Exposure of Galactosylceramide to Galactose Oxidase in Liposomes: Dependence on Lipid Environment and Ceramide Composition†

Robert J. Stewart and Joan M. Boggs*

Department of Clinical Biochemistry, University of Toronto, and Department of Biochemistry, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada

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ABSTRACT: Factors which influence the accessibility, or exposure, of the carbohydrate head group of the glycolipid galactosylceramide (GalCer) at the membrane surface have been examined in lipid model membranes using the technique of galactose oxidase-tritiated sodium borohydride labeling. Both the ceramide composition of GalCer and the lipid composition of its membrane environment were varied. We have shown that GalCer is oxidized in a membrane environment, by purification of the labeled galactosyl moiety of the glycolipid by high-performance anion exchange chromatography. Using semisynthetic molecular species of GalCer with acyl chain lengths ranging from 16 to 26 carbons, incorporated into liposome membranes of egg phosphatidylcholine (PC), and reverse-phase HPLC separation of mixtures of the molecular species, we have shown that increasing the fatty acid chain length of GalCer increases its oxidation by galactose oxidase. In addition, the degree of oxidation is reduced when the fatty acid chain of GalCer is hydroxylated. GalCer incorporated into liposomes containing synthetic species of PC with different fatty acid chain lengths (together with cholesterol) was oxidized less as the PC acyl chain length, and hence the bilayer thickness, was increased. The oxidation of GalCer in liposomes composed of sphingomyelin/cholesterol was reduced compared to its oxidation in PC liposomes. Furthermore, changes in the fatty acid chain length of GalCer had no effect on its oxidation in sphingomyelin liposomes. These findings indicate that the ceramide composition and lipid membrane environment can influence the exposure of the lipid carbohydrate, and hence, they could modulate the receptor activity of glycolipids at the membrane surface.

The carbohydrate head groups of glycolipids have been shown to be involved in a wide variety of cell surface recognition phenomena including cell adhesion (Eggens et al., 1989), interactions with microorganisms (Karlsson, 1989), and interactions with biological ligands (Aruffo et al., 1991). An immune response to glycolipids may contribute to the pathology in some autoimmune diseases (Quarles et al., 1986). Although the membrane concentration and carbohydrate composition of glycolipids can influence cell surface recognition phenomena, the membrane environment and the ceramide composition of glycolipids may also alter their expression on the cell surface, as detectable by specific antibody. For example, during oncogenesis, the expression of glycolipids can be altered without any significant change in glycolipid chemical quantity, and these changes have been associated, at least in part, with changes in glycolipid ceramide composition (Hakomori, 1984; Kannagi et al., 1982, 1983). In addition, developmental changes (O'Brien et al., 1964; Svennerholm & Stallberg-Stenhagen, 1968; Shimomura & Kishimoto, 1983; Palestini et al., 1990) and tissue specific differences in glycolipid ceramide composition (Ogawa-Goto et al., 1990) together suggest a functional role for the ceramide structure. An increase in the content of very long chain fatty acid species (C25 and C26) occurs in membrane sphingolipids in the demyelinating disease adrenoleukodystrophy (Kishimoto et al., 1985). These findings imply further that these changes may modulate cell contact, cell growth and regulation, susceptibility to infection, or immune response, as a result of changes in the expression of the glycolipid carbohydrate. Therefore, in order to completely understand glycolipid function, determination of the effects of changes in both membrane and ceramide composition on glycolipid expression is required.

Liposomes containing glycolipids are a useful model system in which to study the influence of changes in both the membrane environment and the glycolipid ceramide fatty acid composition because the composition of the membrane can be strictly defined and changes can be examined in a systematic and independent manner. Previous studies in our laboratory (Crook et al., 1986; Stewart & Boggs, 1990), as well as others [*cf.* Kinsky and Nicolotti (1977), McConnell (1978), and Alving and Richards (1983) for reviews], have employed liposomes for the study of the recognition of glycolipid haptens by antibody. These studies have shown that the recognition of glycolipids by antibody is modulated by the membrane environment, by changes in the haptenic density, and by the ceramide composition, including the length and hydroxylation of the acyl chain, of the glycolipid itself. However, the possibility that the antibody may recognize the lipid portion of the glycolipid cannot be eliminated. Antibodies to glycolipids have been reported for which the epitope may include part of the acyl chain or the fatty acid hydroxyl group (Fredman et al., 1988; Nakamura et al., 1989). Recent NMR studies indicate that an increase in fatty acid chain length or hydroxylation of the fatty acid of glycolipids have little or no effect on the motion or conformation of the galactose head group in a bilayer environment (Singh et al., 1992a), contrary to the result expected if these changes affect exposure of the head group. Evidence that the long fatty acid chain of the C24 species of glycolipids in a phosphatidylcholine bilayer interdigitates into the other side of the bilayer has been reported

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* To whom correspondence should be addressed at the Biochemistry Department, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada, M5G 1X8.

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(Mehlhorn et al., 1988). If so, this should minimize any effect on exposure of the head group.

Therefore we have examined the effect of the length and hydroxylation of the fatty acid chain of the glycolipid, galactosylceramide (GalCer),[1] and of changes in its membrane environment on exposure of the galactose head group at the bilayer surface by measuring its oxidation by galactose oxidase. Galactose oxidase (EC 1.1.3.9) converts the C-6-OH group of galactose and its derivatives into an aldehyde (Amaral et al., 1968). By subsequently reducing with tritiated sodium borohydride ($NaB[^3H]H_4$), the galactose C-6 alcohol is restored, leaving a specific radioactive tag (Radin et al., 1969). This enzyme has been used to radiolabel various glycoproteins and glycolipids for further analyses or to probe their exposure or accessibility at the cell surface or in liposomes (Suzuki & Suzuki, 1972; Gahmberg & Hakomori, 1975; Lis et al., 1982; Gattegno et al., 1982; Lampio et al., 1984, 1986, 1988). However, it has not previously been used to examine the effect of lipid composition on exposure in a liposome system. One advantage of using galactose oxidase is that its specificity is well characterized (Amaral et al., 1968; Schlegel et al., 1968; Maradufu et al., 1971) and it does not have any reactivity with the lipidic portion of glycolipids. In addition, galactose oxidase can recognize the terminal galactosyl (or galactosylaminyl) residues of many different glycolipids (Suzuki & Suzuki, 1972), and its reaction with the carbohydrate on lipids can be distinguished from that with similar carbohydrate on proteins. Furthermore, because the galactose oxidase/$NaB[^3H]H_4$ technique actually labels molecules which interact with the enzyme, the effect of the glycolipid ceramide group composition can be examined in natural mixtures by comparing specific activities of the different molecular species of the glycolipid. It is thus more generally useful than antibodies for studying glycolipid expression in biological membranes.

In the current study, we have employed the technique of galactose oxidase/$NaB[^3H]H_4$ labeling to examine the effects of changes in the ceramide group of GalCer on exposure of galactose at the surface of phospholipid liposomes, by using semisynthetic species of GalCer, each with a defined fatty acid chain. In addition, by using reverse-phase HPLC to separate the molecular species of GalCer, after oxidation and labeling of mixtures, the relative labeling of individual species was determined. The membrane environment of GalCer was varied by incorporating it into liposomes of phosphatidylcholine of varying fatty acid chain length or into sphingomyelin, together with cholesterol.

MATERIALS AND METHODS

Reagents and Solvents. All general chemicals were of reagent grade. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N,N'*-4-dimethylaminopyridine were purchased from Sigma. Benzoic anhydride, pyridine, *N*-hydroxysuccinimide, and tetrahydrofuran (THF) were obtained from Aldrich. HPLC grade methanol and water were obtained from Caledon Laboratories. Tritiated $NaBH_4$ ($NaB[^3H]H_4$) was obtained from New England Nuclear at a specific activity of 7–9 Ci/mmol. Galactose oxidase was obtained from Sigma, and it was purified on a Sepharose 6B column as described by Hatton and Rogoeczi, (1982). Only 5% of the protein applied to the column was eluted in the fractions containing galactose oxidase activity, and 95% was eluted earlier in a single peak. The activity of the enzyme was determined by the chromagen–horseradish peroxidase assay of Amaral et al., (1968). It increased from 406 units/mg of protein to 2656 units/mg of protein after purification. It gave a single band of MW 65 000 ± 5000 by gel electrophoresis on a 8% polyacrylamide SDS gel in 0.025 M Tris-glycine buffer, pH 8.5. This is similar to the molecular weight reported for galactose oxidase purified by DEAE- and CM-cellulose chromatography by Kosman et al. (1974).

Lipids. Bovine brain galactosylceramide (nGalCer), egg phosphatidylcholine (egg PC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), bovine brain sphingomyelin (bSM), and cholesterol were purchased from Avanti Polar Lipids. Galactosylsphingosine (psychosine), sphingosine, semisynthetic species of GalCer, *N*-palmitoylgalactosylceramide (C16:0-GalCer), *N*-stearoylgalactosylceramide (C18:0-GalCer), and *N*-nervonylgalactosylceramide (C24:1-GalCer) were purchased from Sigma. All lipids were chromatographically pure and were used as supplied. Hydroxy and non-hydroxy fractions of nGalCer (HFA- and NFA-nGalCer, respectively) were purified by TLC with chloroform/methanol/aqueous NH_4OH, 65:25:4 (v/v/v), as the developing solvent, and the individual HFA- and NFA fractions were eluted with chloroform/methanol/water, 7:7:1 (v/v/v) (Radin, 1972). Lignoceric (C24:0) and hexacosanoic (C26:0) fatty acids were obtained from Fluka. The pure D-(+)-2-OH-C24:0 fatty acid was synthesized and purified, and the C24:0h-GalCer molecular species was synthesized as described (Boggs et al., 1988). The semisynthetic C24:0 and C26:0 molecular species of GalCer used in this study were synthesized and purified as described by Koshy and Boggs (1983) and Boggs et al. (1984), with a few modifications. Briefly, instead of preparing the acid chlorides of the fatty acids for reaction with bovine brain psychosine, *N*-hydroxysuccinimide esters of C24:0 and C26:0 fatty acids were prepared by the condensation of *N*-hydroxysuccinimide with the fatty acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, followed by recrystallization of the *N*-hydroxysuccinimide ester from ethanol, according to the method of Lapidot et al. (1967). The GalCer molecular species were purified as described, and the purity was checked by TLC on Merck TLC plates with chloroform/methanol/aqueous NH_4OH, 65:25:4 (v/v/v), as the developing solvent. GalCer bands were visualized by iodine staining and orcinol staining (Bevenue & Williams, 1951), and in each case only a single band was detected.

Preparation of Liposomes Containing nGalCer. Multilamellar liposomes were used in order to avoid the high surface curvature of small unilamellar liposomes and the possible exclusion of the GalCer during preparation of large unilamellar liposomes by various extrusion or solvent injection procedures. They were prepared as described previously (Stewart & Boggs, 1990) with a few modifications. The chloroform/methanol (2:1,v/v) solutions of the lipid mixtures were added to a screw-capped glass tube, and the solvent was evaporated under a stream of nitrogen and dried further under vacuum for at least 2 h. A few glass beads were added to the tube. The lipids were then dispersed in 0.1 M phosphate buffer, pH 7.0, at a concentration of 10 mM phospholipid and incubated at 90–95 °C for 5 min with frequent vortexing. The liposomes

[1] Abbreviations: bSM, bovine brain sphingomyelin; CBS, galactosylceramide-I^3-sulfate; CHOL, cholesterol; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; egg PC, egg yolk phosphatidylcholine; GalCer, galactosylceramide; nGalCer, bovine brain galactosylceramide; HFA-nGalCer, fraction of nGalCer containing hydroxy fatty acids; NFA-nGalCer, fraction of nGalCer containing non-hydroxy fatty acids; GG, galactosylglycerolipid; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; TLC, thin layer chromatography; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

typically contained egg PC/GalCer at a mole ratio of 1.0:0.1, respectively. Liposomes containing other phospholipids (DMPC, DSPC, or bSM) also contained cholesterol (CHOL), and the mole ratios were 1.0:0.75:0.1, phospholipid/CHOL/GalCer, respectively.

Labeling of nGalCer by Galactose Oxidase–NaB[^{3}H]H$_4$. Pure nGalCer was labeled in THF/phosphate buffer at high specific activity as described by Radin (1972). nGalCer was dissolved in THF/0.1 M phosphate buffer, pH 7.0, 1:1 (v/v), and galactose oxidase dissolved in 0.01 M phosphate buffer, pH 7.0, was added to a final ratio of 100 units enzyme/μmol of nGalCer, and THF was added to bring the final ratio of THF/buffer to 1:1. The mixture was incubated at room temperature for 24 h. The mixture was then washed several times by Folch partition (Folch et al., 1957), dried under nitrogen, and redissolved in a minimal volume of THF. One microcurie of NaB[^{3}H]H$_4$ in 40 μl of 0.01 N NaOH was added, and the sample was incubated overnight in a fumehood cabinet. Five milligrams of solid (unlabeled) NaBH$_4$ was added to complete the reduction of the glycolipid. Excess NaBH$_4$ was consumed by the drop-wise addition of 1.0 N HCl until all gas had evolved. The sample was neutralized with 1.0 N NaOH, and the labeled nGalCer ([6-^{3}H]GalCer) was then extracted with chloroform/methanol (2:1,v/v), washed several times by Folch partition, and was purified by preparative TLC.

GalCer (nGalCer or semisynthetic molecular species) was labeled in liposomes by incubating the liposomes at a concentration of 0.5 μmol of GalCer/mL with 100 units of galactose oxidase/μmol of GalCer at 20 °C for varying times. The liposomes were washed several times with 0.1 M phosphate buffer by bench-top centrifugation in an Eppendorf centrifuge. The liposomes were then resuspended in 0.01 N NaOH, and 1 mCi of NaB[^{3}H]H$_4$ in 40 μL of 0.01 N NaOH was added. The samples were incubated overnight in the fumehood cabinet, and the labeled GalCer was treated with cold NaBH$_4$ and isolated as described for the THF/phosphate buffer samples above. Control samples were reduced with NaB[^{3}H]H$_4$ without prior oxidation with galactose oxidase and isolated as described above. The radioactivity of all samples was determined by counting an appropriate aliquot by liquid scintillation in Beckman Ready Value scintillation fluid on a Beckman L5 60001C scintillation counter. The NaB[^{3}H]H$_4$ is an inherently unstable reagent, and the specific activity obtained varied between different batches of NaB[^{3}H]H$_4$ and with time for individual batches. Therefore, experiments conducted at different times could not be compared directly in terms of absolute specific activity, but only in relative terms. The specific activity for the different molecular species was normalized by dividing the value for each species by that for the C24:0 sample to allow averaging of values from a number of experiments. The C24:0 species was chosen because its oxidation was less variable than the C24:1 species. It was normalized to the C26:0 species for estimation of its standard deviation.

Measurement of nGalCer Oxidation by Tritium Release from Liposomes. nGalCer liposomes were prepared as described above, but a known amount of [6-^{3}H]GalCer was included in the mixture. High specific activity [6-^{3}H]GalCer, prepared from samples oxidized and labeled in THF/phosphate buffer, was used, and the final specific activity in each set of liposomes was about 500 000 cpm/μmol nGalCer. The final mole ratio of the lipids in the liposomes was the same as described above. The extent of oxidation of [6-^{3}H]GalCer in liposomes by galactose oxidase was determined from the amount of tritium released (as H_2O_2) into the aqueous environment (Radin & Evangelatos, 1981). Aliquots of liposomes were incubated with 300 units of galactose oxidase/μmole GalCer, and the reaction was stopped by the addition of 0.1 M KCN. The liposomes were then sedimented by centrifugation for 30 min in an Eppendorf bench-top centrifuge, and an aliquot of the supernatant was taken for liquid scintillation counting. Control samples were incubated with galactose oxidase that had been previously inactivated by a 20-min incubation with 0.1 M KCN. In order to compare the oxidation of HFA-nGalCer to NFA-nGalCer, HFA- and NFA-nGalCer fractions of both native and labeled lipids were purified by TLC. The radioactive purity of the [6-^{3}H]HFA- and NFA-nGalCer fractions was confirmed by autoradiography of a TLC plate, before addition to the unlabeled HFA- and NFA-nGalCer fractions. The labeled and unlabeled HFA- or NFA-nGalCer fractions were then combined to achieve the same final specific activity for each. For kinetic analysis, samples of liposomes containing [6-^{3}H]nGalCer were serially diluted in triplicate from 100 nmol/mL to 5 nmol/mL, and, to each aliquot, 2.24 units of galactose oxidase was added. The reaction was stopped after 45 min by the addition of 0.1 M KCN. Control samples, incubated with KCN-inactivated galactose oxidase, were done for each concentration, and the net radioactivity released was determined by subtraction of counts in the control sample from those of the oxidized sample. The data were analyzed by Michaelis–Menten kinetics, and the inverse Lineweaver-Burk plots were fitted by linear regression analysis. Correlation coefficients were greater than 0.98 in all cases. The amount of nGalCer oxidized in these experiments was estimated by dividing the total radioactivity released into the aqueous environment by the specific activity of the nGalCer used and multiplying this value by a factor of 2 (Radin & Evangelatos, 1981). Although the dehydrogenation of nGalCer by galactose oxidase is both stereospecific (removal of the *pro-S* hydrogen only) and demonstrates a significant kinetic isotope effect (Maradufu et al., 1971), there is no reason to assume that the kinetic isotope effect would be intrinsically different for HFA- and NFA-nGalCer fractions or for NFA-nGalCer in different membrane environments; therefore, the nanomoles of nGalCer oxidized calculated from the release of tritium for each experiment should reflect the true relative differences in oxidation.

HPLC Separation of GalCer Molecular Species. After oxidation and reduction in THF or liposomes, the GalCer was purified by preparative TLC, and the GalCer molecular species were separated into individual components by reverse-phase HPLC of the per-*o*-benzoylated derivatives following the procedure of Yahara et al., (1980b). The per-*o*-benzoylated derivatives were prepared as described (McCluer & Ullman, 1980) by reaction of GalCer with benzoic anhydride with *N,N'*-4-dimethylaminopyridine as a catalyst in pyridine. After the reaction was completed, the sample was redissolved in hexane and washed three times with methanol/water (4:1, v/v) containing saturating amounts of Na_2CO_3, followed by three washes with methanol/water (4:1, v/v). The derivatized GalCer molecular species were then redissolved in dichloroethane or carbon tetrachloride.

Mixtures of semisynthetic GalCer benzoylated derivatives were separated into individual components by HPLC of the dichloroethane or carbon tetrachloride solutions following the method of Yahara et al. (1980b) using a 3.2 × 300 mm Waters μBondapak C18 column. The molecular species were eluted isocratically using an eluting solvent of methanol/water, 95:5

(v/v), at a flow rate of 0.8 mL/min, and the effluent was monitored by absorption at 230 nm. Fractions were collected at 5-min intervals, and peaks corresponding to each of the five molecular species were pooled and concentrated for further analysis of the specific activity.

Autoradiography. Autoradiography of nGalCer was done essentially as described by Schwarzmann (1978). The TLC plate was developed in chloroform/methanol/NH_4OH, 65:25:4 (v/v/v), and dried for 15 min in a 100 °C oven. The plate was lightly sprayed with En^{3}Hance, dried in a fumehood cabinet, and then sprayed again. After drying, the TLC plate was overlaid with Kodak X-OMAT AR Imaging Film and placed in a sealed container, and the autoradiogram was incubated at −70 °C for 72 h. The film was developed using a KODAK X-OMAT M35 Processor automatic developer.

Determination of GalCer Specific Activity. The specific activity of [6-^{3}H]GalCer was determined by measuring either the radioactivity specifically associated with its galactosyl moiety or by measuring the amount of radioactivity associated with the intact glycolipid after subtraction of the radioactivity due to reduction without oxidation. The galactose was quantitated directly by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using the Dionex chromatographic system equipped with a 4 × 250 mm IONPAC anion exchange column, essentially as described (Hardy et al., 1988). GalCer samples were dried under nitrogen from their C/M solutions. The samples were hydrolyzed in 0.5 mL of 2 N trifluoroacetic acid (TFA) in a screw-capped test tube in a heating block at 100 °C, with constant stirring. The TFA was evaporated under nitrogen and the remaining aqueous solution lyophilized. The sample was redissolved in 0.5 mL of water and washed three times with hexane to remove hydrophobic products, and the aqueous phase was again lyophilized to dryness. The samples were redissolved in an appropriate volume of HPLC grade water, and particulate matter was removed by centrifugation in an Eppendorf centrifuge. The final aqueous solution was chromatographed on the IONPAC column, and the galactose was eluted isocratically with 20 mM NaOH at a flow rate of 1.0 mL/min over a 20-min interval. The eluate from the column was collected at 1-min intervals, and the radioactivity in each fraction was determined by liquid scintillation counting. The galactose was identified by comparing the retention time with standard galactose solutions, and the samples were quantitated by interpolation of the area from a galactose standard curve.

When the per-*o*-benzoylated GalCer derivatives were hydrolyzed to release the galactosyl moiety for HPAEC-PAD chromatography, the tritium was (surprisingly) stripped from the C-6 position of galactose (R. J. Stewart and J. M. Boggs, unpublished results), and the specific radioactivity of the galactose could not be determined. Thus, in order to determine the specific activity of benzoylated GalCer derivatives, the purified molecular species were quantitated by a sphingosine assay (Naoi et al., 1974). Control samples were treated with NaB[^{3}H]H_4 only, and the specific activity was subtracted from that of the samples oxidized with galactose oxidase.

RESULTS

Oxidation of GalCer in a Membrane Environment. The specific labeling of nGalCer by the galactose oxidase/NaB[^{3}H]H_4 method has been previously demonstrated to occur in THF/phosphate buffer (Radin, 1972). However, there has been some doubt as to whether GalCer can be labeled in a membrane environment (Yahara et al., 1980a; Linington & Rumsby, 1980). This uncertainty stems, at least in part, from the relatively high nonspecific labeling of nGalCer by this method. In order to verify that nGalCer was indeed oxidized in a membrane environment, the specific activity of nGalCer oxidized and labeled in multilamellar egg PC liposomes was assessed by directly determining the specific activity of the galactosyl moiety of the glycolipid by HPAEC-PAD. Figure 1 shows the HPAEC-PAD separation of galactose and the resultant radiochromatograms from GalCer oxidized and labeled with NaB[^{3}H]H_4 as well as a control sample treated with NaB[^{3}H]H_4 only. The figure shows clearly that when GalCer is oxidized and labeled, significant radioactivity coelutes with the galactose moiety. The control sample of GalCer, on the other hand, contained no significant radioactivity in the galactose fractions.

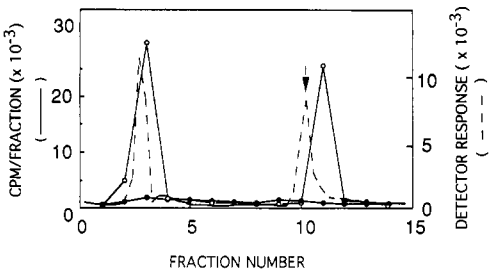


FIGURE 1: HPAEC-PAD radiochromatogram of galactosyl residues of GalCer oxidized with galactose oxidase in egg PC liposomes and reduced with NaB[^{3}H]H_4. The galactose was separated on an IONPAC column after hydrolysis of GalCer in 2 N TFA. Fractions were collected at 1-min intervals and counted by liquid scintillation. (– – –) Pulsed amperometric detector response at 1000 pAmp response range; (○) cpm/fraction of sample oxidized and reduced with NaB[^{3}H]H_4; (●) cpm/fraction of control sample treated with NaB[^{3}H]H_4 only. An arrow indicates the position of galactose. The curves from the detector response and cpm/fraction are displaced from each other because of a delay in collecting the fraction and because of the size of the fractions collected. The initial peak of radioactivity probably contains breakdown products of ^{3}H-labeled galactose; 75–85% of galactose is destroyed by the hydrolysis procedure regardless of whether free galactose or GalCer is hydrolyzed.

Although no specific labeling of the galactosyl moiety of GalCer occurs when it is treated with NaB[^{3}H]H_4 only, there is extensive nonspecific labeling of the intact lipid as well as the other lipids in the liposomes. Figure 2 shows an autoradiogram of a TLC plate of GalCer oxidized and reduced in egg PC for varying periods of time up to 24 h. Even the sample which was not oxidized had significant radioactivity associated with the GalCer. Furthermore, there is also extensive radioactivity present at the origin, in PC, and above the GalCer bands. This is true in both the oxidized and control samples, despite extraction and washing of the lipids present in the sample. There is, however, no detectable evidence of lipid breakdown products when the TLC plate is stained with iodine vapor (Figure 2A). Thus reliable assessment of the specific labeling of nGalCer (and other glycolipids) requires purification of the glycolipid and a determination of the specific activity of the galactose and/or the nonspecific radiolabeling.

The amount of labeling of the GalCer bands shown in Figure 2 appears to increase with the oxidation time. The specific activity of the galactose group of GalCer at each time point was thus quantitated by HPAEC-PAD analysis. The results indicated that the highest specific activity was obtained after 11 h of oxidation (data not shown); this amount of time was thus used to examine the oxidation in most of the subsequent experiments.

The oxidation of GalCer in liposomes was compared to its oxidation in THF/phosphate buffer, which results in maximal radiolabeling of the glycolipid (Radin, 1972). The specific

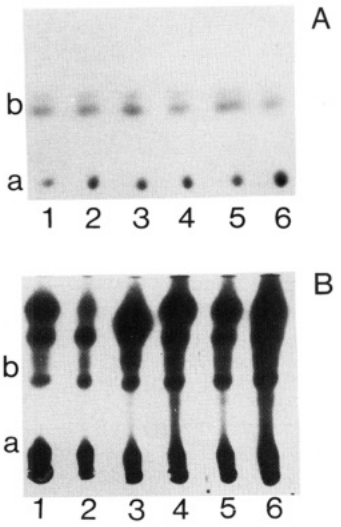


FIGURE 2: TLC purification of nGalCer oxidized and reduced in egg PC liposomes. GalCer was oxidized in egg PC liposomes for varying times up to 24 h and subsequently reduced with NaB[^{3}H]H$_4$. An aliquot of the sample at each time point containing 15 nmol of nGalCer was separated by TLC with chloroform/methanol/ aqueous NH_4OH (65:25:4, v/v/v) as the developing solvent. (A) Iodine stain of TLC plate. (B) 72-h autoradiogram of same plate. Time of oxidation with galactose oxidase: lane 1, no enzyme; lane 2, 2 h; lane 3, 4 h; lane 4, 7 h; lane 5, 11 h; lane 6, 24 h. The letters a and b denote the position of egg PC and nGalCer, respectively, on the TLC plate.

activity of the THF/phosphate buffer-oxidized sample was about 60-fold higher than that of GalCer incorporated into liposomes (not shown). Considering that at most only about 10% of the lipids present in the multilamellar liposomes are actually exposed to the outer aqueous environment (Stewart & Boggs, 1990), the expected difference in the specific activity of the two samples should be 10-fold. The 6-fold greater difference observed, therefore, indicates that the membrane environment significantly impairs the ability of galactose oxidase to oxidize nGalCer compared to THF/buffer.

Effect of GalCer Fatty Acid Chain Length on Oxidation by Galactose Oxidase. Semisynthetic molecular species of GalCer containing 16:0, 18:0, 24:1, 24:0, and 26:0 acyl chains were employed to determine the effect of the fatty acid chain length on oxidation by galactose oxidase. All five molecular species were similarly labeled in THF/phosphate buffer, indicating that the fatty acid chain length did not influence the "intrinsic" activity of the enzyme for GalCer (Table I). This point was important to establish because previous results by Lingwood (1979) showed that galactosylglycerolipid (GG) could not be oxidized by galactose oxidase in THF/phosphate buffer, despite its having a terminal galactosyl residue. The resistance of GG to galactose oxidase oxidation was probably due to an effect of its lipid moiety which is an alkylacyglycerol, as opposed to the ceramide group of GalCer. Thus, it was conceivable that the fatty acyl chain length of GalCer could perhaps influence the intrinsic activity of the enzyme.

These species were either incorporated individually in liposomes or mixed together in an equimolar ratio, and the oxidation of the different molecular species was compared. In all cases the total proportion of GalCer in egg PC was 10 mol %. When the molecular species were incorporated individually in liposomes, the C16:0- and C18:0-GalCer species were oxidized significantly less than the longer chain species (Table II). Similar results were obtained when these species were mixed together in liposomes, oxidized, and separated into their individual components by reverse-phase HPLC of the per-*o*-benzoylated derivatives for determination of the specific

Table I: Comparison of Oxidation of Different Molecular Species of GalCer in THF/Phosphate Buffer[a]

molecular species	relative specific activity[b]
C16:0-GalCer	1.11 ± 0.10
C18:0-GalCer	1.04 ± 0.16
C24:1-GalCer	0.99 ± 0.06
C24:0-GalCer	1.00
C26:0-GalCer	1.17 ± 0.06
NFA-nGalCer	1.27 ± 0.42
HFA-nGalCer	1.00

[a] GalCer was dissolved in THF/phosphate buffer, pH 7.0, 1:1 (v/v). Samples were oxidized with 100 units of galactose oxidase per μmol of GalCer for 11 h. The specific activity for the natural species was determined by HPAEC-PAD analysis of galactosyl residues. That of the semisynthetic species was determined by HPLC separation of the molecular species and quantitation by sphingosine assay, after substraction of background labeling. [b] Values for each molecular species are normalized relative to the specific activity of C24:0-GalCer and are expressed as the mean ± standard deviation, $n = 3$. A standard deviation of ±0.05 for the C24:0 species was calculated by normalizing the values to those of the C24:1 species. Values for the NFA-nGalCer fraction are normalized relative to those for the HFA-nGalCer fraction and are expressed as the mean ± standard deviation, $n = 5$. The specific activity of nGalCer obtained in THF/buffer was 2.3×10^4 cpm/nmol in comparison to a value of 4×10^2 cpm/nmol for nGalCer in egg PC liposomes.

Table II: Comparison of Oxidation of GalCer Molecular Species in Egg PC Liposomes

molecular species	relative specific activity[a]		
	equimolar mixture	individual	all[b]
16:0	0.12 ± 0.09*,++ $n = 3$	0.11 ± 0.11*,+ $n = 8$	0.11 ± 0.11*,+ $n = 11$
18:0	0.25 ± 0.18*,+++ $n = 3$	0.21 ± 0.22*,+ $n = 7$	0.22 ± 0.21*,+ $n = 10$
24:1	2.6 ± 3.1[c] $n = 3$		
	0.40 ± 0.32 $n = 2$	0.65 ± 0.55 $n = 6$	0.59 ± 0.52*** $n = 8$
24:0	1.0 $n = 3$	1.0 $n = 8$	1.0 $n = 11$
26:0	0.69 ± 0.29 $n = 3$	0.79 ± 0.22*** $n = 6$	0.76 ± 0.25** $n = 9$

[a] Values for each molecular species are normalized relative to the specific activity of C24:0-GalCer and are expressed as the mean ± standard deviation. The samples were oxidized in egg PC liposomes either as an equimolar mixture of the five molecular species or individually with only one species present in each sample of liposomes. A standard deviation for the C24:0 species was determined by noramalizing the values for the C24:0 species by dividing by the values for the C26:0 species. This gave values for C24:0/C26:0 of 1.73 ± 0.71 ($n = 3$) for the experiments where the species were mixed, 1.40 ± 0.47 ($n = 6$) for the experiments where each species was measured individually, and 1.51 ± 0.58 ($n = 9$) for the total of all experiments. The specific activities of the equimolar mixtures were determined by HPLC separation of the molecular species, quantitation by sphingosine assay, and determination of the cpm/nmol of each, after subtraction of the cpm/nmol of control samples which were not treated with galactose oxidase. The specific activities of the samples oxidized as individual molecular species were determined by HPAEC of the galactose moiety and determination of the cpm bound to galactose. Statistical analysis by Student's *t*-test: (*) significantly different from C24:0, $p < 0.005$; (**) significantly different from C24:0, $p < 0.025$; (***) significantly different from C24:0, $p < 0.05$; (+) significantly different from C26:0, $p < 0.005$; (++) significantly different from C26:0, $p < 0.025$; (+++) significantly different from C26:0, $p < 0.05$. [b] Mean of all experiments where species were mixed in liposomes and where they were present individually in separate liposomes. [c] The oxidation of C24:1 was unusually high in one experiment. This value was omitted for the $n = 2$ determination of the mean and also in determination of the mean for all experiments ($n = 8$).

activities (Table II). A typical HPLC chromatogram of a mixture of five per-*o*-benzoylated GalCer molecular species is shown in Figure 3 and demonstrates that the five species were effectively separated. The similar results obtained for

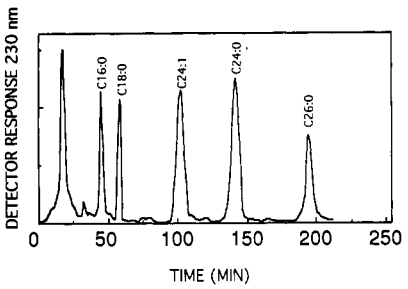


FIGURE 3: HPLC chromatogram of an equimolar mixture of five semisynthetic molecular species of GalCer, as per-*o*-benzoylated derivatives. About 100 nmol of glycolipid were injected, and the detector response range was 0.25 absorption units full scale. Each peak was identified by chromatography of the individual molecular species.

Table III: Comparison of the Effect of Hydroxylation and Membrane Environment on the Oxidation of nGalCer in Liposomes

	relative oxidation[a]	
HFA-nGalCer/NFA-nGalCer	0.21 ± 0.04*	$n = 5$
C24:Oh-GalCer/C24:0-GalCer	0.50 ± 0.05*	$n = 3$
nGalCer in SM-CHOL/nGalCer in PC	0.22 ± 0.13*	$n = 5$

[a] Values after oxidation for 1 h are shown. Normalized values from n different experiments were averaged and expressed as the mean ± SD. Statistical analysis by Student's t-test: (*) The ratio is significantly different from 1, $p < 0.005$. Labeled HFA-nGalCer and NFA-nGal were oxidized in egg PC liposomes, and oxidation was determined from the amount of $^3H_2O_2$ released from labeled substrate. Values after oxidation for 45 min are shown. C24:Oh-GalCer and C24:0-GalCer were oxidized in egg PC liposomes as described in the caption to Figure 4. Values after oxidation for 1 h are shown. NFA-nGalCer was oxidized in egg PC or bSM/CHOL liposomes as described in the caption to Figure 5.

the mixtures indicate that the differences observed with the individual species are not due to differences in properties or surface area of the liposomes. It also indicates that the use of this technique on natural mixtures is feasible.

Although the results varied for C24:1, it was oxidized significantly more than the shorter chain species in most experiments and even more than the C24:0 species in some experiments. However, the mean oxidation of C24:1 averaged over all experiments was less than that of C24:0 and the difference was statistically significant. The results were not as variable for the C24:0 and C26:0 species. The C26:0 species was oxidized somewhat less than the C24:0 species despite its greater length. Although small, the difference was statistically significant. In 8 of 11 total experiments where the species were either mixed or present individually, oxidation of the C16:0 species was less than 20% of that of the C24:0 species, and in 9 of 10 experiments the oxidation of the C18 species was less than 40% of that of the C24:0 species.

Effect of Fatty Acid Hydroxylation of GalCer. Because the specific activity of GalCer oxidized and labeled in egg PC was low, the oxidation of HFA and NFA fractions of nGalCer were compared by an alternate method to conserve material for kinetic analyses. Liposomes containing either [6-^{3}H] HFA- or [6-^{3}H]NFA-GalCer, each prepared with identical specific activities, were oxidized with galactose oxidase, and the amount of radioactivity released (as $^3H_2O_2$) was used as a measure of oxidation. The oxidation of NFA-nGalCer was found to be 5 times greater than that of HFA-nGalCer after 45 min of oxidation (Table III). The dependence of velocity of oxidation on substrate concentration was determined in order to examine the effect of hydroxylation further. Increasing concentrations of egg PC liposomes containing HFA- or NFA-nGalCer were incubated for 45 min with a constant amount of enzyme. The kinetic parameters K_m and V_{max} were determined from Lineweaver–Burk plots of the data and are shown for a representative experiment in Table IV. The activity of the enzyme for HFA-nGalCer, determined by dividing V_{max} by K_m, was 37% of that for NFA-nGalCer. When values from three experiments were averaged, the relative activity of galactose oxidase for HFA-nGalCer was only 0.3 ± 0.1 times that of NFA-nGalCer (Table IV). Thus hydroxylation of the fatty acid apparently reduced the ability of the enzyme to oxidize the galactosyl residue at the membrane surface. However, the fatty acid compositions of the HFA and NFA fractions of nGalCer are different (Jones et al., 1990). Therefore, the effect of hydroxylation on the oxidation of GalCer molecular species with very long fatty acid chains (C24:0 only) was examined using semisynthetic species. The specific activities of C24:0- and C24:0h-GalCer labeled by NaB[^{3}H]H_4 after galactose oxidase oxidation, shown in Figure 4, confirm that hydroxylation reduces the oxidation of the GalCer even when the fatty acid chain of both molecular species is very long and saturated. After 1 h of oxidation, C24:0-GalCer was oxidized twice as much as C24:Oh-GalCer (Table III).

Effect of Membrane Environment. The effect of increasing the PC chain length, and hence bilayer thickness (McIntosh, 1978), on GalCer oxidation was determined by comparison of the relative oxidation of NFA-nGalCer in DMPC/CHOL, DSPC/CHOL, and in egg PC with and without CHOL. The apparent K_m and V_{max} values were both affected by changes in the membrane environment (Table IV). The activity = V_{max}/K_m was determined, normalized to that of DSPC/CHOL, and averaged for three experiments. NFA-nGalCer was most reactive when incorporated into egg PC liposomes, and the presence of CHOL did not have a significant effect. However, the relative activity of the enzyme for NFA-nGalCer in egg PC was 10 times higher than that found for DSPC/CHOL liposomes, indicating that GalCer was not oxidized as well in DSPC/CHOL as in egg PC. NFA-nGalCer incorporated into DMPC/CHOL was oxidized 3.8 ± 0.4 times better than in DSPC/CHOL.

The rate of oxidation of GalCer was also examined in bSM/CHOL liposomes. The data shown in Figure 5A indicate that the oxidation of NFA-nGalCer was much slower in bSM/CHOL vesicles than in egg PC. After 1 h of oxidation, nGalCer was oxidized almost 5 times more in PC than in bSM/CHOL vesicles (Table III). In addition, Figure 5B shows that when two semisynthetic molecular species with C16:0 and C24:0 fatty acids were oxidized in bSM/CHOL liposomes, no difference in specific activity was observed over a 3-h period. This is in distinct contrast to the situation in egg PC liposomes, where increasing the fatty acid chain length of GalCer significantly improved its oxidation by galactose oxidase even after a longer incubation time (Table II).

DISCUSSION

This study resolves a previous controversy (Linington & Rumsby, 1980; Yahara et al., 1980b) about whether GalCer in a membrane environment can be oxidized by galactose oxidase. We have examined the labeling directly by anion exchange chromatography of the galactosyl residue itself and shown unequivocally that tritium is associated with the galactosyl moiety. However, the extent of GalCer oxidation in a membrane environment is significantly less than in THF/buffer, indicating that the membrane environment impairs the ability of galactose oxidase to act on the lipid. This may be due to reduced accessibility of the galactose at the bilayer

Table IV: Comparison of the Kinetic Parameters for the Activity of Galactose Oxidase on HFA- and NFA-nGalCer and on nGalCer in Different Membrane Environments

liposomes[a]	K_m (mM)[b]	V_{max}[b]	activity[b]	normalized relative activity[c] (mean of three experiments)
		Effect of Hydroxylation[d]		
egg PC/NFA-nGalCer	0.123	0.059	0.48	1.0
egg PC/HFA-nGalCer	0.088	0.016	0.18	0.3 ± 0.1*
		Effect of Membrane Environment[d]		
egg PC/NFA-nGalCer	0.116	0.399	3.44	11.2 ± 1.7*
egg PC/CHOL/NFA-nGalCer	0.145	0.395	2.72	10.1 ± 0.2*
DMPC/CHOL/NFA-nGalCer	0.288	0.332	1.15	3.8 ± 0.4*
DSPC/CHOL/NFA-nGalCer	0.073	0.019	0.26	1.0

[a] Liposomes contained egg PC/GalCer at a mole ratio of 1.0:0.1 or PC/CHOL/GalCer at a mole ratio of 1.0:0.75:0.1. [b] V_{max} is expressed as nmol of GalCer oxidized/(min·unit of enzyme). V_{max} and K_m values are from a representative experiment and were determined from Lineweaver–Burk plots of velocity vs substrate concentration curves, with each concentration point an average of triplicates (standard deviation <5% of the value). The correlation coefficient of each line was 0.98 or greater. The activity is V_{max}/K_m. [c] The normalized relative activity of the enzyme is determined by dividing V_{max}/K_m for each set of liposomes by the V_{max}/K_m value of egg PC/NFA-nGalCer in the upper part of the table or by the V_{max}/K_m value of DSPC/CHOL/NFA-nGal in the lower part of the table. Normalized relative activity values from three different experiments (each in triplicate) were averaged, and the values are given as the mean ± standard deviation. [d] The representative experiments shown for the effect of hydroxylation and membrane environment were done with different batches of enzyme and cannot be compared directly. Statistical significance by Student's *t*-test: (*) Values are significantly different from 1, $p < 0.005$.

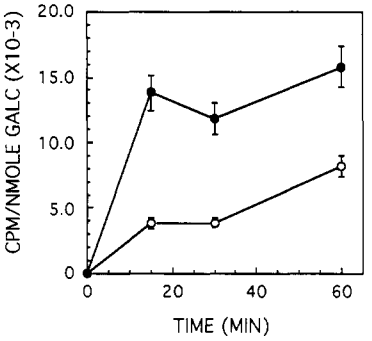
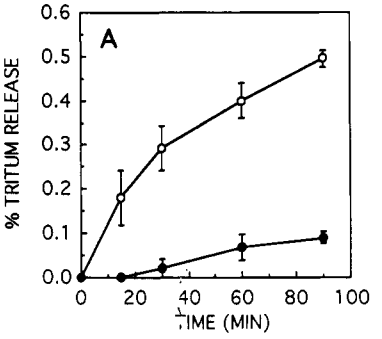


FIGURE 4: Comparison of the rate of oxidation of C24:0- (●) and C24:0h-GalCer (○) in egg PC. Samples, in duplicate, were treated with galactose oxidase and subsequently reduced with NaB[^{3}H]H$_4$. Specific activity was determined by sphingosine analysis. Control values from unoxidized samples treated only with NaB[^{3}H]H$_4$ were subtracted from each point. Error bars indicate the standard deviation.



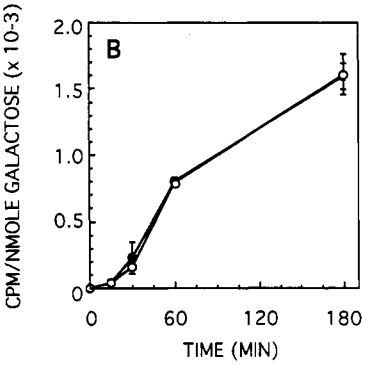


FIGURE 5: Oxidation of GalCer in bSM/CHOL liposomes. (A) Time course of NFA-nGalCer oxidation in egg PC (○) or bSM/CHOL (●) liposomes by measurement of $^3H_2O_2$ release. (B) Time course of the oxidation of C24:0-GalCer (●) and C16:0-GalCer (○) in bSM/CHOL liposomes. Specific activity was determined by HPAEC analysis of galactose. All time points were done in duplicate. The two curves are superimposable at most time points. Error bars indicate the standard deviation. Repeated experiments gave similar results.

surface, consistent with studies of Lampio et al. (1988), who showed that the oxidation of a number of glycolipids in liposomes increased with the size of the carbohydrate head group.

Indeed, we have shown further that the oxidation of GalCer in PC membranes by galactose oxidase is improved by increasing the fatty acid chain length of GalCer and by decreasing the fatty acid chain length of the auxiliary lipid in the surrounding bilayer. Hydroxylation of the fatty acid reduces its oxidation, even for long chain fatty acid species. These results agree with previous studies from our and other laboratories, using antibodies to glycolipids. These studies showed that glycolipids with long chain fatty acids are recognized by antibody better than those with short fatty acids and that the recognition decreases with an increase in the chain length of the PC in its environment (Alving & Richards, 1977; Crook et al., 1986; Stewart & Boggs, 1990). In addition, a glycolipid with hydroxylated fatty acids was recognized less well than that with non-hydroxy fatty acids (Crook et al., 1986; Stewart & Boggs, 1990). However, it could be argued that the effects of ceramide composition on antibody recognition were due to participation of the ceramide acyl chain in the antigenic epitope recognized by the antibody. This possibility is eliminated using galactose oxidase. The similar results obtained with the latter technique strengthen the conclusion that the results are due to altered recognition of the carbohydrate on the bilayer surface.

We have also demonstrated in this study that mixtures of GalCer molecular species containing a variety of different fatty acid chains can be oxidized and labeled in liposomes, and the specific activity of the individual molecular species can be determined following HPLC separation of the individual components. Galactose oxidase can be used to examine the exposure of different glycolipids in cellular membranes (Gahmberg & Hakomori, 1975; Lampio et al., 1984, 1986),

after purification of the individual classes of glycolipids by standard techniques. HPLC separation of the individual molecular species of each glycolipid class would then allow a comparison of their degree of oxidation in order to determine the dependence of exposure on ceramide composition.

The changes in the fatty acid composition of GalCer and in the membrane environment could affect the miscibility of the glycolipid in the bilayer, the orientation and conformation of the galactosyl head group, or the exposure of the head group at the membrane surface. However, both short and long fatty acid chain species of glycolipids have been shown to be miscible in the liquid-crystalline phase of unsaturated and saturated forms of PC, at the low concentrations used here (Bunow & Levin, 1988; Gardam & Silvius, 1989; Morrow et al., 1992). GalCer is miscible with SM even in the gel phase and in the absence of cholesterol (Johnston & Chapman, 1988). In the presence of cholesterol at 20 °C, all of the PC species used and SM should be in a similar phase intermediate between the gel and liquid-crystalline phases (McMullen et al., 1993). Although phase diagrams of DSPC/CHOL/GalCer have not been reported, Ruocco and Shipley (1984) have shown that, at concentrations below 20 mol %, GalCer is completely miscible at 22 °C in 1:1 DPPC/CHOL. However, clustering of GalCer in small microdomains in PC or SM, even in the intermediate or liquid-crystalline phases, cannot be ruled out. Spin-labeled GalCer has been reported to cluster in 1:1 DPPC/CHOL at 25 °C even at low GalCer concentrations (Suzuki et al., 1981). On the other hand, fluorescent GalCer probes were miscible in 1:0.8 PC/CHOL at low GalCer concentrations (less than 15 mol %) (Molotkovsky et al., 1991; Silvius, 1992). Cholesterol significantly enhanced long-lived (1 week at room temperature) metastable solubility of higher concentrations of GalCer in PC after cooling from a high temperature (Johnston & Chapman, 1988; Silvius, 1992). Since our samples were oxidized with galactose oxidase at 20 °C shortly after preparation by hydrating at a high temperature, we think it is most likely that all molecular species of GalCer are mixed with the other lipids in all cases.

Hydroxylation of the fatty acid of GalCer reduced its oxidation by galactose oxidase in PC bilayers. The crystal structure of the hydroxy fatty acid species of GalCer indicates that the NH group hydrogen bonds with both the fatty acid hydroxyl group and with the oxygen of the glycosidic linkage (Pascher & Sundell, 1977). This results in an orientation of the galactose parallel to the bilayer. Since the glucose of the non-hydroxy fatty acid form of hydrated glucosylceramide has been shown by NMR to have an extended conformation perpendicular to the bilayer (Skarjune & Oldfield, 1982), this has led to the suggestion that the fatty acid hydroxyl group may cause an altered conformation of the sugar (Calander et al., 1988). This might explain the fact that some strains of bacteria (Stromberg et al., 1988) and some monoclonal antibodies (Kannagi et al., 1983) bind preferentially to the HFA form of certain glycolipids. However, comparison of the galactose conformation of the HFA and NFA species of GalCer itself in the same environment is necessary to support this conclusion. NMR spectroscopy of HFA and NFA GalCer molecular species, deuterated at the C-6 position of the galactose, detected only a minor effect of hydroxylation of the GalCer fatty acid on the galactose orientation and conformation (Singh et al., 1992a,b). Nyholm et al. (1990) point out that the parallel sugar conformation could expose polar parts of the ceramide moiety such as the fatty acid hydroxyl group, thus allowing those lectins which bind preferentially to the HFA species to interact directly with the hydroxyl group. This, rather than an altered carbohydrate conformation of the HFA relative to the NFA species, might account for this preferential binding.

Thus, altered exposure of the carbohydrate at the membrane surface due to changes in fatty acid chain length or hydroxylation of the glycolipid is the most likely explanation for the observed results. A decrease in exposure of the carbohydrate head group of the HFA species of glycolipids, rather than a change in conformation, is also supported by our previous study of antibody binding to semisynthetic species of CBS (Stewart & Boggs, 1990). We found that antibodies purified from liposomes containing CBS with C16:0h fatty acids were able to recognize C24:0-CBS in liposomes even better than C16:0h-CBS. This indicated that the antibodies which bound to C16:0h-CBS did not do so because of a unique conformation of the sugar caused by the fatty acid hydroxyl group nor because the OH group formed part of the antigenic epitope.

Altered exposure might also be expected to result in altered motion or orientation of the sugar head group. However, NMR studies of C-6 galactose-deuterated GalCer showed that although the motion of the galactose head group was restricted in PC bilayers, increasing the GalCer fatty acid chain length from C18:0 to C24:0 did not have a significant effect on its conformation (Singh et al., 1992a; Jarrell et al., 1992). Nevertheless, as shown here, these modifications have significant effects on the recognition of the carbohydrate head groups. Possibly the altered exposure is of a transient and dynamic nature which is rapid on the NMR time scale, due to vertical movement of the glycolipid up and down in the bilayer.

The long chain species of GalCer have a significantly different hydrocarbon structure from the surrounding PC molecules, whether egg PC, DMPC or DSPC. Due to the configuration of the amide region of the ceramide group, the sphingosine chain of GalCer has an effective bilayer penetrating length of only 13–14 carbons, thus making the ceramide moiety of the long chain species highly asymmetric (Dahlen & Pascher, 1979). The long fatty acid chain species could accommodate to the PC bilayers if the acyl chain either interdigitates across the bilayer center, bends or kinks within its own monolayer due to increased gauche isomerization, or terminates at the bilayer center in a more trans conformation thus forcing the head group to extend further above the plane of the membrane surface. Mehlhorn et al. (1988) have shown that, in PC bilayers, the motion of a GalCer spin probe with a spin-labeled C24:0 chain was more restricted than that with a spin-labeled C18:0 chain (both spin labeled at carbon 16), suggesting that the long fatty acid might be interdigitated. However, Morrow et al. (1992), employing GalCer with perdeuterated C24:0 fatty acids and NMR spectroscopy, have shown that, in PC membranes, GalCer with C24:0 fatty acyl chains has highly disordered methyl termini. Thus, if interdigitation occurs, it may be short lived. If the acyl chain of the glycolipid is not long enough to span the PC bilayer, it would disrupt van der Waals interactions between the PC acyl chains, thus decreasing the probability of interdigitation.

A theoretical study of the packing of dissimilar chain length lipids predicts that they are more likely to pack with the acyl chain terminal methyl groups aligned on the same plane in the center of the bilayer than with the polar head groups all on the same plane (Jacobs et al., 1977). This might then cause the PC molecules surrounding long chain GalCer species to have more trans character resulting in more extended fatty acid chains and the long GalCer fatty acid chains to have more gauche character and less extended chains in order to

decrease the mismatch between the chain lengths and expose less of the hydrophobic region of the long fatty acid chains of GalCer above the apolar–polar interface. This has been reported to occur in binary mixtures of PC's of different chain lengths (Sankaram & Thompson, 1992). Indeed, cholesterol has similar effects on the fatty acid chains of PC resulting in a decrease in the bilayer thickness of DSPC and an increase for DMPC (McIntosh, 1978; Sankaram & Thompson, 1990). This would result in a difference in the order and character of both the surrounding PC molecules and the GalCer molecules in mixtures with a short chain PC compared to a longer chain PC. This alone could conceivably account for differences in the binding or activity of galactose oxidase and other ligands to glycolipids. However, it seems unlikely that complete matching of chain lengths of such greatly dissimilar PC and GalCer species as those used in this study could be achieved by this mechanism and that altered exposure of the glycolipid carbohydrate must account for at least some of the difference in recognition of different molecular species in the same or different environments by the enzyme and by antibodies. A further increase in GalCer acyl chain length from 24 to 26 carbons did not increase oxidation of GalCer in PC and in fact decreased it somewhat. This may be due to increased interdigitation of the longer C26 chain which may be better able to span the PC bilayer than the C24 chain. Altered exposure of the carbohydrate of C26 species compared to the C24 species of glycolipids could affect cell recognition phenomena and be involved in the pathogenesis of adrenoleukodystrophy.

The acyl chain hydroxyl group may promote intermolecular hydrogen bonding of other hydrogen-bonding groups in the ceramide moiety of the HFA species with the carbonyls of phosphatidylcholine. This, in turn, may cause the HFA species of glycolipid to become further embedded or to remain embedded within the bilayer for a longer time and thereby reduce exposure of the carbohydrate. Hydroxylation of the fatty acid of cerebroside sulfate (CBS) increases the phase transition temperature, suggesting that it promotes intermolecular hydrogen bonding within a pure CBS bilayer (Boggs, 1987; Boggs et al., 1984). This is supported by a recent FTIR study on the HFA and NFA species of CBS and GalCer (Tupper et al., 1992).

The oxidation of GalCer in bSM liposomes was reduced compared to egg PC. In addition, and in distinct contrast to the results with PC liposomes, increasing the fatty acid chain length of GalCer from 16 to 24 carbons had no effect on the oxidation. This is in agreement with our previous study using antibody to CBS where we found that changes in either the chain length or hydroxylation of CBS did not alter its antigenic recognition in bSM bilayers (Stewart & Boggs, 1990). A number of other studies have reported that bSM reduces the exposure of glycolipid antigens to antibody relative to PC (Alving & Richards, 1977; Inoue et al., 1971), and this has been attributed to the longer acyl chains generally found in bSM (Calhoun & Shipley, 1979). However, we have found that the fatty acid chain length of SM has only a minor effect on the recognition of CBS by antibody (Stewart & Boggs, 1990). Although SM and PC share the same phosphorylcholine head group, the hydrophobic moieties are different. PC contains diacylglycerol whereas SM contains ceramide. It is likely that the lack of effect of the fatty acid chain of GalCer on its exposure to galactose oxidase in bSM is due to the presence of the ceramide moiety of SM. The hydrogen-bonding capacity of the amide linkage and sphingosine hydroxyl of SM may result in a greater penetration of the glycolipid into the bilayer through interactions of SM with similar groups on the glycolipid molecule. This may mean that the GalCer may be presented in a different way in SM than in PC. Indeed, we have found that CBS forms distinct antigenic structures in bSM when compared to PC and that these antigenic structures are recognized by different populations of antibody within a polyclonal anti-CBS antiserum (Stewart & Boggs, 1990). Thus both the lipid composition of the membrane environment and the ceramide composition of glycolipids could control their receptor activity.

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