The Topological Orientation of N,N'-Diacylchitobiosylpyrophosphoryldolichol in Artificial and Natural Membranes*

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Purified N,N'-diacylchitobiosylpyrophosphoryldolichol (chitobiosyl-lipid) in the presence of detergent was shown to act as a substrate for the soluble enzyme galactosyltransferase. The nature of this transfer reaction and the galactose-containing trisaccharide-lipid product have been partially characterized. Using galactosyltransferase as a probe, the topological arrangement of chitobiosyl-lipid in both artificial and natural membranes has been examined. When incorporated into unilamellar liposomes made from phosphatidylcholine, the disaccharide residue of chitobiosyl-lipid adopts a random transbilayer orientation. Furthermore, no significant mobility in the transverse plane of the membrane (i.e. flip-flop) is detectable. Using both sealed total microsomes or isolated rough microsomes from hen oviduct, the topology of chitobiosyl-lipid after its biosynthesis in the membrane has been determined. The results suggest that, once formed, chitobiosyl-lipid is a relatively static component of the membrane and is oriented with its disaccharide residue facing the lumen of the endoplasmic reticulum.

Relatively little attention has been paid to the question of lateral or transverse movement of polyisoprenoid carriers of glycose units within the hydrophobic interior of membranes. It is known that the C15 polyprenoids which participate in the synthesis of bacterial cell wall polymers are able to move in the plane of the membrane (1). Further, the involvement of these polyprenoids in the vectorial transfer of fluorescently labeled undecaprenyl diphasphate-N-acetyluramylpentapeptide to nascent peptidoglycan external to the bacterial cell membranes has recently been investigated (2). However, no work has been reported on the membrane orientation or dynamics of the saccharide-lipid intermediates involved in the assembly of the oligosaccharide chain of N-linked glycopolypeptides in eukaryotes (3-10). The lipid moiety of these intermediates, dolichol phosphate, differs from the shorter plant and bacterial isoprenoids in two respects. First, it has a saturated α-isoprene residue, and second, the total number of isoprene residues ranges from 16 to 20 (11). Because the total length of an extended form of this molecule (100 Å), estimated from measurements of molecular models, exceeds the thickness of a phospholipid bilayer (60 to 70 Å), its topological orientation within this membrane and its interaction with other components of membranes is especially interesting.

Although recently it has been demonstrated that the transferases involved in synthesis of the dolichol-linked intermediates in the oviduct are localized in the rough endoplasmic reticulum (12), the topological distribution of these enzymes and their products within this organelle are unknown. As a first step in analyzing the behavior of dolichol-saccharide intermediates in membranes, we have examined the transbilayer topology of N,N'-diacylchitobiosylpyrophosphoryldolichol (chitobiosyl-lipid) both in liposomes and in microsomes from hen oviduct. The structure of this lipid-linked disaccharide, which is formed in a two-step process from dolichol phosphate and UDP-N-acetylglucosamine (3, 9, 11), is shown in Structure 1.

It was found that soluble galactosyltransferase could be used as a membrane impermeable probe to investigate the orientation of N,N'-diacylchitobiosylpyrophosphoryldolichol, since the enzyme was observed to catalyze the reaction:

\[
\text{UDP-Gal} + \text{(GlcNAc)}_2 \rightarrow \text{P-P-dolichol}
\]

\[
\text{Mn}^{+} \rightarrow \text{Gal-(GlcNAc)}_2 \rightarrow \text{P-P-dolichol + dolichol-P + UDP}
\]

Using this enzymatic probe and phosphatidylcholine liposomes prepared in the presence of trace amounts of labeled N,N'-diacylchitobiosylpyrophosphoryldolichol, it was found that approximately equal amounts of the disaccharide-lipid are located at each face of the membrane. The results also indicate the absence of significant transverse movement of the saccharide-lipid within a 3-h period. Application of this enzymatic probe to sealed microsomes from hen oviduct indicates that chitobiosyl-lipid is a relatively static component of the endoplasmic reticulum membrane and is oriented with its disaccharide residue facing the lumen of this organelle. A brief report of some aspects of this work has appeared elsewhere (13).

EXPERIMENTAL PROCEDURES

Materials and Methods

UDP-N-acetyl[^14]C]glucosamine (300 mCi/mmole), UDP-N-acetyl[^1]H]glucosamine (6.6 Ci/m mole), N4[^1]H]acetylglucosamine (500 mCi/m mole), and UDP-N-acetyl[^1]H]galactosamine (14.6 Ci/m mole) were purchased from Amersham (Arlington Heights, Ill.). Partially purified galactosyltransferase (4.5 units/mg) from bovine milk, soy bean trypsin inhibitor, ribonuclease type XII-B (81 units/mg), and α-chymotrypsin type YS-143 units/mg were purchased from Sigma Chemical Co. (St. Louis, Mo.). Trypsin (250 units/mg) was obtained from Worthington Biochemicals (Freehold, N. J.). Highly purified galactosyltransferase (600 units/mg) was a generous gift from Dr. Joel
Knockout of the Johnston's Hopkins University School of Medicine. Ery phospholipid-interaction was performed on Seracon Research Laboratories (London, Ontario, Canada). β-Galactosidase from bovine liver and Escherichia coli was purchased from Sigma and Boehringer-Mannheim Biochemicals (Indianapolis, Ind., respectively). Phosphatidyl-β-D-galactosyltransferase was purchased from Grant. Phosphatidyl-β-D-galactosyltransferase was obtained from Boehringer Mannheim and converted to the Na+ form by ion exchange on AG 50W-X4 (200 to 400 mesh H form) purchased from Bio-Rad Laboratories, Richmond, Cal., respectively. The solution was brought to pH 7.5 with NaOH. Macrosephosphate assays were carried out by modification of the method of Mann (24). β-Galactosidase assays were carried out by the method of Salter et al. (35). When peptide-polyester substrates were used, assays were carried out by a modification of the method of Lee (25). The enzyme source for preparation of highly purified N,N-diacylphosphorylcholine was a crude membrane fraction prepared from the maximum portion of bovine liver as previously described (17). Protein was assayed by the method of Lowry (15) or by the BioRad Protein assay (ovalbumin standard). (BioRad Laboratories). (15). Liquid phosphatidylcholine was estimated by the method of Haubert (20). Authentic N,N-diacylcholines were supplied by Dr. W. Stone. Chloroform was prepared as described previously (19).

**Chromatographic Procedures**

Descending paper chromatography was performed on Whatman No. 3MM paper using Solvent A, butanol/pyridine/water (48:40:20). Radioactivity on paper chromatograms was detected by scanning with a Packard Model 7300 Radiographic Scanner. Sugars were detected by staining with amine-diphenylamine (22). Thin layer chromatography was performed on 0.5-mm Silica Gel 60 precoated plates (EM Laboratories) in the following solvent systems: R, CHCl3/CH3OH/H2O (60:35:5); R, CHCl3/CH3OH/H2O (80:20:5); R, CHCl3/H2O (90:10:3). Radioactivity was detected by scraping the 0.25-mm segments off the plate, eluting with Solvent B, detecting the solvent, and counting in a Packard liquid scintillation counter.

**Preparation and Partial Purification of N-Acetylq-Cl-glucosamine-containing Diacylphosphorylcholine and N,N-Diacylq-Cl-cholines containing Diacylphosphorylcholine**

Large quantities of N-acetylq-Cl-glucosamine-containing diacylphosphorylcholine were prepared and purified by a modification of the method of Moxon and Leshner (25). Two 5-ml (or 10-fold) portions of 250 ml of ovocid membrane preparation (45 mg/ml), 15 ml of 1 M Na2PO4, 5% sucrose, 0.1% NaN3, 1% EDTA, 1% diisothiocyanate, and 50 ml of Tris/HCl (pH 7.2) in a final volume of 200 ml were incubated for 15 min at 37°C. Total N-acetylq-Cl-glucosamine-containing lipids (4.4 x 109 cpm) were extracted with CHCl3/CH3OH (2:1) as described previously (10). The extract was concentrated by rotary evaporation and chromatographed on a DEAE-cellulose column (1.5 x 15 cm) in CHCl3/CH3OH (2:1). Labeled material was eluted with 100 ml ammonium formate and appeared as a single peak of radioactivity (3 x 1010 cpm). After desalting as described previously (22), the material was chromatographed on a DEAE-cellulose column and eluted with a linear gradient from 0 to 100 mM ammonium formate. A single peak was recovered at 95 mM ammonium formate. This fraction was desalted as before, concentrated by rotary evaporation, and further purified by preparative thin layer chromatography in Solvent B. Bands of two radioactive components (RF values of 0.29 and 0.40) were separated and eluted from the gel with Solvent D. After elution from the gel, the carbohydrate moiety was removed from each of these components by hydrolysis in tetrahydrofuran 0.5 M HCl (4:1) at 30°C for 90 min. The carbohydrate moiety of the material with an RF of 0.29 in Solvent B was shown to comigrate with authentic unlabeled N,N-diacylcholines upon paper chromatography in Solvent A. The carbohydrate derived from the material with an RF of 0.45 in Solvent B was shown to be identical with N-acetyl-glucosamine. The intact labeled glycolipids were further characterized by analytical thin layer chromatography. The material identified as N-acetylq-Cl-glucosamine containing diacylphosphorylcholine was shown to have RF values of 0.17 and 0.74 in Solvents C and D, respectively. In the same two solvents the material identified as N,N-diacylq-Cl-glucosamine containing diacylphosphorylcholine was observed to have RF values of 0.31 and 0.62, respectively. Total recovery of N,N-diacylq-Cl-diacylphosphorylcholine and N-acetylq-Cl-glucosamine was found to be 1.5 x 109 cpm and 0.3 x 109 cpm, respectively.

**Preparation of Unialkylated Liposomes with Incorporated N,N-Diacylcholines**

The method used for incorporation of the purified N,N-diacylq-Cl-cholines into unilamellar liposomes is a modification of the procedure of Kremser et al. (26) and Hassig (22). An aliquot containing 560,000 cpm of the glucosylated 6.5 ml of CHCl3/CH3OH (2:1) was evaporated to dryness under N2, and dissolved in CHCl3 (1 ml). Ery phospholipid containing 10 mg in 0.3 ml of chloroform) was added to this material and the mixture was evaporated to dryness under N2. The lipids were then dissolved in absolute ethanol (150 ml) for large liposomes, 900 ml for small liposomes). Using a 1-ml microcentrifuge syringe equipped with a 29 gauge needle, the ethanol solution was injected into 50 ml of a solution stirred at 30°C containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.0 (Tris-NaCl buffer). The resulting suspension was concentrated by Amicon filtration (UM 10 membrane) to 1 ml and chromatographed on a Sephadex G-10 column (1.5 x 25 cm) prepared and eluted with Tris-NaCl buffer. Fractions of 1.8 ml each were collected and subsequently assayed by removing 100-μl aliquots for total radioactivity using a Packard liquid scintillation counter. The total recovery was 5.5 μl/ml of phospholipid and 90,000 cpm/glycospides. Fractions were pooled and concentrated to 500 ml. The labeled liposomes were stored at 4°C for no longer than 12 h prior to use.

**Electron Microscopy of Liposomes**

An aliquot of a mixture was dehydrated in a 1.5% 20% ethanolic solution of glycospides, and 0.3% 1.5% glutaraldehyde. The specimen was embedded in a Paraplast coated electron microscopy grid. Specimens were examined in a Siemens Elmiskop I electron microscope.

**Galactosyltransferase Assays on Isolated Cholineslipid**

When dextran-oligomerized N,N-diacylq-Cl-cholines containing diacylphosphorylcholine was used as a substrate, the lipids were suspended in 100 ml of a buffer containing 0.3% Triton X-100 in Tris-NaCl buffer. Incubation mixtures also contained 50 mM MgCl2 and varying concentrations of UDP-galactose (10 to 400 μM) and galactosyltransferase (0 to 200 μM of protein). Incubation was carried out at 37°C in a total volume of 250 ml. The reaction was terminated at appropriate time intervals by the addition of 4 ml of CHCl3/CH3OH (2:1), and the products analyzed as described above. In assays using the glycosylated incorporated into liposomes as substrate, 2.5 ml of the liposome suspension (2.5 μmol of total phospholipid, 4.6 x 109 cpm were incubated at 37°C in a buffer containing 100 mM Tris-NaCl buffer with or without 0.15% Triton X-100. Subsequently, 3 ml of MnCl2 (5 mM final concentration), UDP-galactose, and galactosyltransferase in Tris-NaCl buffer were added to the suspension to bring the total volume to 40 ml. The reaction was stopped by removing an aliquot of the suspension and adding it to 1.0 ml of CHCl3/CH3OH (2:1). After addition of 1.5 ml of 0.9% saline to separate the phases, the organic phase containing the glycosylated was evaporated to dryness under N2. The carbohydrate moiety was eluted from the lipid by hydrolysis to tetrahydrofuran 0.5 M HCl (4:1, 15 min at 37°C) and analyzed by thin layer chromatography in Solvent A. The relative amounts of the product (disaccharide) and reactant (disaccharide) was determined by either counting and counting of the resultant chromatograms, or by integration of the area of the tracing of the radiochromatogram scanner by cutout and weighing the paper on an analytical balance. These methods were found to agree within 2%.
β-Galactosidase Assays

When attempts were made to digest the intact galactosylated glycolipid, incubations were performed by suspending the glycolipid in a solution containing 0.15% Triton X-100 and Tris-NaCl buffer (E. coli 0.1 unit) or both ligands (1 unit β-galactosidase was added to the Tris-NaCl buffer and the reaction allowed to proceed for 3 h at 37°C). Zn²⁺ (10 mM) was present when E. coli β-galactosidase was used. The products were analyzed by paper chromatography after mild acid hydrolysis as described above.

β-Galactosidase digestion of the free carbohydrate moiety of the galactosylated glycolipid was performed in a 100-μl reaction mixture consisting of 10 mM Tris, 10 mM ZnCl₂, and 0.1 unit of E. coli β-galactosidase. Product analysis was carried out by paper chromatography as described above. The enzyme activities of the galactosidases used in these experiments were determined both in the absence or presence of 0.15% Triton X-100 using p-nitrophenyl-β-D-galactopyranoside as substrate.

Preparation of Total Microsomes and Isolation of Rough Microsomes

Total microsomes and rough microsomes were isolated by a modification of the method of Czichi and Lennarz (12). The magnet portion of ovocytes from freshly killed laying hens was cleared of connective tissue and minced with scissors in 40 ml of 10 mM Tris/HCl, pH 7.5, containing 0.05% ovalbumin, 60 μg/ml of heparin, 22% sucrose, 33% mannitol (Buffer 1). The mixture was then homogenized in Buffer 1 (4.5 ml/g, wet weight) with a Dounce homogenizer (Vitro) using pestle B. The pellet after centrifugation for 10 min at 13,333 × g was resuspended (46 ml/g, wet weight) in a buffer of 2 M KCl, pH 7.5, containing 11% sucrose, 33% mannitol, 0.05% ovalbumin, and 60 μg/ml of heparin (Buffer 2). This material was homogenized with 10 strokes of the Teflon pestle and subjected to centrifugation at 5,000 × g for 10 min. This postmitochondrial supernatant was then passed through a cheesecloth.

Total microsomes were obtained by subjecting the postmitochondrial supernatant to centrifugation at 27,000 × g for 30 min. The pellet from this step was resuspended (1 to 10 mg/ml) in a buffer containing 50 mM Tris/HCl pH 7.5, 25 mM NaCl, 10 mM MgCl₂, and 5% sucrose (Buffer 3). This material was centrifuged at 27,000 × g for 10 min and the pellet was resuspended in Buffer 3 at a concentration of 7 to 10 mg/ml. This membrane fraction had a nucleic acid content of roughly 17% as estimated by the relative absorbance at 280 and 260 nm.

Isolated rough microsomes were desired, 10 ml of the postmitochondrial supernatant solution were layered over a discontinuous sucrose gradient and handled as described previously (12). Microsomal fractions from the gradient were characterized by electron microscopy, nucleic acid content, and marker enzymes as described previously (12). The pellet recovered from the gradient (rough microsomes) contained a membrane fraction devoid of endogenous galactosyltransferase activity, and having a nucleic acid content greater than 25% as estimated by the relative absorbance at 260 and 280 nm. Electron microscopy of the isolated rough microsomes revealed a nearly homogeneous population of ribosome-studded vesicles (data not shown). Virtually no smooth membranes were observed in this fraction. The specific activities of both mannosyltransferase and N-acetylglucosaminyltransferase were 2 to 3-fold higher in this membrane fraction than in total microsomes. Rough microsomes were resuspended in Buffer 3 at a concentration of 5 to 7 mg/ml prior to use in enzyme assays.

Formation of Labelled Chitobiosyl-Lipid in Microosomal Membranes

Chitobiosyl-lipid was generated in the microsomal membranes by incubation with radiolabeled UDP-N-acetylglucosamine. Total microsomes (2.8 to 3.5 mg of membrane protein) or rough microsomes (0.6 to 1.4 mg of protein) were incubated with either 1 μCi of UDP-N-acetyl[14C]glucosamine or 1 μCi of UDP-N-acetylmannosamine. Incubation was carried out in a total volume of 420 μl (Buffer 3) for up to 30 min at 37°C. When membranes were not to be treated subsequently, the reaction was monitored by stopping at appropriate time intervals with CHCl₃/CH₃OH (2:1) and analyzed as described under "Galactosyltransferase Assays on Isolated Chitobiosyl-Lipid." If membranes were to be used to assess chitobiosyl-lipid accessibility, the reaction was stopped by cooling the tubes on ice and subjecting them to centrifugation at 27,000 × g for 10 min (4°C). The clear supernatant was removed and the pellet carefully resuspended in 150 μl of a buffer containing 50 mM Tris/HCl (pH 7.5), 25 mM NaCl, 10 mM MnCl₂, and 5% sucrose (Buffer 4).

Pretreatment of Microsomal Fractions

After formation of chitobiosyl lipid in the microosomal membrane, the resuspended fractions were either pretreated with those agents listed in Table II or assayed directly using galactosyltransferase. When the membranes were pretreated the following conditions were employed. Detergents (Triton X-100 or taurocholate) were added just prior to treatment with galactosyltransferase or measurement of latency. Paranitrophenyl-KCl treatment to remove ribosomes was carried out essentially as described previously (25), except it was done for 20 min at room temperature. This treatment removed roughly 60% of attached ribosomes as evidenced by release of nucleic acid and decrease in the relative absorbances at 260 and 280 nm. Ribonuclease B-tritium (150 μg/ml, 1 h, 37°C) released roughly 20% of the total nucleic acid from microsomal membranes (data not shown). Treatments with proteases were carried out as described in Table II. All protease treatments were shown to release protein from the surface of the microsomes. This was demonstrated by a significant release of protein (12 to 17% of the total) into the supernatant after centrifugation.

Galactosyltransferase Assays on Microsomal Fractions

To determine whether preformed chitobiosyl-lipid was accessible to galactosyltransferase in sealed microsomes the following types of experiments were carried out. To pretreated membrane fractions (± detergent), 10 μl (1.3 μg, 0.8 unit) of a highly purified preparation of galactosyltransferase (greater than 900 units/mg) were added. UDP-galactose was added to a final concentration of 400 μM. Incubations were carried out for 20 min at 37°C. The reaction was terminated with CHCl₃/CH₃OH (2:1) and the products were analyzed as described under "Galactosyltransferase Assays on Isolated Chitobiosyl-Lipid." The accessibility of chitobiosyl lipid to galactosyltransferase was derived from the relative amounts of trisaccharide-lipid formed and disaccharide-lipid remaining after galactosyltransferase treatment. Control incubations were run for the small amount (less than 10%) of endogenous mannose-containing trisaccharide-lipid present after these incubations. The galactosyltransferase reaction was shown to be totally dependent on the addition of exogenous galactosyltransferase.

Latency Assays

In all microsome experiments, parallel tubes were carried through the microsome "treatment" and centrifugation steps. After those treatments, the integrity of the microsomal membranes was assessed by two independent methods.

1. Mannose 6-phosphatase

The enzyme mannose 6-phosphatase has been used in earlier studies to quantitatively estimate the intactness of microsomes from other sources (26, 27). The microsomes prepared in this study have been previously shown to possess a latent mannose 6-phosphatase activity (28). To assay for this activity, the incubation mixture consisted of 2 mM mannose 6-phosphate and 2.8 to 3.5 mg of total microsomal protein in a total volume of 220 μl (Buffer 4). Boiled membranes (2 min, 100°C) served as controls. After incubation at 37°C, the reaction was stopped with 2 ml of 1-butanol and the amount of released phosphate determined as described under "Experimental Procedures." Optimal activity (0.35 μg of P released/min/mg) was observed at pH 6.5. However, to insure that latency assays were exactly comparable with galactosyltransferase assays, the activity (0.15 μg of P, released/min/mg) was monitored at pH 7.5.

2. β-Glucuronidase

The enzyme β-glucuronidase has been characterized in other systems and shown to be a latent enzyme that is associated with microsomes as well as lysosomes (27, 29, 30). The enzyme was also shown to be present as a latent enzyme in the microsomal preparation described here. Incubation conditions for measuring β-glucuronidase activity were identical with those described for mannose 6-phosphatase assays, except that 2 mM p-nitrophenyl-β-glucuronide or 2 mM phenolphthalein-β-glucuronide were used as substrates. Assays were terminated with the appropriate stopping solutions and the substrate cleaved by centrifugation at 27,000 × g for 15 min. Spectrophotometric analysis was carried out as described under "Experimental Procedures." Specific activities of the enzyme in total and isolated rough microsomes were nearly identical indicating that the preparation was not extensively contaminated with lysosomes. Optimal activity (0.1 μmol of phenolphthalein
RESULTS

Isolation and Purification of N-Acetyl[14C]glucosaminylpyrophosphoryldolichol and N,N'-Diacyetyl[14C]chitobiosylpyrophosphoryldolichol—The two N-acetyl[14C]glucosamine-containing lipids isolated and partially purified in this study have chromatographic characteristics similar to those observed previously (3). Both lipids bound tightly to DEAE-cellulose and were eluted only with ammonium formate concentrations exceeding 95 mM. The results of analysis by thin layer chromatography in three solvent systems (see "Experimental Procedures") are consistent with the results of previous studies (3, 7-9). After mild acid hydrolysis, the saccharide moieties of the two N-acetyl[14C]glucosamine-containing lipids were analyzed by paper chromatography. As shown in Fig. 1, the radioisotopic purity of the carbohydrate moieties of each of the isolated lipids is greater than 95%. It is also apparent that the saccharide residues of the isolated mono- and disaccharide-lipids comigrate with authentic standards of N-acetylgalactosamine and N,N'-diacetyltchitobiose, respectively. With regard to chemical purity, 1 x 10^4 cpm of the purified N,N'-diacetyltchitobiosylpyrophosphoryldolichol contained an undetectable level of lipid phosphate in an assay sensitive to 1 nmol (data not shown).

**N,N'-Diacyetyl[14C]chitobiosylpyrophosphoryldolichol as a Substrate for Galactosyltransferase—**The soluble enzyme galactosyltransferase has been previously shown to catalyze the transfer of galactose to N-acetylgalactosamine derivatives, forming Gal-β-(1→4)-GlcNAc residues (31-33). In searching for an enzymatic probe specific for the hexosamine residues of N,N'-diacetyltchitobiose and [14C]trisaccharide released upon mild acid hydrolysis. In Fig. 2, a typical series of chromatograms is obtained (350 mCi/mmol) is only slightly higher (instead of 2-

![Fig. 1. Paper chromatography of the carbohydrate moiety of purified N,N'-diacyethyl[14C]chitobiosylpyrophosphoryldolichol (A) and N-acetyl[14C]glucosaminylpyrophosphoryldolichol (B). Authentic unlabeled standards (cross-hatched) were chromatographed (Solvent A) in separate lanes and detected with aniline-diphenylamine. In other experiments in which internal standards were mixed with the radioactive product exact coincidence of the labeled and unlabeled compounds was observed.](http://www.jbc.org/content/264/24/9240/F1)

![Fig. 2. Paper chromatographic analysis of carbohydrate moiety of the galactosylated product formed from N,N'-diacetyltchitobiosylpyrophosphoryldolichol. The reaction was stopped and analyzed at 0 (A), 40 (B), and 100 min (C) as described under "Experimental Procedures." In this example of the results of product analysis, the N,N'-diacetyltchitobiosylpyrophosphoryldolichol was incorporated into small liposomes which were subsequently disrupted with 0.15% Triton X-100 prior to incubation (see Fig. 7A for details).](http://www.jbc.org/content/264/24/9240/F2)
Transbilayer Orientation of a Dolichol-linked Disaccharide

Fig. 3. Formation of galactose-containing trisaccharide-lipid from chitobiosyl-lipid. Except as indicated below, the incubation conditions were those described under "Experimental Procedures." A, dependence on time (164 μg/ml of protein, 0.36 mM UDP-galactose); B, UDP-galactose concentration dependence (164 μg/ml of protein, 60 min); C, dependence on concentration of galactosyltransferase (time, 10 min, 0.36 mM UDP-galactose) and presence of α-lactalbumin at 60 μg/ml (Δ).

Fig. 4. Susceptibility of carbohydrate moiety of galactose-containing trisaccharide-lipid to β-galactosidase. Paper chromatographic analysis in Solvent A of trisaccharide incubated for 2 h at 37°C with 50 μg of bovine serum albumin (A) and with 50 μg (0.1 unit) of E. coli β-galactosidase (B).

This is consistent with the earlier observation (6) that most of the label in N,N'-diacetylchitobiosylpyrophosphoryldolichol synthesized in hen oviduct membranes is on the N-acetylglucosamine residue at the nonreduced end of the disaccharide, presumably because the membrane contains a large pool of the monosaccharide lipid, N-acetylglucosaminylpyrophosphoryldolichol.

Characterization of Unilamellar Liposomes with Incorporated N,N'-Diacetyl[14C]chitobiosylpyrophosphoryldolichol—Phosphatidylcholine liposomes containing N,N'-diacetylchitobiosylpyrophosphoryldolichol were presented by injection of an ethanol solution containing both glycolipid and egg phosphatidylcholine into an aqueous saline media (23). This method was chosen because it does not degrade the phospholipid and it causes little or no hydrolysis of N,N'-diacetyl[14C]chitobiosylpyrophosphoryldolichol. Further, the technique can be used to obtain a fairly uniform population of vesicles of controlled size by varying the injection conditions. Subsequent to the formation of the liposomes by ethanolic injection the suspension was fractionated by Sepharose 4B chromatography. As shown in Fig. 5, the labeled N,N'-diacetylchitobiosylpyrophosphoryldolichol is associated with the phosphatidylcholine vesicles. The radioactivity coincides with lipid phosphorus, and both of the components are found in a single, included peak. The observed elution characteristics for these liposomes are consistent with those reported elsewhere (24). Larger vesicles were formed by the same general method using a decreased amount of ethanol in the injection step. Electron microscopy of negatively stained preparations of small (Fig. 6, top) and large (Fig. 6, bottom) vesicles reveal unilamellar structures with average diameters of 220 Å and 600 Å, respectively. The diameters observed are those expected from the injection conditions used for their formation (23).

Galactosyltransferase as a Probe for Monitoring the Transverse Mobility of N,N'-Diacetyl[14C]chitobiosylpyrophosphoryldolichol—Phosphatidylcholine liposomes containing incorporated N,N'-diacetyl[14C]chitobiosylpyrophosphoryldolichol were presented by injection of an ethanol solution containing both glycolipid and egg phosphatidylcholine into an aqueous saline media (23). This method was chosen because it does not degrade the phospholipid and it causes little or no hydrolysis of N,N'-diacetyl[14C]chitobiosylpyrophosphoryldolichol. Further, the technique can be used to obtain a fairly uniform population of vesicles of controlled size by varying the injection conditions. Subsequent to the formation of the liposomes by ethanolic injection the suspension was fractionated by Sepharose 4B chromatography. As shown in Fig. 5, the labeled N,N'-diacetylchitobiosylpyrophosphoryldolichol is associated with the phosphatidylcholine vesicles. The radioactivity coincides with lipid phosphorus, and both of the components are found in a single, included peak. The observed elution characteristics for these liposomes are consistent with those reported elsewhere (24). Larger vesicles were formed by the same general method using a decreased amount of ethanol in the injection step. Electron microscopy of negatively stained preparations of small (Fig. 6, top) and large (Fig. 6, bottom) vesicles reveal unilamellar structures with average diameters of 220 Å and 600 Å, respectively. The diameters observed are those expected from the injection conditions used for their formation (23).

Fig. 5. Sepharose 4B gel filtration profile of small unilamellar liposomes containing incorporated N,N'-diacetyl[14C]chitobiosylpyrophosphoryldolichol. Lipid phosphorus (Δ—Δ) and radioactivity (○—○) were monitored as described under "Experimental Procedures." The positions of blue dextran (V0) and acetyltryptophan (V1) are indicated. When injected alone, chitobiosyl-lipid elutes as a narrow peak just ahead of V0.

Fig. 6. Electron micrographs of negatively stained preparations of small (top) and large (bottom) unilamellar liposomes. The average size of the particles as determined from electron micrographs are 220 Å and 600 Å, respectively. Magnification. × 70,000.
phosphoryldolichol in Liposomes—As demonstrated in Fig. 7A, 30% of the $\text{N,N'}$-diacetyl$[^{14}\text{C}]$chitobiosylphosphoryl-dolichol incorporated into small unilamellar liposomes is not galactosylated, even after a 2-h incubation. Simultaneous treatment of a second aliquot of liposomes treated with 0.15% Triton X-100 to disrupt the vesicles resulted in 100% conversion to trisaccharide-lipid. The amount of enzyme used in this experiment was much greater than that used in Fig. 3 so that the galactosylation would be dependent solely upon substrate availability. UDP-galactose is present in these assays at saturating concentrations. Further, the addition of more galactosyltransferase after the reaction had apparently gone to completion (indicated by arrows) did not increase the extent of galactosylation. The enhanced galactosylation in the presence of Triton X-100 must result from disruption of the bilayer and subsequent accessibility of the unreacted $\text{N,N'}$-diacetyl$[^{14}\text{C}]$chitobiosylphosphoryldolichol, rather than mere activation of the galactosyltransferase, since control experiments with $\text{N}$-acetyl$[^{15}\text{H}]$glucosamine as substrate showed that the activity of the transferase was unaffected by Triton X-100 (data not shown).

The results in Fig. 7B indicate that the diameter of the glycolipid-containing liposomes has an effect on the extent of galactosylation. In large vesicles (600 Å) galactosylation is limited to 58%, while in small liposomes (220 Å) the reaction proceeds to 70%. Furthermore, the addition of more enzyme (indicated by arrows) after the reaction was apparently complete did not increase the extent of galactosylation. As was the case in Fig. 7A, when the latency of the liposomes is disrupted by Triton X-100, galactosylation goes to completion.

In another experiment (Fig. 7C), large liposomes were treated with galactosyltransferase and UDP-galactose for 90 min, at which time galactosylation had proceeded to approximately 55%. At this time UDP-galactose was added to double its original concentration (indicated by arrows). No increase in the extent of galactosylation was observed in a subsequent 15-min incubation. Next, enzyme was added to double its original concentration, and the reaction allowed to proceed for an additional 15 min. Again, no increase in the amount of trisaccharide-lipid was observed. However, when Triton X-100 was added to a final concentration of 0.1%, disruption of the bilayer resulted in virtually complete galactosylation in 30 min. These data, in conjunction with the experiments presented in Fig. 7, A and B, clearly show that the observed protection of a fraction of the $\text{N,N'}$-diacetyl$[^{14}\text{C}]$chitobiosylpyrophosphoryldolichol molecules is due to the permeability barrier presented by the bilayer of the liposomes.

To determine the physical state of the vesicles during the reaction, the absorbance at 300 nm was followed on aliquots of the liposomes under conditions identical with those present in the incubation mixture. The specific turbidity (absorbance/μmol of lipid) did not change during the course of the reaction, indicating that very little alteration in size distribution of the vesicles took place during the incubation (34). Further, electron microscopy of the liposomes subsequent to the enzyme assay indicated that the morphology was essentially unchanged from that shown in Fig. 6.

**Kinetics of Formation of Chitobiosyl-Lipid in Sealed Microsomes**—Using sealed microsomes from hen oviduct it was possible to accumulate chitobiosyl-lipid in the membrane. In Fig. 8, the kinetics of isotope incorporation from labeled sugar nucleotide into membrane-associated chitobiosyl-lipid is shown. The incorporation was nearly linear with time for up to 30 min. At this time (indicated by the arrow), the membranes were washed to remove labeled sugar nucleotide, and were then incubated at 37°C in the presence or absence of GDP-mannose for an additional 60 min. In the absence of unlabeled GDP-mannose, the amount of chitobiosyl-lipid remains nearly constant during the incubation, while in the presence of GDP-mannose the amount of membrane-associated chitobiosyl-lipid rapidly decreases. Further, only in the presence of GDP-mannose was significant radiolabeled material recovered in oligosaccharide-lipid or endogenous protein acceptors (data not shown). These data indicate that in the absence of GDP-mannose, the disaccharide unit of chitobiosyl-lipid is not rapidly transferred to protein. This allowed us to examine the topological arrangement of chitobiosyl-lipid in the membrane during this time interval. The data further suggests that the preformed chitobiosyl-lipid examined in this study can be nearly quantitatively chased into a larger oligosaccharide-lipid with GDP-mannose. It is, therefore, not a...
“dead end” intermediate in the glycoprotein biosynthetic pathway.

Accessibility of Chitobiosyl-Lipid to Galactosyltransferase—To determine the topology of preformed chitobiosyl-lipid in microsomal membranes, the membranes were treated with UDP-galactose and highly purified galactosyltransferase, in the presence or absence of the ionic detergent taurocholate. As Table I demonstrates, the galactosylation reaction is apparently complete at 10 min for both untreated and detergent-treated membranes. Further, enzyme is clearly not limiting, since the galactosylation reaction is virtually independent of enzyme addition above 0.5 units. Most interestingly, chitobiosyl-lipid is almost entirely inaccessible to galactosyltransferase in the absence of detergent. Using two criteria for microsomal integrity, 85 to 90% of the membrane vesicles used in this experiment were sealed (see below).

To establish whether the increased accessibility of chitobiosyl-lipid is correlated with the loss of the microsomal permeability barrier, the experiments shown in Fig. 9 were carried out. In Fig. 9A the increase in the accessibility of chitobiosyl-lipid in microsomes as a function of detergent concentration is shown. It is apparent that, at most, 10% of the glycolipid is accessible in untreated microsomes. With increasing detergent concentrations the accessibility increases sharply at detergent concentrations exceeding 0.03% taurocholate. As shown in Fig. 9B, similar results are obtained using isolated rough microsomes. When the latencies of mannose-6-phosphatase (Fig. 9C) or β-glucuronidase (Fig. 9D) are assessed as a function of detergent concentration, it is apparent that there is a good correlation between loss of latency and the increased accessibility of chitobiosyl-lipid.

The strong correlation between loss of microsomal membrane integrity and increased accessibility of chitobiosyl lipid is highly suggestive of protection by the bilayer of the microsomes. However, alternative interpretations of these findings can be offered. Chitobiosyl-lipid may be simply solubilized from the membrane by the detergent and then act as a soluble acceptor. Alternatively, a microsomal membrane component could protect chitobiosyl-lipid from the exogenously added transferase. In an attempt to rule out the possibility that solubilization of chitobiosyl-lipid accounts for its increased accessibility, microsomes containing labeled chitobiosyl-lipid

Table I

Galactosylation of chitobiosyl-lipid as a function of time and galactosyltransferase concentration

Total microsomes were used to generate [14C]chitobiosyl-lipid in microsomal membranes as described under “Experimental Procedures.” The membranes were then treated with galactosyltransferase at the concentrations and for the period of time indicated in the presence or absence of 0.2% taurocholate. The amount of accessible chitobiosyl-lipid was then assessed. The microsomes used in this experiment were 85 to 90% sealed vesicles as determined from parallel measurements of the activities of the latent enzymes, mannose-6-phosphatase and β-glucuronidase, in untreated and detergent-treated membranes.

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Accessible chitobiosyl-lipid*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Untreated</td>
</tr>
<tr>
<td>5 min</td>
<td>12</td>
</tr>
<tr>
<td>10 min</td>
<td>14</td>
</tr>
<tr>
<td>20 min</td>
<td>14</td>
</tr>
<tr>
<td>30 min</td>
<td>14</td>
</tr>
</tbody>
</table>

Galactosyltransferase added

<table>
<thead>
<tr>
<th>units</th>
<th>% Untreated</th>
<th>% Detergent-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>13</td>
<td>92</td>
</tr>
<tr>
<td>1.6</td>
<td>14</td>
<td>96</td>
</tr>
<tr>
<td>2.4</td>
<td>14</td>
<td>96</td>
</tr>
</tbody>
</table>

* Determinations are ±3%.

Fig. 9. Correlation between chitobiosyl-lipid accessibility and loss of microsomal membrane integrity. A, accessibility of chitobiosyl-lipid in total microsomes as a function of taurocholate concentration. B, accessibility of chitobiosyl-lipid in highly purified rough microsomes as a function of detergent concentration. C, latency of the enzyme mannose-6-phosphate as a function of taurocholate concentration (maximum activity was 0.15 μg of P, released/min/mg). D, latency of β-glucuronidase as a function of the concentration of taurocholate (maximum activity was 0.4 μg of phenolphthalein released/mg/min).

Fig. 10. Lack of solubilization of chitobiosyl-lipid from microsomal membranes. Microsomes in which [3H]chitobiosyl-lipid had been accumulated were incubated 30 min at 37°C in Buffer 4 containing the indicated concentrations of taurocholate. Membranes were sedimented and the amount of membrane-associated and solubilized chitobiosyl-lipid were assessed as described under “Experimental Procedures.” Membranes were incubated with concentrations of taurocholate up to 1.0% for 30 min at 37°C and subjected to centrifugation. The amounts of solubilized and membrane-associated chitobiosyl-lipid were then assessed. It is apparent from Fig. 10 that in the range of detergent required for complete accessibility of chitobiosyl-lipid to galactosyltransferase (0.05 to 0.15%), very little solubilization of the glycolipid occurs. Only at taurocholate concentrations above 0.5% does significant solubilization take place (data not shown).
Transbilayer Orientation of a Dolichol-linked Disaccharide

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Decrease in latency</th>
<th>Accessibility of chitobiosyl-lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>10%</td>
</tr>
<tr>
<td>0.2% taurocholate</td>
<td>100</td>
<td>94%</td>
</tr>
<tr>
<td>0.2% Triton X-100</td>
<td>96</td>
<td>90%</td>
</tr>
<tr>
<td>Ribonuclease B</td>
<td>16</td>
<td>17%</td>
</tr>
<tr>
<td>Puromycin-KC1</td>
<td>14</td>
<td>17%</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>22</td>
<td>20%</td>
</tr>
<tr>
<td>Trypsinα</td>
<td>13</td>
<td>20%</td>
</tr>
<tr>
<td>Trypsinβ</td>
<td>48</td>
<td>38%</td>
</tr>
</tbody>
</table>

*Values ranged from 4 to 17% in different experiments.

In an effort to exclude the possibility that a microsomal membrane component protects the chitobiosyl lipid from enzymatic modification, microsomes were pretreated with agents known to remove or alter membrane surface components (Table I). Proteases, although effective in removing protein from the surface of the microsomes, had little effect on the accessibility of the chitobiosyl-lipid. Only when the vesicles became leaky by trypsin digestion at an elevated temperature was significant accessibility observed. Similarly, removal of ribosomes with puromycin-KC1 treatment or by incubation with RNase caused no detectable increase in chitobiosyl-lipid accessibility. These data, taken together, strongly argue that the disaccharide residue of chitobiosyl-lipid is protected from the transference by the microsomal membrane.

**DISCUSSION**

Defining the dynamics and orientation of polyisoprenyl derivatives in the bilayer is a prerequisite to understanding the nature of their involvement in the assembly of oligosaccharide chains. We have initially investigated N,N'-diacetylchitobiosylphosphoryldolichol in this context for two reasons. First, it is an early, key intermediate in the assembly of the oligosaccharide chain of N-linked glycoproteins. A second, practical reason for studying chitobiosyl-lipid was the preliminary finding that this lipid intermediate serves as a substrate for soluble galactosyltransferase and is converted to a galactose-containing trisaccharide-lipid. Since galactosyltransferase is a large molecule (~45,000 Mw), and catalyzes the transfer from a hydrophilic sugar nucleotide to a membrane component, it would be expected to act only on chitobiosyl-lipid at the external face of a sealed vesicle. In fact, this enzyme has been used previously as a probe for other non-lipid, membrane-associated molecules (35, 36).

Prior to use as a probe, the transferase reaction using N,N'-diacetylchitobiosylphosphoryldolichol as an acceptor was partially characterized using the glycolipid solubilized in detergent. Because chitobiosyl-lipid was present in these enzyme assays in tracer amounts, at concentrations below the reported K\textsubscript{m} values for both N-acetylglucosamine (8.3 mM) and N,N'-diacetylchitobiose (0.62 mM) (32), direct comparison of the initial rates of this reaction with others catalyzed by galactosyltransferase was not possible. However, it was found that several basic characteristics of the reaction are similar to those observed previously using other GlcNAc-containing substrates. First, the stimulation of enzyme activity in the presence of 60 μg/ml of α-lactalbumin has also been observed when GlcNAc is used as an acceptor well below the K\textsubscript{m} value (31). Further, the absolute dependence upon Mn\textsuperscript{2+}, and the observation that UDP-galactose become limiting below 0.2 mM are consistent with the reported nature of the reaction (31, 32).

We next turned to unilamellar phosphatidylcholine vesicles prepared in the presence of [14C]chitobiosyl-lipid. Such liposomes provide a simple model system in which to study the interaction of chitobiosyl-lipid with lipid bilayers. It seemed possible that by measuring the accessibility of liposome-associated chitobiosyl-lipid to the soluble galactosyltransferase, the percentage of molecules on the external surface of the bilayer could be estimated. Complete conversion of the lipid to the trisaccharide-lipid product would indicate either preferential association of the disaccharide-lipid with the outer surface of the bilayer, or rapid flip-flop in the transverse plane of the bilayer. Alternatively, galactosylation of only a fraction of the disaccharide would be expected if there is random distribution of the glycolipid and very slow transverse movement. Under these latter circumstances the extent of protection of the internally oriented N,N'-diacetylchitobiosylphosphoryldolichol molecules should be governed by the ratio of external to total surface area. Based on the measured diameters of the two liposome preparations used in this study, the ratio of external to total surface areas of large and small liposomes should be 58:100 and 68:100, respectively (37). The values for the extent of galactosylation of the glycolipid observed experimentally were 58% and 70%, for the small and the large liposomes, respectively. Clearly, these values agree well with the calculated ratio of the external to total surface area of liposomes. In addition, when the barrier to the interior was destroyed by addition of detergent, almost 100% of the chitobiosyl lipid was converted to the trisaccharide lipid. On the basis of these data, we conclude that chitobiosyl-lipid is incorporated into the lipid bilayer of liposomes in such a way as to make the carbohydrate moiety of only those disaccharide-lipid molecules that are on the external leaflet of the bilayers susceptible to enzymatic modification. Furthermore, these data indicate that there is no preferential association of the glycolipid with one face of the liposome. Therefore, it appears that geometric packing constraints probably play little role in determining the transbilayer distribution of the glycolipid in the liposomes (37).

The observed protection of a fraction of the chitobiosyl-lipid from galactosylation also indicates that, above the phase transition of the phosphatidylcholine vesicles, no apparent change in the transbilayer distribution of molecules of the lipid intermediate takes place during a 3 h incubation. The absence of detectable flip-flop of the molecule in 3 h suggests that chitobiosyl-lipid does not undergo transbilayer translocation at a rate faster than that observed for other lipids in liposomes, where half-lives of days or more are observed (38-40). It is clear that the novel structural features of chitobiosyl lipid, as compared to diacylphosphoglycerides, do not per se render this lipid intermediate uniquely mobile in the lipid bilayer of unilamellar liposomes.

Although these results provide little insight into how the extraordinarily long polyisoprenoid chain of chitobiosyl-lipid is oriented in lipid bilayers, the fact that a soluble galactosyltransferase can interact with the carbohydrate moiety of chitobiosyl-lipid clearly demonstrates the accessibility of the terminal saccharide unit of this molecule to the aqueous phase. The observed resistance of the galactose-containing trisaccharide-lipid to β-galactosidase digestion may be due to steric hindrance, since *E. coli* β-galactosidase (500,000 Mw) is a much larger enzyme than galactosyltransferase (45,000 Mw) (41, 42). Also, since galactosyltransferase has been shown to...
have a higher affinity for N-acetylglucosamine-containing substrates bearing a hydrophobic aglycone (43), this property of the enzyme may account for its ability to act near the surface of a bilayer or upon the detergent-solubilized disaccharide-lipid. Taken together, these observations suggest that the carbohydrate moiety of the chitobiosyl-lipid molecule is in a hydrophilic-hydrophobic boundary very close to the surface of the bilayer. Quite similar conclusions were reached regarding the microenvironment of undecaprenyl-phosphate-N-acetylmuramylpentapeptide in membrane fragments of *Staphylococcus aureus* (2). In the bacterial system the data suggest that the lipid intermediate is immobilized such that the spin label on the pentapeptide chain is within 4 to 6 Å of the lipid matrix.

Having established that galactosyltransferase could be utilized as a probe of the topology of chitobiosyl-lipid in artificial membranes, we next used this enzyme to study the orientation of chitobiosyl-lipid after it had been synthesized in microsomal vesicles. If chitobiosyl-lipid is randomly oriented after its biosynthesis in the membrane, it would be expected that roughly 50% of the molecules would be galactosylated by the transferase. Alternatively, if the molecules were oriented with the disaccharide residue facing the lumen of the microsomes, none of them should be accessible to galactosyltransferase. Lastly, if chitobiosyl-lipid undergoes flip-flop, or is externally oriented, complete galactosylation would be expected. The results obtained clearly indicate that the chitobiosyl-lipid is inaccessible to galactosylation in sealed microsomal vesicles. In view of these results it became important to rule out detergent solubilization as an alternative explanation for the fact that significant galactosylation was observed only when detergent was added to microsomes. This was excluded by the finding that the increased accessibility of chitobiosyl lipid occurs at a concentration of detergent at which little or no solubilization of this lipid from the membrane occurs. Further, the increased accessibility of chitobiosyl-lipid can be closely correlated with an increase in the leakage of the microsomal membranes as measured by the increased activity of two latent enzymes. To further substantiate the idea that the glycolipid is inaccessible to galactosyltransferase because of its orientation in the bilayer, and not merely protected by surface components of the membrane, microsomal surface components were removed or altered, with little effect on the accessibility of chitobiosyl-lipid. Thus, all the data presented are consistent with a model in which chitobiosyl-lipid is asymmetrically oriented in the bilayer of the endoplasmic reticulum and faces the lumen of this organelle after its biosynthesis.  

Synthesis of chitobiosyl lipid is accomplished by two distinct enzymatic reactions. The first involves transfer of N-acetylglucosaminyl-1-phosphate from UDP-N-acetylglucosamine to dolichol phosphate, while the second involves transfer of N-acetylglucosamine from its nucleotide derivative to N-acetylglucosaminylpyrophosphoryldolichol. The results in this study provide no information on the topological orientation of the product of the first reaction; they simply indicate the product of the second reaction is oriented toward the lumen. At this time the mechanism by which this orientation is brought about is unknown. Clearly, the results in microsomes and liposomes indicate that chitobiosyl-lipid does not undergo rapid flip-flop. However, it is possible that, after two-step assembly on the outer face of the membrane vesicle the chitobiosyl-lipid undergoes spontaneous unidirectional transverse movement to the inner face, although it is difficult to envision what the driving force for such movement would be. A second possibility is that UDP-N-acetylglucosamine enters the lumen, where it reacts first with dolichol phosphate and then with N-acetylglucosaminylpyrophosphoryldolichol to produce chitobiosyl-lipid. Given the size and polarity of sugar nucleotides and, the fact that mannose-6-phosphate cannot enter the lumen of these membrane vesicles, this seems only possible if a specific transport mechanism exists for UDP-N-acetylglucosamine. Alternatively, transmembrane movement of N-acetylglucosaminyl-1-phosphate and N-acetylglucosamine (rather than UDP-N-acetylglucosamine per se) could be accomplished by the membrane-associated glycosyltransferases involved in assembly of the two saccharide-lipids. That is, the transmembrane movement of the N-acetylglucosaminyl-1-phosphate and N-acetylglucosamine units could be an integral part of the enzymatic reaction leading to their attachment to the lipid. This possibility is consistent with earlier findings with impermeant inhibitors that were found to block synthesis of chitobiosyl-lipid (29), suggesting that at least a portion of the enzyme system involved in chitobiosyl-lipid formation is externally oriented. Clearly, further studies to elucidate the mechanism by which the asymmetry of chitobiosyl-lipid is established are warranted.

Acknowledgments—We are grateful to Glenn L. Decker for his expert assistance with the electron microscopy, to Dr. Winston W. Chen for the gift of authentic N,N-diacyltrehalaminiose and for his initial observation regarding the galactosyltransferase acceptor activity of chitobiosyl-lipid, and to Dr. Joel Shaper for the gift of highly purified galactosyltransferase. We also thank Drs. Ursula Czichi and Gerald W. Hart for their valuable advice. The assistance of Michael McKinney in assigning marker enzymes and the assistance of Ms. Ann Fuhr in the preparation of this manuscript is gratefully acknowledged.

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Transbilayer Orientation of a Dolichol-linked Disaccharide

The topological orientation of N,N'-diacetyldichitobiosylpyrophosphoryldolichol in artificial and natural membranes.
J A Hanover and W J Lennarz


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