fractions (containing 1 μmol phosphorus) of vesicles, composed of egg PC (○), small, unilamellar vesicles of DPMC (▽), and unilamellar vesicles of DPPC (□, small; ●, fused), each in 50 mM Tris-HCl, pH 7.0, were mixed with sulfatide (0.250 μmol, 0.2 μCi) adsorbed onto 15 mg of cellulose filter paper. After 30 min at the temperatures described in the figure, the tubes were centrifuged and the supernatants applied to Sepharose 4B as described in the legend to Fig. 1A.

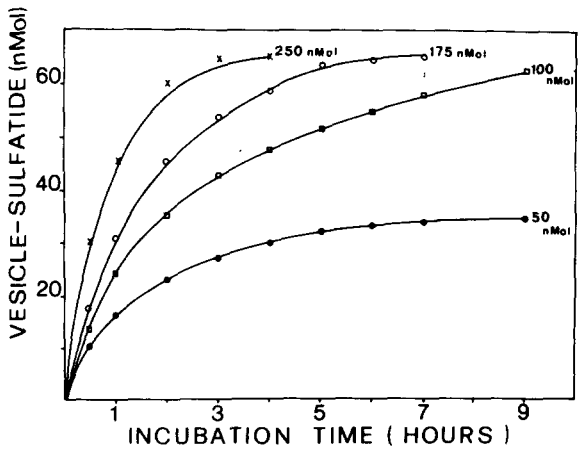

Fig. 3. Effect of incubation time and sulfatide concentration on the rates of its transfer to PC vesicles. 0.5-ml fractions of small unilamellar vesicles of egg PC, containing 1 μmol phosphorus in 50 mM Tris-HCl, pH 7.0 were incubated with 15 mg of cellulose filter paper containing varying quantities of sulfatides, as shown in the figure. After the respective incubation time, at 25°C, the tubes were centrifuged and supernatants applied to Sepharose 4B columns as described in the legend to Fig. 1A.

these respective two phospholipids. Above each of these respective temperatures, sulfatide transfer increased in a seemingly linear relation to the incubation temperature. In contrast, the transfer to unilamellar vesicles of egg PC was linear between 5 and 45°C.

Effect of incubation time and sulfatide concentration on its transfer to vesicles

Fig. 3 shows the results of an experiment in which the incubation time as well as the quantity of sulfatide adsorbed into the solid cellulose support were varied. The rate of transfer was linear for 1–2 h and maximal incorporation into the vesicles was obtained after incubating for about 6 h. The total quantity of sulfatide transferred to the vesicles (whose content was a fixed quantity of 1000 nmol PC) varied from about 75–80% when 50 nmol sulfatide were adsorbed onto the cellulose support to only 25% when 250 nmol sulfatide were used. This provides a final concentration of 4–6% sulfatide relative to the total phospholipid content of the vesicle or about 7–10% of the phospholipids in the outer layer.

Effect of lipids and ions on sulfatide transfer to PC vesicles

Incorporating a positively-charged lipid (octadecylamine, 5–10 mol%) into DPPC vesicles resulted in a small increase (about 10%) in the nmol sulfatide associated with these vesicles. In contrast, incorporation of a negatively charged lipid (dicetyl phosphate, 5–10 mol%) or a hydrophobic component (cholesterol, 10–30 mol%) decreased sulfatide incorporation by about 20%, each. Fig. 4 shows the effect of ions on the incorporation of sulfatide to PC vesicles. Calcium or magnesium chloride markedly inhibited sulfatide transfer while monovalent ions showed a lesser inhibitory effect. It is of interest that, while transfer of sulfatide was inhibited by sodium ions, once incorporated into the vesicle it could not be removed even by 1 M NaCl. The same was true when 1 M urea was used. This suggested that dissociation of either ionic or hydrogen bounds could not reverse the association of the sulfatide with the PC vesicle.

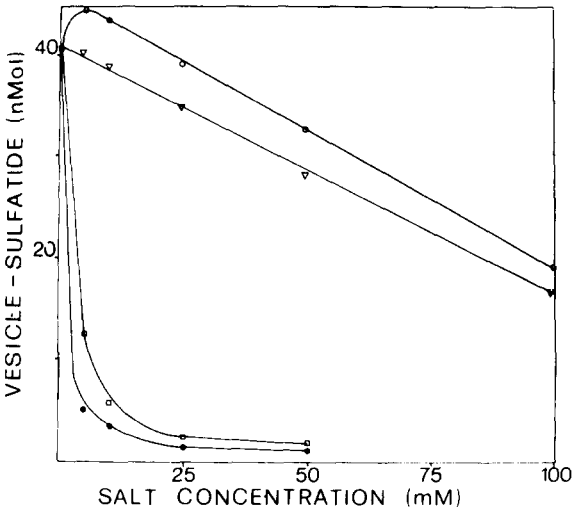

Fig. 4. Effect of salt concentration on the rates of transfer of sulfatide to PC vesicles. 0.5-ml fractions of small unilamellar vesicles of egg PC containing 1 μmol phosphorus in 50 mM Tris-HCl, pH 7.0 and added salts as shown in the figure, were incubated with sulfatide (0.250 μmol, 0.2 μCi) adsorbed onto 15 mg of cellulose filter paper. After 1 h at 25°C the tubes were centrifuged and the supernatant applied to Sepharose 4B columns as described in the legend to Fig. 1A. $MgCl_2$ (□); $CaCl_2$ (●); NaCl (○); KCl (▽).

Discussion

This paper describes a spontaneous transfer of cerebroside sulfate from a solid support (cellulose filter paper) to unilamellar vesicles of phosphatidylcholine. Once transferred, the sulfatide molecules are intimately associated with the vesicle and could not be removed by gel filtration, 1 M NaCl or 1 M urea. This, as well as changes in the enthalpy of the phase transition of DPPC vesicles and quenching of the absorbance of acridine orange suggest that the sulfatide molecules are indeed inserted into the outer leaflet of the bilayered-PC vesicles. Incorporation of the sulfatide did not affect the integrity of the vesicles. Sulfatide transfer was proportional to the time of incubation as well as sulfatide contents. Maximal incorporation, obtained after about 6 h was about 60–65 nmol sulfatide per μmol PC, namely about 10 mol% of the phospholipid content of the outer leaflet. It is possible that the negative charge, acquired by the vesicle once this concentration is reached prevents further incorporation of negatively-charged sulfatide molecules. Incorporation of sulfatide occurred above the transition temperature of the lipid, and was similar using small or large unilamellar vesicles. Since these have diameters of 200 and 700 Å, respectively, this suggests but little effect of the curvature of the liposomes on the rate of transfer of the sulfatide from the cellulose support to these vesicles. Sulfatide transfer and incorporation probably follows a two-stage course. First, the sulfatide molecules which are strongly charged, are torn away from the cellulose support, as monomers into the aqueous medium (probably by means of ionic interaction with the polar phospholipid heads protruding from the vesicles); then, these monomers are taken up and inserted into the outer layer of the vesicles. The latter is probably facilitated by hydrophobic interaction between the ceramide portion of the sulfatide and the diacylglycerol residues of PC. The involvement of the monomeric form of sulfatide in this transfer process is further supported by the following considerations: (1) cellulose-bound sulfatides are very likely to be monomers, having been dried from a chloroform-methanol solution (see Methods); (2) addition of micellar sulfatide rather than cellulose-bound sulfatide to vesicles gave rise to a sudden increase in turbidity followed by flocculation of lipid dispersion. Moreover, this lipid coagulation, made useless the following attempts to isolate unilamellar vesicles containing incorporated sulfatide.

Incorporation of 6.5 mol% sulfatide transferred from cellulose support, had but little effect on the transition temperature of the phospholipid of the vesicles but decreased the enthalpy of the phase transition. A similar observation was made by Di Silvestro et al. [38] who used symmetrically-distributed sulfatide-DPPC vesicles prepared by sonic irradiation. These data support the conclusion that the sulfatide molecules are indeed inserted into the lipid layer and become an integral component of the outer leaflet of the vesicle.

The technique here described provides a rapid and easy procedure for preparating asymmetric vesicles of sulfatide and lecithin in which the sulfatide molecules are located only in the outer layer. This produces vesicles which are electrically unbalanced and which might be useful for studies of transport as well trans-bilayer migration of the sulfatide ('flip-flop'). The asymmetric vesicles might be viewed as a model for biological membranes which contain components distributed asymmetrically between the two leaflets of the bilayer. Like sulfatides another negatively-charged sphingolipid class, the gangliosides are present only in the outer layer of biological membranes [15]. Felgner et al. have recently shown that ganglioside monomers can be inserted in the outer layer of phospholipid vesicles [20]. It is possible that these glycolipids (ie, ganglioside, sulfatide, etc.) are transferred to the biological membrane by a mechanism similar to that presented in this paper, namely, a transfer from a support (e.g. the Golgi membrane) to the acceptor biological membrane.

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