Nonspecific lipid transfer protein in the assay of a membrane-bound enzyme CMP-N-acetylneuraminic:lactosylceramide sialyltransferase

Hiroko Kadowaki, Lori A. Symanski, and Raymond S. Koff

Department of Medicine, Framingham Union Hospital, Framingham, MA 01701, and Division of Medicine and Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

Abstract  CMP-N-acetylneuraminic:lactosylceramide α-2, 3-sialyltransferase is tightly associated with the luminal side of the Golgi membrane as is its lipid substrate, lactosylceramide. In order to understand the kinetics, properties, and regulation of this enzyme, it is necessary to alter the amount and type of substrate in the membrane while minimizing changes in the membrane environment or in the conformation of the enzyme. Therefore, nonspecific lipid transfer protein, which accelerates the transfer of phospholipids, cholesterol, and glycosphingolipids between membranes was used to study the properties and kinetics of rat liver CMP-N-acetylneuraminic:lactosylceramide sialyltransferase. These results are compared to those obtained in parallel experiments using detergent-solubilized substrate. Enzyme activity was increased four- to fivefold by transfer protein and was consistently higher than the activity measured in the presence of detergents. In contrast to the results obtained with detergents, the enzyme activity increased linearly with both the apparent Km values for the water-soluble substrate, CMP-neuraminic acid, and for the lipophilic substrate, lactosylceramide, were markedly different when determined in the presence of transfer protein (0.23 mM) or detergents (0.27 mM). On the other hand, the apparent Km values for the lipophilic substrate, lactosylceramide, were virtually identical when determined in the presence of transfer protein (0.23 mM) or detergents (0.27 mM). These observations suggest that transfer protein is a useful tool to study the properties and kinetics of membrane-bound enzymes when both the enzyme and substrate are components of the same membrane.

Supplementary key words  lipid transfer protein • sialyltransferase • membrane enzyme assay

In mammalian cells, glycosphingolipids are present predominantly in the outer leaflet of the plasma membrane with their carbohydrate portions extending into the external environment. It has been suggested that glycosphingolipids are involved in the regulation of cellular differentiation, development, and growth control (1–3). In addition, gangliosides, acidic glycosphingolipids, are known to participate in cell surface-related events as receptors for a variety of molecules including neurotransmitters (4), bacterial toxins (5–7), interferon (8), and viruses (9). The oligosaccharide portion of glycosphingolipids that are presumed to be responsible for their biological activity is synthesized by the sequential addition of monosaccharides from sugar nucleotide donors to appropriate glycosphingolipid acceptors. These reactions are catalyzed by a series of specific glycosyltransferases (10, 11). As in the case of glycosylation of glycoproteins, glycosyltransferases involved in the biosynthesis of gangliosides are found predominantly in the Golgi apparatus and to some extent in the endoplasmic reticulum (12). These enzymes are tightly associated with the luminal side of the Golgi apparatus membranes (13–15), and are not solubilized by ultrasonic treatment (16, 17).

One of the major problems in studying glycosphingolipid-metabolizing enzymes, such as CMP-N-acetylneuraminic:lactosylceramide α-2, 3-sialyltransferase (EC 2.4.99.9) (CMP-NeuAc:LacCer sialyltransferase) which catalyzes the transfer of N-acetylneuraminic acid (NeuAc) from CMP-NeuAc to the glycosphingolipid acceptor, lactosylceramide (LacCer), to form GM₃ ganglioside, is that both the enzyme and one of the substrates, LacCer, are membrane components. In order to study the kinetics and properties of a membrane enzyme, where one of the substrates is also an integral component of the membrane, it is necessary to incorporate the lipid substrate into the membrane enzyme assay.

Abbreviations used: lactosylceramide, Galβ₁-4Glc-ceramide; trihexosylceramide, Galα₁-4Galβ₁-4Glc-ceramide; globoside, GalNAcβ₁-3Galα₁-4Galβ₁-4Glc-ceramide; GM₃, NeuAcα₂-3Galβ₁-4Glc-ceramide; GMD, NeuAcα₂-3Galβ₁-4Glc-ceramide; GM₄, Galβ₁-3GalNAcβ₁-4(NeuAcα₂-3)Galβ₁-4Glc-ceramide; GM₅, Galβ₁-3GalNAcβ₁-4(NeuAcα₂-3)Galβ₁-4Glc-ceramide; GD₃, NeuAcα₂-3Galβ₁-4Glc-ceramide; G₀, NeuAcα₂-2Galβ₁-4Glc-ceramide; GD₁, NeuAcα₂-3Galβ₁-4Glc-ceramide; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography.
same membrane as the enzyme. Previous studies have attempted to resolve this problem in a number of ways, chiefly by the use of liposomes containing the substrate (18, 19), dispersing the substrate with celite (20), or dissolving the substrate in detergents (21, 22). These procedures all have limitations in that they either fail to achieve equilibration of the substrate in the membranes where the enzyme is located (liposomes or celite) or they disrupt the enzyme structure and/or the environment of the enzyme in the membrane (detergent).

An alternative method for incorporating glycosphingolipid substrates into the membrane containing the enzyme is by the use of unilamellar vesicles containing the glycosphingolipid substrate and a lipid transfer protein, such as glycosphingolipid-specific transfer protein or non specific lipid transfer protein. Glycosphingolipid transfer protein, which accelerates the translocation of glucosylceramide, galactosylceramide and, to some extent, LactCer between membranes, has been purified from bovine spleen (23) and porcine brain (24). These glycosphingolipid-specific transfer proteins, however, do not accelerate the transfer of ceramide, trihexosylceramide, or globoside. No studies have been conducted on gangliosides using these glycosphingolipid-specific transfer proteins. A protein that does accelerate the transfer of GM~1, GM~2, and GD1a, and GD1b gangliosides between membranes has been isolated from human kidney, but this protein is only marginally active with GM3 ganglioside and asialo GM2 (25). On the other hand, a nonspecific lipid transfer protein, which accelerates the intermembrane transfer of both gangliosides and neutral glycosphingolipids (26) as well as sphingomyelin, phospholipids, and cholesterol, has been purified from bovine (27) and rat livers (28), and its properties have been extensively studied by a number of investigators (26, 29, 30).

In the present study, we describe the application of nonspecific lipid transfer protein to the study of CMP-NeuAc:LacCer sialyltransferase. Comparisons are drawn with a parallel experiment using detergent-solubilized substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NeuAc), β-N-acetyl glucosaminidase, Tween 80, and Triton CF-54 were obtained from Sigma (St. Louis, MO); CMP-[4,5,6,7,8,9,14C]NeuAc (258 mCi/mmol) was from New England Nuclear (Boston, MA); globoside was from Supelco (Bellefonte, PA); and α-galactosidase was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Analytical and HPLC grade solvents were obtained from Fisher Scientific (Medford, MA). DEAE-Sephadex A-25 was purchased from Pharmacia (Piscataway, NJ); Unisil was from Clarkson Chemicals (Williamsport, PA); and C18 reversed-phase column (Bond-Elut) was from Analytichem International (Harbor City, CA).

**Preparation of Golgi-rich fraction**

Adult (2-3 months old) male Sprague-Dawley rats (Charles River, Kingston, NY) were anesthetized with sodium pentobarbital and the livers were perfused with 100 ml of saline through the hepatic portal vein to remove residual blood. Livers were quickly excised and homogenized in 0.5 M sucrose containing 0.1 M Tris-HCl (pH 7.6), 0.01 M magnesium chloride, and 1% dextran (mol wt 2 × 10^6). A Golgi-rich fraction was prepared by discontinuous sucrose density gradient centrifugation according to the procedure of Schachter et al. (31) except that the volume of each sucrose solution was 10 ml and the amount of homogenate loaded onto each tube was equivalent to 2 g of liver. The centrifugation was performed with Spinco head SW 28 at 22,000 rpm for 45 min. The Golgi-rich fraction was collected from the interface between the 0.7 M and 1.3 M sucrose gradient layers and diluted fourfold in 0.05 M Tris-HCl (pH 7.4) and recentrifuged at 105,000 g for 60 min. The Golgi pellet was suspended in 0.05 M Tris-HCl (pH 7.4) and sonicated on ice for a total of 2 min at a power setting of 25-30 W using a Microtip sonicator (Bronson, Danbury, CT). On the average, 29 mg of protein was recovered in the Golgi fraction from 10 g of rat liver (wet weight). Sialyltransferase activity was stable at ~20°C for at least 5 days.

**Preparation of microsomes**

Livers with residual blood removed as described above were homogenized in 0.25 M sucrose containing 0.05 M Tris-HCl (pH 7.4), 0.25 M sucrose, and 1 mM EDTA, and successively centrifuged at 700 g for 5 min and then 12,000 g for 15 min. The final supernatant was removed and centrifuged at 105,000 g for 60 min. The resulting microsomal pellet was washed once with the homogenization buffer and recentrifuged. The pellet was suspended in 0.05 M Tris-HCl buffer (pH 7.4).

**Preparation of glycolipid substrate**

LacCer was prepared from globoside by treatment with β-N-acetyl glucosaminidase and α-galactosidase. The incubation condition for cleavage of the sugars from globoside was: 2 mg of taurodeoxycholate, 1 mg of globoside, 2.5 units of β-N-acetyl glucosaminidase, 1 unit of α-galactosidase, and 0.2 M citrate buffer (pH 5.0) in a total volume of 0.5 ml. After incubation at 37°C overnight, the reaction was stopped with 9.5 ml of chloroform–methanol 2:1 and partitioned with 0.2 volume of saline as described by Folch, Lees, and Sloane Stanley (32). This resulted in two solvent phases and the enzymatic products were obtained.
from the lower phase. Although the major neutral glycosphingolipid resulting from the incubation was LacCer, small amounts of glycosylceramide were also formed since these enzymes contain a trace of other acid hydrolases including β-galactosidase. In order to remove the glucosylceramide, the lower chloroform phase (after partitioning) was dried under a stream of nitrogen gas, redissolved in 5 ml of chloroform, and applied to a Unisil (100–200 mesh) column (0.5 x 3 cm). The column was washed with 10 ml of chloroform and then the neutral glycosphingolipids were eluted with a step gradient (15 ml each) of increasing proportions of methanol in chloroform (1% interval). An aliquot of each fraction was monitored by HPTLC on plates developed with chloroform–methanol–water 60:35:8 and visualized by orcinol spray. The fractions containing LacCer were pooled and dried under a stream of nitrogen gas. LacCer was redissolved in ethanol and an appropriate aliquot was perbenzoylated as described previously (33) and quantitated by HPLC. 

Preparation of unilamellar liposomes containing LacCer

Liposomes were prepared as described previously (27) with microsomal lipids and LacCer. The microsomal lipids were prepared by extraction of microsomes in chloroform–methanol 2:1 and partitioned with 0.2 volume of saline as described by Folch et al. (32). The entire lower phase was used since the concentration of LacCer in the microsomal fraction of rat liver was negligible. Lipid phosphorus concentration was determined as described by Bartlett (35). Unless otherwise stated, LacCer and microsomal lipids (25 nmol of LacCer/200 nmol microsomal lipid phosphorus) were evaporated to dryness. The dried lipid mixtures were suspended in 0.05 M Tris–HCl (pH 7.4) containing 0.02% sodium azide and sonicated under a nitrogen atmosphere for 30 min at 20–25°C in a bath sonicator.

Preparation of transfer protein

Nonspecific lipid transfer protein was purified from bovine liver as described by Crain and Zilversmit (27), except that the final octylagarose and second CM-cellulose column chromatography steps were omitted. Therefore, this transfer protein preparation was not pure and contained both the CM-I and CM-II transfer proteins. An aliquot of the first CM-cellulose eluate was heated at 90°C for 5 min without changing the pH. Denatured protein was removed by centrifugation and the supernatant, which contained transfer protein, was concentrated just prior to use by Amicon YM5 membrane.

Separation of Golgi vesicles from liposomes

Liposomes containing LacCer, Golgi fraction, and transfer protein were incubated at 37°C for various times and chilled on ice to stop further transfer. The samples were layered on ice-cold sucrose gradients consisting of 0.5 ml of 1.3 M, 0.5 ml of 0.7 M, 3.0 ml of 0.5 M, and 0.5 ml of 0.25 M sucrose. After centrifugation at 2–4°C with Spinco head SW50.1 at 32,000 rpm for 60 min, Golgi vesicles were found between the 0.7 and 1.3 M sucrose layers and liposomes between the 0.25 and 0.5 M sucrose layers. Tubes were immediately transferred to dry ice and, after freezing, were cut by blade to obtain Golgi vesicle and liposome fractions. An aliquot of the Golgi fraction was analyzed for protein concentration. Lipids were extracted from both fractions with chloroform–methanol as described by Folch et al. (32). The neutral glycosphingolipids were isolated as previously described (33) and LacCer was quantitated by HPLC after perbenzoylation as mentioned above.

Enzyme assays

Sialyltransferase activity was assayed in a final volume of 45 μl by two incubation methods, a) with transfer protein and b) with detergents.

Incubation with transfer protein. The incubation mixture was prepared on ice with transfer protein, liposomes containing LacCer, sonicated Golgi fraction, and sodium cacodylate buffer, and the reaction was initiated by adding CMP-[14C]NeuAc. The final sodium cacodylate buffer concentration was 0.055 M and the pH of the reaction mixture was 6.2 unless otherwise stated.

Incubation with detergents. The assay procedure with detergents was adapted from the method described by Richardson, Keenan, and Morre. (21) LacCer (in ethanol), Triton CF-54, and Tween 80 (both in methanol) were mixed and the solvent was evaporated under a stream of nitrogen. Sonicated Golgi fraction and sodium cacodylate buffer were added and the reaction was initiated by adding CMP-[14C]NeuAc. The final sodium cacodylate buffer concentration was 0.15 M and the pH of the reaction mixture was 6.2 unless otherwise stated.

Exact reaction conditions are given in the figures. Reactions were performed at 37°C and terminated, usually after 60 min unless otherwise stated, by addition of 3 ml of chloroform–methanol 2:1. The amounts of transfer protein and Golgi protein were determined by the method of Lowry et al. (36).

Isolation of product, GM3

The enzyme reactions, stopped with chloroform–methanol, were partitioned with 0.2 volume of water. The
lower phase was repartitioned four times with 0.2 volume of methanol-water 1:1, after which no gangliosides were detected in the lower phase. All upper phases were combined and adjusted to 0.1 M KCl and applied to a C18 reversed-phase column (100 mg) as described previously (37). The column was washed with 20 ml of water and gangliosides were then eluted from the column with 10 ml of methanol. The methanol eluate was applied to a DEAE-Sephadex A-25 (acetate form) column (0.5 x 2 cm). Monosialogangliosides were eluted from the column with 15 ml of 0.02 M ammonium acetate in methanol, and other gangliosides were eluted with 5 ml of 0.8 M ammonium acetate in methanol. The 0.02 M ammonium acetate in methanol eluate was dried under a stream of nitrogen gas and the radioactivity of the enzymatic product, \( \text{G}_{\text{M3}} \), was determined by liquid scintillation counting.

RESULTS

Since this report is concerned only with the conversion of LacCer to the monosialoganglioside \( \text{G}_{\text{M3}} \) and since sialyltransferase is also active with other potential substrates, it was necessary to verify that only \( \text{G}_{\text{M3}} \) was being detected in the assay system used in this study. Panel A of Fig. 1 shows an HPTLC autoradiogram of the methanol eluate from the initial C18 reversed-phase column (lane 1), the 0.02 M ammonium acetate fraction (lane 2), and the 0.8 M ammonium acetate fraction (lane 3) from the final DEAE-Sephadex column. Panel B shows the same chromatogram after resorcinol staining. \( \text{G}_{\text{M3}} \), which chromatographs as a double band, and \( \text{G}_{\text{D1b}} \) are the only radioactive compounds eluting in the methanol fraction of the initial C18 reversed-phase column (panel A, lane 1). The \( \text{G}_{\text{D1b}} \) results from the sialylation of \( \text{G}_{\text{M1}} \) which is one of the major glycolipids in the microsomal fraction of rat liver. There is absolutely no trace of either the radioactive precursor CMP-[\( ^{14} \text{C} \)]NeuAc or free[\( ^{14} \text{C} \)]-NeuAc both of which are either not retained by the C18 reversed-phase column or elute in the water wash. Likewise, the only radioactive compound which elutes from the DEAE-Sephadex column in the 0.02 M ammonium acetate fraction is \( \text{G}_{\text{M3}} \) (lane 2). \( \text{G}_{\text{D1b}} \) as well as the other polysialogangliosides elute in the 0.8 M ammonium acetate fraction (panel A and B, lane 3). When radiolabeled \( \text{G}_{\text{M3}} \) was incubated in the complete (except for the CMP-[\( ^{14} \text{C} \)]NeuAc) incubation mixture and purified as described in Experimental Procedures (extracted in chloroform-methanol, partitioned (32), and chromatographed on both the C18 reversed-phase column and the DEAE-Sephadex column), the recovery of radioactivity was 96.1 ± 4.6% (mean ± SD of four observations).

In order to confirm that the amount of transfer protein was not the rate-limiting factor in the enzyme reaction, various concentrations of nonspecific lipid transfer protein were tested. When 100 \( \mu \)g of Golgi protein was incubated with various amounts of transfer protein, maximum \( \text{G}_{\text{M3}} \) synthesis was achieved with approximately 100 \( \mu \)g of transfer protein preparation (1:1 ratio of Golgi protein to transfer protein) (Fig. 2). Addition of more transfer protein had no effect on the rate of \( \text{G}_{\text{M3}} \) synthesis. The amount of \( \text{G}_{\text{M3}} \) synthesized at saturating levels of transfer protein (2.3 nmol/mg of Golgi protein per hr) was four to five times the amount formed in the absence of transfer protein (0.45 nmol/mg of Golgi protein per hr). Moreover, under these conditions, the amount of \( \text{G}_{\text{M3}} \) formed was directly proportional to the amount of Golgi protein.

As a final verification that it is the properties of sialyltransferase and not the properties of the transfer protein that are responsible for the activity observed under these assay conditions, the pH profile of \( \text{G}_{\text{M3}} \) synthesis was examined. Sialyltransferase is known to have an acidic pH maximum (21, 38). The pH profile observed in the presence of transfer protein (optimum pH of 6.2) was the same as that obtained with detergent-solubilized substrate.

As seen in Fig. 3, when Golgi and liposomes containing LacCer with or without transfer protein were mixed on ice
in the $K_m$ for CMP-NeuAc, the water-soluble substrate. The apparent $K_m$ for CMP-NeuAc calculated from double reciprocal plots (insets in Figs. 5 and 6) was 0.23 ± 0.02 mM (mean ± SD of three separate determinations) with transfer protein and 0.27 mM (average of two determinations) with detergents.

Fig. 7 shows the LacCer concentration curve when assayed with transfer protein. The apparent $K_m$ for the LacCer was found to be 47.9 ± 5.9 pM (mean ± SD of three determinations). This value did not vary with amount of the Golgi protein in the incubation mixture. In contrast, the apparent $K_m$ for LacCer assayed with detergents was 1.2 μM (average of two determinations) (Fig. 8). In both assay systems, substrate inhibition at high LacCer concentrations was clearly evident (0.6 mM and 0.06 mM for transfer protein and detergents, respectively).

DISCUSSION

The participation of glycosphingolipids in cell surface-related events, such as regulatory effects on cellular differentiation and growth control, and as receptors for a variety of molecules has been studied by many investigators. It is known that various tissues contain their own specific patterns of glycosphingolipid classes (39), and that each glycosphingolipid class contains a tissue-specific pattern of ceramide molecular species (40). It has also been reported that the glycosphingolipid molecular species as well as the class composition change under various metabolic conditions (41-43). More specifically, several recent studies have demonstrated that the ceramide molecular
species composition of glycosphingolipids affects the antigenicity of glycosphingolipid, even though their carbohydrate moieties are responsible for the antigenic activity (42-44). The variation in glycosphingolipid molecular species composition among different tissues or in the same tissue under different metabolic conditions may be due to variation in glycosphingolipid synthesis among different cell types within a tissue or to differences in the specificity of the glycosphingolipid synthesizing enzymes for ceramide molecular species. Since both the enzymes that synthesize the glycosphingolipids and the glycosphingolipids themselves are integral components of the same membrane, it is possible, if not probable, that changes in the physical properties of the membrane will affect the molecular species specificitY of the glycosphingolipid-synthesizing enzymes either by differentially affecting the movement of various ceramide molecular species in the membrane or by changes in the conformation of the enzyme. It has been demonstrated that changes in the lipid environment of a membrane can change the conformation of membrane proteins (45, 46).

Thus, there is considerable interest in studying the role of changes in membrane environment on the rate and specificity of the enzymes of glycosphingolipid synthesis. One of the problems in studying membrane-bound enzymes in which one of the substrates is also a component of the membrane, as is the case for the glycosphingolipid-synthesizing enzymes, is introducing exogenous substrate into the membrane where the enzyme is located. Traditionally, this is accomplished by dispersing the substrate with detergents. While this procedure may or may not be valid in any particular case, in general this procedure is likely to be accompanied by changes in enzyme conformation due to the effect of detergent on the enzyme. Moreover, because of the disruptive effect of detergent on the membrane (particularly at high concentration), it is impossible to study the effect of the membrane environment on enzyme activity. In order to overcome the potential problems inherent in the use of detergents, we have examined the potential use of nonspecific lipid transfer protein to facilitate the transfer of the exogenous lipophi-

![Figure 4](image1)

**Fig. 4.** The effect of incubation time on the activity of CMP-NeuAc:LacCer sialyltrasferase in the presence of transfer protein (■■■■) or detergents (○○○○). The reaction mixtures assayed with transfer protein contained 25 nmol of LacCer in liposomes, 25 nmol of CMP-[14C]NeuAc, 50 μg of liver Golgi apparatus protein, and 156 μg of transfer protein. The reaction mixtures assayed with detergents contained 25 nmol of LacCer, 25 nmol of CMP-[14C]NeuAc, 50 μg of liver Golgi apparatus protein, 100 μg of Triton CF-54, and 50 μg of Tween 80. The results are means of duplicate determinations.

![Figure 5](image2)

**Fig. 5.** CMP-NeuAc:LacCer sialyltransferase activity assayed with transfer protein as a function of CMP-NeuAc concentration. The reaction mixtures contained 25 nmol of LacCer in liposomes, various amounts of CMP-[14C]NeuAc as indicated, 50 μg of Golgi protein, 132 μg of transfer protein.
Fig. 6. CMP-NeuAc:LacCer sialyltransferase activity assayed with detergents as a function of CMP-NeuAc concentration. The reaction mixtures contained 25 nmol of LacCer, various amounts of CMP-[14C]NeuAc as indicated, 50 μg of Golgi protein, 100 μg of Triton CF-54, and 50 μg of Tween 80.

Fig. 7. CMP-NeuAc:LacCer sialyltransferase activity assayed with transfer protein as a function of LacCer concentration. The reaction mixtures contained various amounts of LacCer in liposomes as indicated, 50 nmol of CMP-[14C]NeuAc, 50 μg of Golgi protein, and 150 μg of transfer protein. While LacCer concentration was varied, the concentration of microsomal lipids of liposomes was maintained constant in each tube.

For this study, we chose to examine the enzyme (CMP-NeuAc:LacCer sialyltransferase) which catalyzes the transfer of NeuAc to LacCer to form G\textsubscript{M3}. LacCer is an important intermediate in a number of pathways of glycosphingolipid biosynthesis since it is a precursor to trihexosylceramide and asialo G\textsubscript{M2} as well as G\textsubscript{M3} (and the higher gangliosides). It has been suggested that there are several sialyltransferases in the liver Golgi apparatus that catalyze the sialylation of the various glycosphingolipid acceptors (38). Sialyltransferases are believed to be intrinsic membrane proteins with their active site facing the luminal side of the Golgi apparatus membrane (13). It has been proposed that the transport of CMP-NeuAc as well as other nucleotide sugars through the Golgi membrane in vivo is facilitated by a carrier protein (47) and that the mechanism is a 1:1 exchange of nucleotide sugars with corresponding...
nucleoside monophosphates (48). In the incubation system described in this study, the glycosphingolipid substrate, LacCer, is transported into Golgi membrane by nonspecific lipid transfer protein, but this transfer protein does not, so far as is known, facilitate the transfer of the lipids into the inner leaf of a lipid bilayer. Therefore, in order to maximize the interaction between the enzyme and both substrates and to ensure that the transfer of both substrates across the Golgi membrane is not a limiting factor, the Golgi vesicles were sonicated prior to use, thereby converting some of the rightside-out Golgi vesicles to inside-out vesicles. The enzyme's apparent activity for GM₃ synthesis was increased approximately 2.5-fold by sonication.

Since we wanted to study only one specific enzyme activity and since there are a number of potential substrates for the various sialyltransferases in the Golgi membrane, it was necessary to establish that the assay used in this study was measuring only that single enzyme activity, i.e., that our assay procedure is measuring only GM₃. It has been reported that when rat liver Golgi fraction was incubated in the presence of detergents, about 10% of the radiolabeled NeuAc was incorporated into GD₃ (21). No GD₃ was detected in the present study, since the disialogangliosides (GD₃ as well as GD₄) do not elute from the DEAE-Sephadex column with 0.02 M ammonium acetate in methanol. Moreover, the GM₃ which is formed during the incubation was not further metabolized to GM₂ (which does elute in the 0.02 M ammonium acetate fraction), because of the absence of the required sugar nucleotide, UDP-N-acetylglucosamine. This was confirmed experimentally since incubation of labeled GM₃ in complete assay mixture (in the absence of added CMP-[¹⁴C]NeuAc) resulted in complete recovery of the labeled GM₃ (96.1 ± 4.6%, mean ± SD of four determinations). The appearance of two bands on the autoradiogram is due to the fact that GM₃ resolves into two bands on TLC (Fig. 1). It is known that differences in the fatty acid as well as the long chain base are responsible for the double-band appearance of glycosphingolipids on TLC (49, 50). In general, the upper band is enriched in longer chain and/or nonhydroxy fatty acid-containing molecular species, while the lower band contains shorter chain and/or hydroxy fatty acid-containing species.

The results obtained with the nonspecific lipid transfer protein were, in general, similar to those obtained with detergent-solubilized substrate. The pH profiles were similar to each other and are in general agreement with results obtained by other workers, although the activity measured in the presence of detergent is consistently a little less than the activity measured using the transfer protein. In both of the assay methods, high concentration of the lipophilic substrate, LacCer, inhibited the enzyme activity. A similar inhibitory effect of LacCer on sialyltransferase has previously been reported using the detergent Triton X-100 (38). There are other small but relevant differences between the results obtained with the detergent and transfer protein assay systems which indicate potential problems in the use of detergent-solubilized substrate. In particular, the rate of GM₃ formation is not quite linear with incubation time with the detergent-solubilized substrate. Of greater concern is the fact that CMP-NeuAc:LacCer sialyltransferase activity did not increase linearly with increasing amounts of rat liver Golgi protein when assayed with detergents (21).
The $K_m$ for the water-soluble substrate, CMP-NeuAc, was effectively the same as determined by the two methods, being 0.23 mM with transfer protein and 0.27 mM in the presence of detergent. However, these values were much lower than the values previously reported for rat liver Golgi fraction assayed with detergents, i.e., 1.5 mM (38) or 2.7 mM (21). On the other hand, the apparent $K_m$ values for the membrane-bound lipid substrate, LacCer, was markedly different when assayed in the presence of transfer protein (47.9 $\mu$M) or in the presence of detergent (1.2 $\mu$M). In the presence of detergents, the $K_m$ for LacCer using rat liver Golgi fraction as the source of enzyme has variously been reported as 80 $\mu$M (38) or 130 $\mu$M (21). The reason for the discrepancy between the previously published $K_m$ values and our $K_m$ values using detergent-solubilized substrate are most likely due to the fact that we were using a sonicated Golgi preparation. However, differences in the source of LacCer or differences in the methods used for the quantitation of the LacCer or the purification of the product could also be contributing factors.

The difference in $K_m$ for the lipophilic substrate, LacCer, which we observed using the two different methods emphasizes the problems inherent in the detergent assay. As suggested by Schell et al. (51), the classical Michaelis-Menten theory which is applicable to water-soluble (or solubilized) enzyme and substrate may not be applicable to membrane-bound enzymes, particularly if the substrate is also in the membrane. (We did use the classical method to derive our $K_m$ values, however). This is due in part to geometric restrictions when both the enzyme and the substrate are confined to the plane of the membrane. This is presumably the case in vivo and in the studies using transfer protein. On the other hand, the detergent-solubilized substrate with the enzyme has access to at least the entire solution facing the membrane. Other effects of detergents are possible, particularly on the conformation of the enzyme, either directly or through changes in the integrity of the membrane. A possible example of such effects has been reported by Dawson, McLawhon, and Miller (52) and Burczak, Soltyiska, and Sweeley (53). They have proposed that one of the mechanisms regulating the activities of CMP-NeuAc:LacCer sialyltransferase and UDP-Gal-GM$_2$ galactosyltransferase is phosphorylation and dephosphorylation of these enzymes. However, it has been difficult to verify this assertion because of the poor reproducibility of the phosphorylation and dephosphorylation of the enzymes. It has been suggested that the use of detergents in the assay system is responsible for the poor reproducibility (53) owing to changes in the susceptibility of the phosphorylated enzyme to phosphatase(s) that result from exposure to detergents.

Although it is easy to measure sialyltransferase activity with a judicious choice of detergents and with careful attention to the amounts of detergents added, it is not in general the method of choice to study the regulation of the enzyme. Nonspecific lipid transfer protein, on the other hand, by facilitating the transfer of substrate to and product from the membrane where the enzyme is located, is a useful tool to study enzyme kinetics when both the enzyme and substrate are components of the membrane. The reaction rate of a membrane enzyme can also be significantly affected by changes in membrane “fluidity” caused by changes in lipid composition (51, 54). Therefore, in the present study, the lipids for the liposome preparation were isolated from the same membrane fraction as the enzyme in order to minimize the effect of changes in the composition of lipids other than the substrate, LacCer. In general, however, nonspecific lipid transfer protein may also prove to be a convenient way to study the effects of changes in the lipid environment on the activity of membrane enzymes.

We wish to thank Dr. R. H. McCluer, Eunice Kennedy Shriver Center for Mental Retardation, Inc., Boston, MA, for his support in the preliminary experiments. This investigation was supported by the National Institutes of Health grant NS 23354.

REFERENCES

9. Suzuki, Y. M., Matsumura, M., and M. Matsumoto. 1985. N-acetyl neuraminylactosylceramide, GM$_2$-NeuAc, a new influenza A virus receptor which mediates the adsorption-


ceramide composition and coexisting glycolipid on the anti-
genicity of gangliotriaosylceramide in murine lymphoma
44. Crook, S., J. M. Boggs, A. I. Vistnes, and K. M. Koshy.
1986. Factors affecting surface expression of glycolipids: in-
fluence of lipid environment and ceramide composition on
antibody recognition of cerebroside sulfate in liposomes.
Biochemistry. 25: 7488–7494.
45. Wilson, M. L., and F. W. Dahlquist. 1985. Membrane pro-
tein conformational change dependent on the hydrophobic
for changes in the conformational status of rat liver micro-
somal glucose-6-phosphate:phosphohydrolase during deter-
gent-dependent membrane modification. Effect of p-mer-
curibenzoate and organomercurial agarose gel on glucose-
6-phosphatase of native and detergent-modified micro-
CMP-N-acetylenuraminic acid: isolation from and penetra-
of glycosylation and sulfation in the Golgi apparatus: evi-
dence for nucleotide sugar/nucleoside monophosphate and
nucleotide sulfate/nucleoside monophosphate antiports in
81: 7051–7055.
characterization of human liver hematoside. J. Lipid Res.
19: 538–543.
(GM1) is a major ganglioside in chicken embryonic liver. J.
51. Schell, G., E. Acevedo, E. Conzelmann, H. Nehrkorn, and
K. Sandhoff. 1982. Model for the interaction of membrane-
bound substrates and enzymes. Hydrolysis of ganglioside
GD3 by sialidase of neuronal membranes isolated from calf
52. Dawson, G., R. McLawhon, and R. J. Miller. 1980. Inhibi-
tion of sialoglycosphingolipid (ganglioside) biosynthesis in
mouse colonial lines N4TG1 and NG108-15 by 8-endorphin,
Regulation of membrane-bound enzymes of glycosphin-
of phospholipid fatty acids on the kinetics of high and low
64: 1195–1210.