Biosynthesis of Glucosylated Derivatives of Dolichol: Possible Intermediates in the Assembly of White Matter Glycoproteins†

Malka G. Scher, A. Jochen, and C. J. Waechter*

ABSTRACT: Incubation of a particulate, cell-free preparation from the white matter of calf brain with UDP-[^{14}C]glucose in the presence of Mg^{2+} results in the transfer of [^{14}C]glucose into three classes of endogenous acceptors. The enzymatic products are: two ^{14}C-labeled glucolipids recovered in $CHCl_3$–CH_3OH (2:1); a ^{14}C-labeled glucosylated oligosaccharide lipid, insoluble in H_2O or $CHCl_3$–CH_3OH mixtures, but readily extracted with $CHCl_3$–CH_3OH–H_2O (10:10:3) and a labeled glycoprotein fraction remaining in the delipidated membrane residue. The two labeled glucolipids extracted with $CHCl_3$–CH_3OH (2:1) have the hydrolytic and chromatographic characteristics of glucosylceramide and glucosylphosphoryldolichol. Synthesis of the latter glucolipid is stimulated by exogenous dolichyl monophosphate. The synthesis of both glucolipids is inhibited by UDP, but only the formation of glucosylphosphoryldolichol is freely reversible. The kinetics of [^{14}C]glucose transfer are compatible with glucosylphosphoryldolichol and the glucosylated oligosaccharide lipid serving as intermediates in glycoprotein assembly. The chromatographic behavior of the intact oligosaccharide lipid on DEAE-cellulose indicates that the oligosaccharide unit is linked to the carrier lipid by a pyrophosphate bridge. An electrophoretically neutral *Glc*-^{14}C-labeled oligosaccharide unit is liberated by mild acid hydrolysis. Two positively charged products are produced by strong alkaline hydrolysis of the *Glc*-^{14}C-labeled oligosaccharide. The labeled products were converted to neutral compounds by N-acetylation, indicating the presence of two N-acetylated sugars. The free *Glc*-^{14}C-labeled oligosaccharide has been estimated to contain 10 glycose units by paper chromatography and gel filtration. Approximately 75% of the large molecular weight material in the glycoprotein fraction labeled in the presence of Mg^{2+} is extensively degraded by trypsin or Pronase. A ^{14}C-labeled glucopeptide produced by extensive Pronase digestion has a molecular size similar to the lipid-linked *Glc*-^{3}H-labeled oligosaccharide. The addition of 10 mM EDTA to the incubation mixture blocks the incorporation of [^{14}C]glucose into the putative intermediates and the product yielding a labeled glucopeptide. However, glucosylceramide and a product recovered in the glycoprotein fraction, that is sensitive to α- or β-amylase digestion, are labeled in the presence of 10 mM EDTA.

The first oligosaccharide lipid intermediate found in animal tissues was detected by Leloir and his co-workers (Behrens et al., 1971) in rat liver microsomes. Although this oligosaccharide lipid was isotopically labeled with glucose, most of the studies in animal tissues on the biosynthesis and function of polyisoprenoid glycolipids have been conducted on mannose and *N*-acetylglucosamine derivatives of dolichol (for recent reviews, see Lucas and Waechter, 1976; Waechter and Lennarz, 1976). The exact relationship between the glucosylated oligosaccharide lipid and the mannose- and *N*-acetylglucosamine-containing oligosaccharide lipids has been unclear. However, recent studies have demonstrated that slices from bovine thyroid and other tissues (Spiro et al., 1976b,c) and calf pancreas microsomes (Herscovics et al., 1977a) synthesize dolichol-linked oligosaccharides containing mannose, *N*-acetylglucosamine, and glucose. These structures are basically in agreement with the biosynthetic scheme proposed earlier by Behrens et al. (1973) in which the lipid-linked mannosylated oligosaccharide was elongated by the addition of two glucose residues. In a related experiment Robbins et al. (1977) have obtained evidence for the incorporation of glucose into a mannosylated oligosaccharide lipid formed by cell-free preparations from fibroblasts. Enzymatic studies with rat liver microsomes (Parodi et al., 1972) and pulse-chase experiments with thyroid slices (Spiro et al., 1976a) indicate that glucosylated oligosaccharide lipids function as oligosaccharide donors in glycoprotein biosynthesis.

We have previously shown that a mannose-containing oligosaccharide lipid is enzymatically formed by a membrane preparation from calf brain white matter (Waechter et al., 1976) and later provided evidence that its biosynthesis proceeds by the mannosylation of *N,N'*-diacetylchitobiosylpyrophosphoryldolichol (Waechter and Harford, 1977). In this paper we present the first detailed studies on the transfer of glucose from UDP-glucose into glucosylphosphoryldolichol (GPD), a glucosylated oligosaccharide lipid and at least one glucoprotein catalyzed by membranes from central nervous tissue. The kinetic pattern of labeling of endogenous acceptors by UDP-[^{14}C]glucose is consistent with the glucosylated dolichol derivatives serving as intermediates in the formation of glycoprotein. A preliminary report on this work has been presented (Jochen et al., 1977).

Materials and Methods

Materials. UDP-[U-^{14}C]glucose and UDP-[6-^{3}H]glucose were purchased from the Amersham/Searle Corp. UMP, UDP, Pronase (Protease type V from *Streptomyces griseus*), and dolichyl monophosphate were obtained from the Sigma Chemical Co. Porcine pancreas α-amylase (α-1, 4-glucan glucanohydrolase, EC 3.2.1.1) and sweet potato β-amylase (α-1,4-glucanomaltohydrolase, EC 3.2.1.2) were from Boehringer-Mannheim. Trypsin (EC 3.4.21.4) was purchased from the Worthington Biochemical Corp. Glucosylceramide isolated from the spleen of a patient with Gaucher's disease was kindly provided by Dr. Robert Glew, University of Pittsburgh

† From the Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201. *Received May 25, 1977.* This work was supported by U.S. Public Health Service Grant No. NS-12296, an award from the Pangborn Fund of the University of Maryland School of Medicine, and also aided by a Basil O'Connor Starter Research Grant from the National Foundation of the March of Dimes.

School of Medicine. All other materials were purchased from commercial sources.

Preparation of Enzyme. Membranous enzyme was prepared from calf brain white matter as previously described (Waechter and Harford, 1977).

Measurement of Radioactive Samples. All labeled samples were counted in Hydromix (Yorktown Research) in a Packard Tri-Carb scintillation spectrometer. The data presented as counts per minute are average values obtained by counting each sample for 10 min.

Assay for the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into Endogenous Acceptors. The assay for the labeling of endogenous acceptors by UDP-[^{14}C]glucose was essentially the same as the procedure described for measuring the incorporation of [^{14}C]mannose into mannosylphosphoryldolichol, a mannosylated oligosaccharide lipid and mannoproteins (Waechter et al., 1976). Because the $CHCl_3$-CH_3OH extract contained [^{14}C]glucosylceramide and [^{14}C]glucosylphosphoryldolichol, the glycolipids were separated by chromatography on SG-81 paper in solvent mixture B. The chromatogram was dried and exposed to Kodak No-Screen x-ray film. The spots on the autoradiogram were used to locate the radioactive zones on the paper chromatogram. The labeled zones corresponding to the two glycolipids were cut out and counted in 10 mL of Hydromix. The percentage of the total radioactivity was calculated for each lipid. Based on these percentages and the total amount of [^{14}C]glucose in the lipid extract, the amount of each glycolipid labeled was determined.

Preparation of Labeled Products for Chromatographic Analysis. Preparative incubation mixtures consisted of enzyme (9–25 mg of membrane proteins) suspended in 70–90 mM Tris-HCl, pH 8.0, 0.7–0.9 mM EDTA, 0.17–0.24 M sucrose, 7–17 mM $CaCl_2$, $MgCl_2$, or 10 mM EDTA, 2–4 mM AMP, and either 5–9 μM UDP-[^{14}C]glucose (482 cpm/pmol) or UDP-[^{3}H]glucose (2071 cpm/pmol) in a total volume of 1–2 mL. Following an incubation period of 10 min at 37 °C, the reaction was terminated by the addition of 20 volumes of $CHCl_3$-CH_3OH (2:1). After 5–10 min at room temperature the suspensions were centrifuged at approximately 1000 rpm in a clinical centrifuge. The lipid extract was saved and the pellets were washed three times to extract the ^{14}C-labeled glucolipids. All lipid extracts were pooled and washed with 0.20 volume of 0.9% NaCl. The organic phase (lower) was washed again with CH_3OH–0.9% NaCl (1:1). The upper phases were discarded and the lipid extract containing both ^{14}C-labeled glucolipids was concentrated by evaporation under vacuum. The two glucolipids were resolved by chromatography on either a Unisil column or DEAE-cellulose.

The remaining pellets were then dried by a stream of N_2 and resuspended in 0.9% NaCl by ultrasonication. The suspension was sedimented by centrifugation and the pellet washed again with 0.9% NaCl, and three times with water to remove residual labeled sugar nucleotide. The radiolabeled oligosaccharide lipids were extracted with a minimal volume of $CHCl_3$-CH_3OH-H_2O (10:10:3). In some preparations it was necessary to add a few drops of CH_3OH to produce a single phase. The $CHCl_3$-CH_3OH-H_2O extracts were dried by evaporation under vacuum or by a stream of N_2. The labeled oligosaccharide units were released from the carrier lipid by treatment with 0.1 N HCl in 80% tetrahydrofuran at 50 °C for 1 h. Lipid-linked oligosaccharide labeled by GDP[1]-[^{14}C]mannose was prepared in the presence of 10 mM $CaCl_2$ under the same conditions used for *Glc*-^{3}H-labeled oligosaccharide lipid. The labeled products remaining in delipidated membrane residues were designated as the glycoprotein fraction. The glycoprotein fraction was digested with trypsin or Pronase as described elsewhere (Waechter and Harford, 1977).

Treatment of Labeled Products with α- or β-Amylase. Labeled products were incubated with 0.1 mg of either α- or β-amylase for 2 h at 37 °C. The digestion products were analyzed by paper chromatography using solvent system H.

Hydrolytic Procedures. The kinetics of mild acid hydrolysis of [^{14}C]glucosylphosphoryldolichol in 0.1 N HCl in 50% 1-propanol at 50 °C were assayed by the procedure described for mannosylphosphoryldolichol (Waechter et al., 1973). Determination of the time course for the mild acid hydrolysis of the ^{14}C-labeled glucosylated oligosaccharide lipid was the same as the method outlined for the hen oviduct mannosylated oligosaccharide lipid (Lucas et al., 1975). Glucosylceramide was hydrolyzed by suspending the lipid in 3 N HCl by ultrasonication and heating the suspension at 100 °C for 6 h. The HCl was removed by drying under a stream of N_2. The labeled glycoprotein fraction was suspended in 3 N HCl and heated at 100 °C for 6 h. The hydrolyzate was neutralized by the addition of Ag_2CO_3. Upon cessation of the evolution of CO_2, the mixture was centrifuged in a clinical centrifuge to separate the insoluble residue from the water-soluble product. For strong alkaline hydrolysis of the *Glc*-^{14}C-labeled oligosaccharide approximately 40 000 cpm was treated with 1 M sodium borohydride at room temperature for 15 min. The sample was then hydrolyzed in 1 N NaOH at 100 °C for 18 h. The hydrolyzate was neutralized with glacial acetic acid and desalted by gel filtration on a Sephadex G-25 column equilibrated and eluted with 0.1 N acetic acid. The radioactive fractions were pooled and dried by rotoevaporation under vacuum. A portion of the alkaline hydrolyzed material was then treated under conditions described for N-acetylation (Carlson, 1967).

Paper Chromatographic Methods. The ^{14}C-labeled glucolipids were chromatographed on SG-81 paper pretreated with EDTA (Steiner and Lester, 1972) using the following solvent mixtures: (A) $CHCl_3$-CH_3OH-H_2O (60:25:4); (B) $CHCl_3$-CH_3OH-H_2O (75:25:4); (C) $CHCl_3$-CH_3OH-concentrated NH_4OH (36:13:3); and (D) diisobutyl ketone-glacial acetic acid-H_2O (60:45:6), or on SG-81 paper pretreated with 0.1 M sodium borate, pH 9.0, developed with (E) $CHCl_3$-CH_3OH-H_2O (65:25:4); (F) 1-propanol-H_2O-concentrated NH_4OH (70:13:17); or (G) 1-propanol-H_2O (7:3). Unlabeled glucosylceramide was detected by spraying with a 0.001% solution of rhodamine 6G. Descending chromatography of the intact glucosylated oligosaccharide lipid and water-soluble compounds was performed on Whatman No. 3MM paper developed with (H) isobutyric acid-concentrated NH_4OH-H_2O (57:4:39); (I) ethyl acetate-pyridine-H_2O (12:5:4); (J) 1-butanol-pyridine-H_2O (6:4:3); (K) 1-butanol-pyridine-0.1 N HCl (5:3:2). Glucose and sorbitol were resolved by developing with (L) ethyl acetate-pyridine-0.1 M boric acid (12:5:4). Unlabeled sugars and sorbitol were detected with a periodate dip (Wawszkiewicz, 1961).

Unisil Chromatography of ^{14}C-Labeled Glucolipids. A crude lipid extract (0.1 mL of $CHCl_3$) containing 3000 cpm was applied to a Unisil column (1.45 × 8.0 cm) developed in $CHCl_3$. The column was then washed with 50 mL of $CHCl_3$. All radioactivity remained adsorbed to the column. The ^{14}C-labeled glucolipids were recovered by sequentially eluting with 50 mL of acetone and 50 mL of $CHCl_3$-CH_3OH (1:1).

Size Estimation of Lipid-Linked Glc-^{14}C-Labeled Oligosaccharide. The chain length of the *Glc*-^{14}C-labeled oligo-

[1] Abbreviation: GPD, glucosylphosphoryldolichol.

TABLE I: Effect of Various Divalent Metal Ions on the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into Endogenous Acceptors.[a]

Metal ion added 10 mM	[^{14}C]Glucose incorp into (cpm)			
	GPD	Oligosaccharide lipid	Glycoprotein fraction	Glc-Cer
None	3	59	1491	820
Mg^{2+}	1601	2578	2332	1419
Mn^{2+}	419	1139	1495	1910
Ca^{2+}	1097	1448	2284	1013
Co^{2+}	1590	1079	1575	246
Ni^{2+}	2152	858	1138	1108
Zn^{2+}	ND	68	392	ND
Hg^{2+}	ND	45	1687	ND
EDTA	ND	33	1514	810

[a] All incubation mixtures contained enzyme (2.1 mg of membrane protein) in 0.05 M Tris-HCl, pH 8.0, 0.125 M sucrose, 0.05 mM EDTA, 2.5 mM AMP, and 5 μM UDP-[^{14}C]glucose (482 cpm/pmol) in a total volume of 0.20 mL. Where indicated divalent metal ions (chloride salts) were included. Following incubation at 37 °C for 5 min the amount of labeled products formed was assayed as described in Materials and Methods. ND, not detectable; Glc-Cer = glucosylceramide; GPD = glucosylphosphoryldolichol.

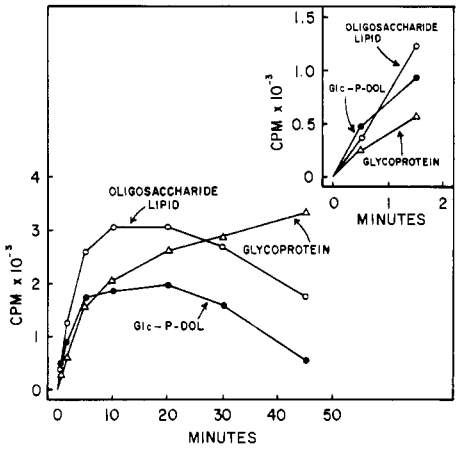

FIGURE 1: Time course of transfer of [^{14}C]glucose from UDP-[^{14}C]-glucose into endogenous acceptors of calf brain white matter preparations. The reaction mixture contained enzyme (20 mg of protein), 93 mM Tris-HCl, pH 8.0; 230 mM sucrose; 0.93 mM EDTA; 2.3 mM AMP; 7.5 mM $MgCl_2$; 5.3 μM UDP-[^{14}C]glucose (482 cpm/pmol) in 1.07 mL. The mixture was incubated at 37 °C and at the indicated times 100-μL samples were withdrawn and transferred directly into 2.0 mL of $CHCl_3$-CH_3OH (2:1). The amount of each labeled product formed was assayed as described in Materials and Methods. The values shown in the insert panel for 30 and 90 s are the averages of four experiments.

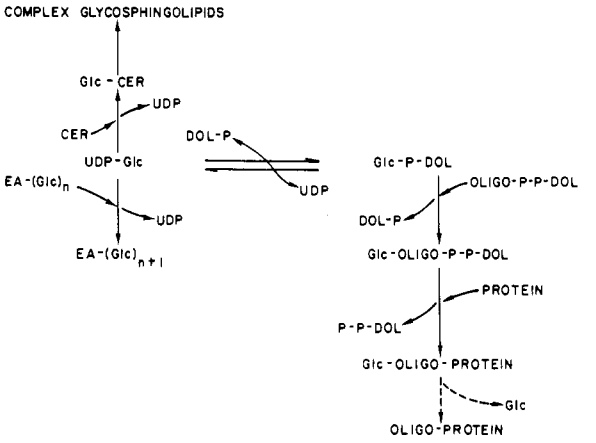

FIGURE 2: Postulated reaction scheme for glucosyl transfer reactions catalyzed by white matter membranes; EA = endogenous acceptor; CER = ceramide; Glc-CER = glucosylceramide.

saccharide was estimated by descending paper chromatography on Whatman No. 3MM developed for 48 h with solvent system H. The calibration markers were G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, stachyose; and G_8, *Man*-^{14}C-labeled oligosaccharide from hen oviduct oligosaccharide lipid (Lucas et al., 1975). When the log of the distances migrated by the calibration markers was plotted against the number of glycose units, a straight line was obtained. Extrapolation of this line to the distance migrated by the white matter *Glc*-^{14}C-labeled oligosaccharide yielded an estimated chain length of ten glycose units. The same calibration markers without maltotriose were used to estimate the molecular weight of the free *Glc*-^{14}C-labeled oligosaccharide on a Bio-Gel P-4 column eluted with 0.1 M NaCl. The apparent molecular weight was calculated by the method of Bhatti and Clamp (1968).

Results

Time Course for the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into Endogenous Acceptors. When white matter membranes were incubated with UDP-[^{14}C]glucose and Mg^{2+}, [^{14}C]glucose was most rapidly incorporated into GPD and the oligosaccharide lipid and more slowly into the glycoprotein fraction (Figure 1). The initial rates of labeling (insert, Figure 1) were in the order GPD > oligosaccharide lipid > glycoprotein. This relative order is compatible with the postulated general reaction scheme shown in Figure 2. The loss of label from the lipid intermediates after 20 min while isotope accumulates in the glycoprotein fraction is also in agreement with this reaction sequence. The kinetic pattern of labeling does not, however, eliminate the possibility that some glucosyl units in the oligosaccharide lipid or glycoprotein fraction are donated directly by UDP-glucose. Because glucosylceramide does not appear to be glucosylated via lipid intermediates, its labeling pattern was not included in this experiment.

Enzymatic Conditions for the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into Endogenous Acceptors. The incorporation of [^{14}C]glucose into GPD, the glucosylated oligosaccharide lipid, and the glycoprotein fraction is stimulated by the addition of several divalent metal ions. The order of effectiveness for GPD labeling is Ni^{2+} > Mg^{2+} > Co^{2+} > Ca^{2+} > Mn^{2+}. No labeled GPD was detected following incubations with Hg^{2+} or Zn^{2+}. EDTA also markedly inhibited the formation of labeled GPD and the oligosaccharide lipid. However, in the presence of EDTA, [^{14}C]glucose was still incorporated into glucosylceramide and, although at a slightly reduced level, into the glycoprotein fraction (Table I). This result indicates that glucosylceramide and at least one product

TABLE II: Effect of Exogenous Dolichyl Monophosphate on the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into [^{14}C] Glucolipids.[a]

Dolichyl monophosphate added (nmol)	[^{14}C]Glucose incorp into (cpm)	
	Glc-P-dolichol	Glc-ceramide
None	2546	1144
46	11050	1228
92	15990	1203
184	20900	1107

[a] Each reaction mixture contained enzyme (2.1 mg of membrane protein) suspended in 0.04 M Tris-HCl (pH 8.0), 0.1 M sucrose, 0.1 mM EDTA, 16 mM $MgCl_2$, 2 mM AMP, 0.032% Triton X-100, and 4 μM UDP-[^{14}C]glucose (482 cpm/pmol) in a total volume of 0.25 mL. Dolichyl monophosphate was dispersed in 0.1% Triton X-100 by ultrasonication and the indicated amount was added to the reaction mixtures. Following incubation at 37 °C for 5 min, the reaction was terminated by the addition of 20 volumes of $CHCl_3$–CH_3OH (2:1) and the amount of radioactivity in each glucolipid was assayed as described in Materials and Methods.

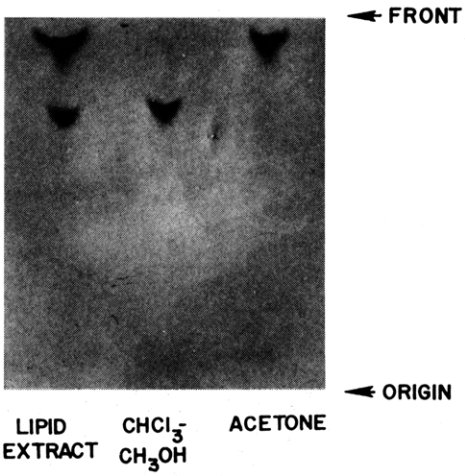

FIGURE 3: SG-81 paper chromatography of [^{14}C]glucolipid fractions from Unisil column. Samples of a crude lipid extract, an acetone fraction from Unisil, and a $CHCl_3$–CH_3OH (1:1) fraction from Unisil (1000 cpm of each) were spotted on a SG-81 paper and developed with solvent mixture A. Kodak No-Screen x-ray film was exposed to the dried chromatogram to produce an autoradiogram. The chromatographic procedure for obtaining the [^{14}C]glucolipid fractions from Unisil is described under Materials and Methods.

in the glycoprotein fraction are glucosylated directly by UDP-[^{14}C]glucose as depicted in Figure 2. Studies discussed later in the text reveal that the product in the glycoprotein fraction, labeled in the presence of EDTA, is a particle-bound glucan.

The incorporation of [^{14}C]glucose into oligosaccharide lipid was stimulated most by the addition of Mg^{2+}. Since the maximum level of labeling of the oligosaccharide lipid and the glycoprotein fraction was achieved in the presence of Mg^{2+}, most enzymatic studies were carried out in the presence of $MgCl_2$. When the effect of varying the concentration of Mg^{2+} on the isotopic labeling of GPD was studied, a broad optimum (10–80 mM) was found. The optimal pH for GPD synthesis was 8.0. When the effect of varying the concentration of UDP-glucose on GPD formation was assayed an apparent $K_m = 5.2 \times 10^{-7}$ M was determined.

Since the glucolipid that is labile to mild acid had the properties of a glucosylphosphorylpolyisoprenol, the effect of adding exogenous dolichyl monophosphate on the formation of this lipid was assessed. The results in Table II show that increasing levels of dolichyl monophosphate led to a corresponding stimulation in the labeling of GPD. The stimulatory effect was specific in that the addition of the polyprenyl monophosphate had no effect on the incorporation of label into glucosylceramide. These data corroborate the conclusion that the glucolipid sensitive to mild acid formed by these white matter preparations is glucosylphosphoryldolichol and indicate that the endogenous level of dolichyl monophosphate is rate limiting. Because the biosynthesis of glucosylceramide by brain membranes has been previously described (see review by Morell and Braun, 1972), this reaction was not examined extensively in this study.

Effect of UMP and UDP on the Biosynthesis of [^{14}C]*Glucolipids.* Enzymatic studies were conducted to determine whether UMP or UDP was the nucleotide product formed during the biosynthesis of GPD and to examine the reversibility of the reaction. While the addition of UMP had no effect on the labeling of GPD or glucosylceramide, UDP inhibited the labeling of both [^{14}C]glucolipids (Table III). To test the reversibility of these biosynthetic reactions, white matter membranes were prelabeled by incubation with UDP-[^{14}C]glucose for 10 min. Then unlabeled UMP or UDP was added and the membranes were incubated for 5 min. The data in Table III show that the addition of UMP to membranes containing endogenous labeled glucolipid has no effect and did not prevent further incorporation of [^{14}C]glucose into either glycolipid. However, the addition of UDP to prelabeled membranes resulted in a loss of over 85% of the radioactivity in GPD. Although UDP prevented further incorporation of [^{14}C]glucose into glucosylceramide, there was no loss of label from the glucosphingolipid. These data indicate that, although UDP inhibits the formation of both glucosylceramide and GPD, only the biosynthesis of GPD is freely reversible. This result is consistent with the presence of an activated linkage in GPD between the glucosyl residue and the carrier lipid as would be expected if GPD served as an intermediary glucosyl donor.

Partial Chemical and Chromatographic Characterization of ^{14}*C-Labeled-Glucolipids Formed by White Matter Membranes.* Incubation of a crude membrane preparation from the white matter of calf brain in the presence of UDP-[^{14}C]glucose and Mg^{2+} led to the labeling of two ^{14}C-labeled glucolipids. The proportions of the two labeled glucolipids varied with the metal ion added and time of incubation. The labeled glucolipids were resolved by chromatography on EDTA-treated SG-81 paper developed with solvent A (Figure 3). Both glucolipids remained adsorbed to a Unisil column washed with $CHCl_3$. One labeled glucolipid was eluted with acetone, the fraction containing glycosphingolipids. The other ^{14}C-labeled glucolipid was recovered by elution with $CHCl_3$–CH_3OH (1:1) indicating that it is a phosphoglucolipid. The ^{14}C-labeled glucolipid recovered in the acetone fraction was stable to alkaline methanolysis (0.1 N KOH in CH_3OH–toluene 3:1, 60 min, 0 °C) and mild acid hydrolysis (0.1 N HCl in 50% 1-propanol, 60 min, 50 °C). These chemical properties distinguish this glucolipid from glucosyl diglycerides and glucosylphosphorylpolyisoprenols, respectively. Treatment with strong acid (3 N HCl, 100 °C, 6 h) released a radioactive water-soluble product that cochromatographed with authentic glucose on paper chromatography in solvent systems I, J, and K. No labeled galactose was observed. The labeled glucolipid recovered from Unisil by elution with acetone also cochromatographed with authentic glucosylceramide on borate-treated SG-81 paper when developed with solvent mixtures E, F, G, and H. The mild acid stable ^{14}C-labeled glucolipid was

TABLE III: Effect of UMP and UDP on the Transfer of [^{14}C]Glucose from UDP-[^{14}C]Glucose into [^{14}C]Glucolipids.[a]

Prelabeling time (min)	Addition prior to incubation period (1 mM)	Incubation time (min)	[^{14}C]Glucose incorp into (cpm)	
			Glc-P-dolichol	Glc-ceramide
	None	5	1400	518
	UMP	5	1478	481
	UDP	5	15	7
10	None		1747	860
10	None	5	2030	1378
10	UMP	5	2034	1305
10	UDP	5	239	898

[a] All reaction mixtures contained enzyme (1.7 mg of membrane protein) suspended in 0.05 M Tris-HCl (pH 8.0), 0.125 M sucrose, 0.5 mM EDTA, 2.5 mM AMP, 10 mM $MgCl_2$, and 5 μM UDP-[^{14}C]glucose (482 cpm/pmol) in a total volume of 0.20 mL. Uridine nucleotides were added where indicated and, after incubation at 37 °C, 20 volumes of $CHCl_3$-CH_3OH (2:1) were added to stop the reaction. The amount and distribution of labeled glucolipids formed were assayed as described in Materials and Methods.

not retained by a DEAE-cellulose column in $CHCl_3$-CH_3OH (7:3). All of these chemical and chromatographic characteristics indicate that the mild acid-stable glucolipid formed by these white matter membranes is glucosylceramide. This tentative identification is supported by the observation that the enzymatic labeling of this glucolipid is enhanced by the addition of exogenous ceramide.

The ^{14}C-labeled glucolipid recovered from a Unisil column in the $CHCl_3$-CH_3OH (1:1) fraction was retained by a DEAE-cellulose column developed with $CHCl_3$-CH_3OH-H_2O (10:10:3) and eluted as a single peak of radioactivity with the developing solvent containing 2 mM ammonium acetate (Figure 4, panel A). This chromatographic property is characteristic of the monosaccharide derivatives of dolichol in which the glycosyl groups are linked to the lipid moiety by a monophosphate bridge. The labeled glucolipid recovered from DEAE-cellulose was stable to mild alkaline methanolysis, but, when treated with mild acid (0.1 N HCl in 50% 1-propanol at 50 °C), free [^{14}C]glucose was released with a $t_{1/2}$ = 15 min. The radioactive water-soluble product was identified as [^{14}C]glucose by paper chromatography in solvent systems I, J, and K. Glucose was the only labeled product detected following complete hydrolysis of the labeled glucolipid. The mild acid sensitive glucolipid also had a mobility similar to mannosylphosphoryldolichol when chromatographed on SG-81 paper with solvent mixtures B, C, and D.

All of the hydrolytic and chromatographic characteristics of the ^{14}C-labeled glucolipid eluted from Unisil with $CHCl_3$-CH_3OH (1:1) are consistent with the conclusion that it has the structure glucosylphosphoryldolichol. This conclusion is corroborated by the observation that its synthesis is stimulated by the presence of exogenous dolichyl monophosphate (Table II).

Partial Characterization of the Glucosylated Oligosaccharide Lipid Labeled in Vitro by White Matter Membranes. The labeled product recovered in $CHCl_3$-CH_3OH-H_2O (10:10:3) is insoluble in H_2O or $CHCl_3$-CH_3OH mixtures. The intact oligosaccharide lipid has a characteristic R_f (0.70–0.75) when chromatographed on Whatman No. 3MM paper and developed with solvent mixture H. It is retained by a DEAE-cellulose column developed with $CHCl_3$-CH_3OH-H_2O (10:10:3) and remains bound to the column during elution with the developing solvent containing 2 mM ammonium acetate, but is recovered as a single peak of radioactivity when the ammonium acetate concentration is increased to 20 mM (Figure 4, panel B). This chromatographic behavior is characteristic of the dolichol derivatives in which the glycosyl

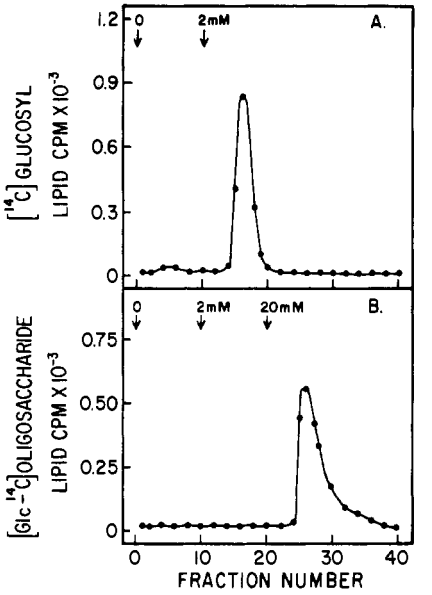

FIGURE 4: Chromatographic comparison of [^{14}C]glucosylphosphoryldolichol and [^{14}C]glucosylated oligosaccharide lipid on DEAE-cellulose. [^{14}C]Glucosylphosphoryldolichol (A) or [*Glc*-^{14}C]oligosaccharide lipid (B) were applied to a DEAE-cellulose column (1.45 × 5.0 cm) equilibrated with $CHCl_3$-CH_3OH-H_2O (10:10:3). The column was sequentially washed with 20 mL of the equilibration solvent containing 2 mM ammonium acetate and 20 mL of the same solvent containing 20 mM ammonium acetate (salt concentrations indicated by arrows). Two-milliliter fractions were collected and the amount of radioactivity eluted was measured.

groups are attached to the lipid carrier by a pyrophosphate bridge. Consistent with this structure is the liberation of a labeled oligosaccharide unit by treatment with 0.1 N HCl in 80% tetrahydrofuran at 50 °C ($t_{1/2}$ = 14 min). Sensitivity to mild acid hydrolysis distinguishes this class of oligosaccharide lipids from the complex glycosphingolipids found abundantly in neural membranes. The *Glc*-^{14}C-labeled oligosaccharide released by mild acid treatment is electrophoretically neutral on Whatman No. 3MM paper with 1.5 M acetic acid–0.5 M formic acid, pH 2.2 (Figure 5, trace A), or pyridinium acetate, pH 6.4. Some preparations contained a very small amount of a positively charged species. Treatment of the neutral oligosaccharide with strong alkali (1 N NaOH–1 M sodium borohydride, 100 °C, 18 h) produced two prominent positively charged products (Figure 5, trace B). It is likely that these two products are produced by the deacetylation of one or two

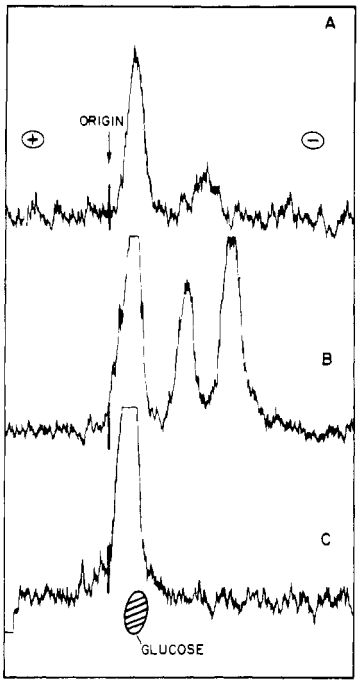

FIGURE 5: Paper electrophoresis of the products formed by strong alkaline hydrolysis of the [*Glc*-^{14}C]oligosaccharide released from its carrier lipid. Approximately 6000 cpm was subjected to electrophoresis on Whatman No. 3MM paper in 1.5 M acetic acid-0.5 M formic acid, pH 2.2, for 3 h at 1900 V. Unlabeled glucose (1 μmol) was added to each sample as a marker for the mobility of neutral molecules. The electrophoretograms were dried and scanned with a Packard radiochromatogram scanner 7201. (Trace A) Untreated [Glc-^{14}C]oligosaccharide; (trace B) [Glc-^{14}C]oligosaccharide treated with 1 N NaOH–1 M sodium borohydride, 100 °C, 18 h; (trace C) [Glc-^{14}C]oligosaccharide treated with strong alkali followed by N-acetylation (Carlson, 1967). Unlabeled glucose was detected with an aniline–diphenylamine dip (Schwimmer and Benvenue, 1956).

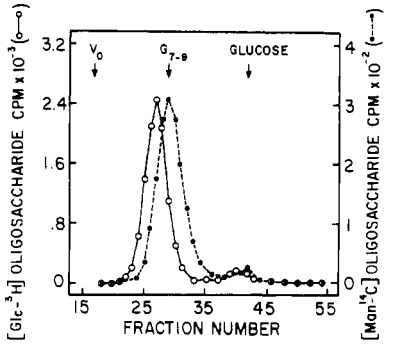

FIGURE 6: Chromatographic comparison on Bio-Gel P-6 of [Glc-^{3}H]-oligosaccharide and [Man-^{14}C]oligosaccharide released from their carrier lipids by mild acid hydrolysis. Samples of each labeled oligosaccharide were mixed and cochromatographed on a Bio-Gel P-6 column (0.9 × 37 cm). The column was eluted with 0.1 M NaCl, and 0.5-mL fractions were collected and counted.

hexosamines. In support of this tentative conclusion the two positively charged compounds were neutralized by treatment under conditions used for N-acetylation (Figure 5, trace C).

Because the glucosylated oligosaccharide lipid may be synthesized in brain by the addition of two glucose residues to the mannosylated oligosaccharide lipid, as originally proposed for liver (Behrens et al., 1973), we have compared the molecular size of the *Glc*-^{3}H-labeled oligosaccharide unit with the *Man*-^{14}C-labeled oligosaccharide unit by gel filtration on Bio-Gel P-6. An analysis of the lipid-linked *Man*-^{14}C-labeled oligosaccharide formed by white matter membranes used in an earlier study revealed two partially resolved fractions (Waechter et al., 1976). However, the membrane preparations used in our current studies incorporate [^{14}C]mannose into a single oligosaccharide fraction (Figure 6). The mannose-labeled oligosaccharide formed by brain is chromatographically identical with the mannosylated oligosaccharide formed by hen oviduct membranes previously estimated to contain approximately 8 glycose units (Lucas et al., 1975) when compared on Bio-Gel P-6 or paper chromatography with solvent system H. The *Glc*-^{3}H-labeled oligosaccharide appears to be slightly larger than the mannosylated oligosaccharide based on its elution volume on Bio-Gel P-6 (Figure 6). When the size was evaluated by paper chromatography, an estimate of 10 glycose units was obtained. In agreement with this value a molecular weight of 1700 was determined by gel filtration (Bhatti and Clamp, 1968) using Bio-Gel P-4.

Treatment of the [*Glc*-^{14}C]oligosaccharide with strong acid (3 N HCl, 100 °C, 6 h) released a labeled product that was chromatographically identical with authentic glucose on paper chromatography with solvent mixtures I and L. No labeled galactose was detected in the hydrolyzate. Glucose does not appear to be at the reducing terminus since treatment of the free oligosaccharide with sodium borohydride prior to strong acid hydrolysis did not yield any glucitol. Under the same reductive conditions labeled glucose was quantitatively converted to glucitol. As yet, there is no information on the identity of the glycose residue at the reducing terminus. However, the lipid-linked glucosylated oligosaccharide formed by white matter membranes appears to contain two hexosamines and has the chemical and chromatographic properties of the glucose-containing oligosaccharide lipid intermediate synthesized by rat liver microsomes (Parodi et al., 1972), and the dolichol-linked oligosaccharides formed by thyroid slices (Spiro et al., 1976b) and calf pancreas microsomes (Herscovics et al., 1977a) that contain mannose, *N*-acetylglucosamine, and glucose. The molecular size of the white matter oligosaccharide is consistent with the proposal that it is synthesized by the addition of two glucosyl residues to the mannosylated oligosaccharide lipid also formed by white matter membranes.

Partial Characterization of Labeled Products Recovered in the Glycoprotein Fraction. When the delipidated membrane residue, labeled by incubation with UDP-[^{14}C]glucose, is treated with strong acid (3 N HCl, 100 °C, 6 h), a water-soluble product is released that cochromatographs with authentic glucose on paper chromatography with solvent mixtures I and K. The glucosylated products can be solubilized with 1% sodium dodecyl sulfate–0.1% mercaptoethanol (100 °C, 5 min). Upon gel filtration on Bio-Gel P-100 or A-1.5M utilizing an elution buffer containing 0.2% sodium dodecyl sulfate–0.1% mercaptoethanol, all of the radioactivity is excluded. Approximately 75% of the large molecular weight material labeled in the presence of Mg^{2+} was extensively degraded by trypsin or Pronase. These results indicate that the membrane residue contains at least one glucoprotein.

The Pronase digest of the glycoprotein fraction labeled in the presence of Mg^{2+} was resolved into two major products by gel filtration on Bio-Gel P-6 (Figure 7, solid line). A minor radioactive peak is also observed occasionally. This product appears to be a small amount of labeled glucose produced by prolonged Pronase digestion. Only the product eluted in the void volume is observed when the glycoprotein fraction is labeled in the presence of 10 mM EDTA and solubilized by Pronase treatment (Figure 7, broken line). This product is also excluded by Bio-Gel P-100 and is digested to a mixture of glucose and maltose by incubation with α-amylase. Treatment with β-amylase yields maltose. Thus this product has the properties of a particle-bound glucan containing α-1,4-linked

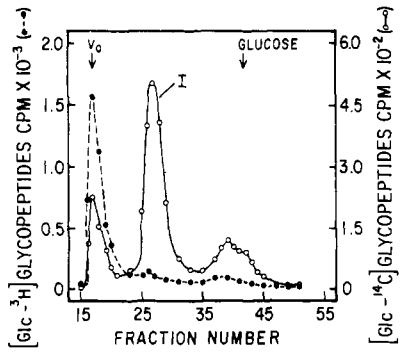

FIGURE 7: Chromatographic comparison of Pronase digest of [^{14}C]glycoprotein fraction labeled in the presence of Mg^{2+} (O—O) and Pronase-digested [^{3}H]glycoprotein fraction labeled in the presence of 10 mM EDTA (●- - - -●). The labeled samples were mixed and applied to a Bio-Gel column (0.9 × 37 cm) equilibrated with 0.1 M NaCl. The column was eluted with 0.1 M NaCl, and 0.5-mL fractions were collected and counted. V_0, blue dextran.

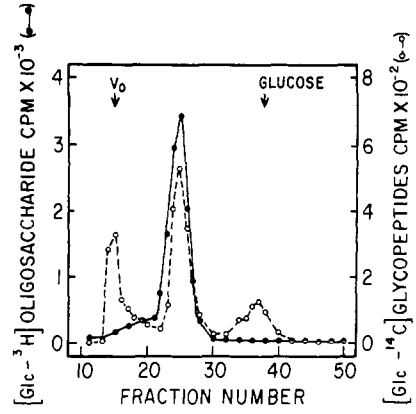

FIGURE 8: Chromatographic comparison of Pronase digest of [^{14}C]glycoprotein fraction labeled in presence of Mg^{2+} (O- - -O) and [Glc-^{3}H]oligosaccharide (●—●) released from carrier lipid by mild acid treatment. The chromatographic procedure was the same as described for Figure 7.

glucosyl residues and may be related to glycogen. It is not yet clear whether this glucan is derived from a proteoglucan similar to the one proposed to be involved in glycogen biosynthesis (Krisman and Barengo, 1975).

The major labeled product, included in Bio-Gel P-6 (Figure 7, fraction I), is not sensitive to amylase treatment, but moves toward the cathode, as expected for a glucopeptide, when subjected to electrophoresis in 1.5 M formic acid, pH 2.0. Because this glucopeptide is labeled under conditions that allow [^{14}C]glucose incorporation into the oligosaccharide lipid, it is possible that the peptide-bound oligosaccharide unit is derived from the carrier lipid. Consistent with this idea, a chromatographic comparison on Bio-Gel P-6 revealed that the [^{14}C]glucopeptide has a molecular size quite similar to the lipid-linked Glc-^{3}H-labeled oligosaccharide (Figure 8). Further studies will be required to conclusively show that the glucoprotein is glycosylated via the glucosylated oligosaccharide lipid.

Discussion

The biosynthesis of glucosylphosphoryldolichol was first detected in animal tissues by Behrens and Leloir (1970). Since then the formation of GPD has been reported for membrane preparations from cotton fibers (Forsee and Elbein, 1973), *Tetrahymena pyriformis* (Keenan et al., 1973), human lymphocytes (Wedgewood et al., 1974), hen oviduct (Waechter et al., 1974), calf pancreas (Herscovics et al., 1977b), insects (Quesada et al., 1975), and yeast (Palamarczyk and Chojnacki, 1973) (Parodi, 1976). Experiments conducted with embryonic chick brain (Breckenridge and Wolfe, 1973) and rat brain (Jankowski and Chojnacki, 1972) have shown that membrane preparations from these tissues catalyze the transfer of labeled glucose from the sugar nucleotide into a lipid fraction, and this transfer was stimulated by the addition of dolichyl monophosphate to the reaction mixture. However, the glucolipids were not further characterized in these studies.

In this paper we present detailed information on the enzymatic transfer of glucose from UDP-glucose into GPD, a glucose-containing oligosaccharide lipid and a glucoprotein fraction catalyzed by a membranous enzyme preparation from the white matter of calf brain. Under the same incubation conditions, glucose was also transferred into a glucolipid, chemically and chromatographically identical with glucosylceramide and a glucan recovered in the glycoprotein fraction. Since the presence of EDTA blocked the formation of GPD and the oligosaccharide lipid, but allowed labeling of glucosylceramide and a particle-bound glucan in the glycoprotein fraction, it is likely that the latter two products are glucosylated directly by UDP-glucose. The product recovered in the glycoprotein fraction, after labeling in the presence of EDTA, is degraded by α- or β-amylase indicating that this product contains α-1,4-linked glucosyl residues, and is possibly related to the proteoglucan believed to be a precursor of glycogen (Krisman and Barengo, 1975). Whelan and his co-workers (Butler et al., 1977) have also recently reported evidence for a protein-bound form of glycogen. The glucopeptide labeled in the presence of Mg^{2+} (Figure 7, fraction I) may be derived from a glucoprotein containing the linkage region for the glycogen primer. The glycogen-like polymer enzymatically glucosylated in these studies also appears to be similar to a high molecular weight glucan that was found in high amount in young rat brain (Krusius et al., 1974; Margolis et al., 1976).

The time course for the labeling of GPD, the glucosylated oligosaccharide lipid, and glycoprotein during incubation of white matter membranes with UDP-[^{14}C]glucose and Mg^{2+} is consistent with a precursor-product relationship existing between the lipophilic glucosyl carriers and glycoprotein as shown in Figure 2. Earlier studies (Waechter et al., 1976) have shown that exogenous mannosylphosphoryldolichol serves as a mannosyl donor in the biosynthesis of a calf brain oligosaccharide lipid. Work now underway is designed to determine if GPD serves a similar function in the biosynthesis of the glucosylated oligosaccharide lipid formed by calf white matter. Similar studies conducted with membrane preparations from rat liver (Behrens et al., 1971), calf pancreas (Herscovics et al., 1977b), and yeast (Parodi, 1976) have shown that exogenous GPD can serve as a glucosyl donor in the formation of glucosylated oligosaccharide lipid.

The hydrolytic and chromatographic characteristics of the glucosylated oligosaccharide formed by white matter membranes indicate that the oligosaccharide unit is bound to the lipid moiety by a pyrophosphate bridge. The lipid-linked oligosaccharide unit has been estimated to contain 10 glycose units by paper chromatographic and gel filtration analyses. We have also presented evidence for the presence of two hexosamine residues. It is possible that the lipid-linked oligosaccharide formed in vitro is incomplete since the lipid-bound oligosaccharide that accumulates in thyroid slices contains 11 mannose, 1-2 glucose, and 2-*N*-acetylglucosamine residues (Spiro et al., 1976b). Although there is no evidence that this

calf brain oligosaccharide lipid contains mannose, it appears quite similar to the dolichol-linked oligosaccharides radiolabeled by thyroid slices and pancreas microsomes (Herscovics et al., 1977a) that contain mannose, glucose, and *N*-acetylglucosamine. Based on the findings that (1) *N,N'*-diacetylchitobiosylpyrophosphoryldolichol serves as a precursor of the mannosylated oligosaccharide lipid in white matter (Waechter and Harford, 1977), (2) the white matter mannosylated oligosaccharide bound to lipid contains approximately 8 glycose units, and (3) that the lipid-linked glucosylated oligosaccharide has been estimated to contain 10 glycose residues, including two hexosamines, the tentative structure shown below can be proposed for the glucosylated oligosaccharide lipid formed in vitro by white matter membranes.

$$(Glc)_2(Man)_6(GlcNAc)_2\text{-P-P-dolichol}$$

If the glucosylated oligosaccharide lipid serves as an oligosaccharide donor in the biosynthesis of membrane glycoproteins in brain, the identification of the glucoprotein product will be an important objective. Although there have been several papers documenting the presence of glucose-containing glycoproteins in brain (Van Nieuw Amerongen et al., 1972; Javaid et al., 1975; Churchill et al., 1976; Simpson et al., 1976; Barclay et al., 1976), the presence of glucose in brain glycoproteins is still a controversial issue. Margolis et al. (1976) have suggested that residual glycogen is responsible for the presence of small amounts of glucose found in glycopeptide fractions. However, our studies (Figure 8) show that the glucan and the glucopeptide labeled by incubating white matter membranes with UDP-[^{14}C]glucose in the presence of Mg^{2+} are chromatographically distinct products.

It will be of great interest to see if cellular membranes from the central nervous system, as well as other animal tissues, contain glycoproteins having oligosaccharide units consisting of glucose, mannose, and *N*-acetylglucosamine as would be expected if the oligosaccharide units are transferred en bloc from the lipid carrier. The possibility that glucose residues serve a regulatory function and are excised by cytoplasmic or membrane-bound glucosidases after the oligosaccharide is linked to the polypeptide acceptor (as shown in Figure 2, dotted line) must also be considered.

Acknowledgments

The authors wish to express their appreciation to Mr. William Reid of George L. Reid, Inc., for his help in obtaining fresh calf brains for these studies. The helpful technical assistance of Carol Cass is also gratefully acknowledged.

References

Barclay, A. N., Letarte-Muirhead, M., Williams, A. F., and Faulkes, R. A. (1976), *Nature (London) 263,* 563–567.

Bartlett, G. R. (1959), *J. Biol. Chem. 234,* 466–468.

Behrens, N. H., Carminatti, H., Staneloni, R. J., Leloir, L. F., and Cantarella, A. I. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3390–3394.

Behrens, N. H., and Leloir, L. F. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 152–159.

Behrens, N. H., Parodi, A. J., and Leloir, L. F. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2857–2860.

Bhatti, T., and Clamp, I. R. (1968), *Biochim. Biophys. Acta 170,* 206–208.

Breckenridge, W. C., and Wolfe, L. S. (1973), *FEBS Lett. 29,* 66–68.

Butler, N. A., Lee, E. Y. C., and Whelan, W. J. (1977), *Carbohyd. Res. 55,* 73–82.

Carlson, D. M. (1967), *Anal. Biochem. 20,* 195–198.

Churchill, L., Cotman, C., Banker, G., Kelly, P., and Shannon, L. (1976), *Biochim. Biophys. Acta 448,* 57–72.

Forsee, W. T., and Elbein, A. D. (1973), *J. Biol. Chem. 248,* 2858–2867.

Herscovics, A., Bugge, B., and Jeanloz, R. W. (1977b), *J. Biol. Chem. 252,* 2271–2277.

Herscovics, A., Golovtchenko, A. M., Warren, C. D., Bugge, B., and Jeanloz, R. W. (1977a), *J. Biol. Chem. 252,* 224–234.

Jankowski, W., and Chojnacki, T. J. (1972), *Biochim. Biophys. Acta 260,* 93–97.

Javaid, J. I., Hof, H., and Brunngraber, E. G. (1975), *Biochim. Biophys. Acta 404,* 74–82.

Jochen, A., Scher, M. G., and Waechter, C. J. (1977), *Trans. Am. Soc. Neurochem. 8,* 189.

Keenan, R. W., Matula, J. M., and Holloman, L. (1973), *Biochim. Biophys. Acta 326,* 84–92.

Krisman, C. R., and Barengo, R. (1975), *Eur. J. Biochem. 52,* 117–123.

Krusius, T., Finne, J., Karkkainen, J., and Jarnefelt, J. (1974), *Biochim. Biophys. Acta 365,* 80–92.

Lucas, J. J., and Waechter, C. J. (1976), *Mol. Cell Biochem. 11,* 67–78.

Lucas, J. J., and Waechter, C. J., and Lennarz, W. J. (1975), *J. Biol. Chem. 250,* 1992–2002.

Margolis, R. K., Preti, G., Lai, O., and Margolis, R. V. (1976), *Brain Res. 112,* 363–369.

Morell, P., and Braun, P. (1972), *J. Lipid Res. 13,* 293–310.

Palamarczyk, G., and Chojnacki, T. J. (1973), *FEBS Lett. 34,* 201–203.

Parodi, A. J. (1976), *FEBS Lett. 71,* 283–286.

Parodi, A. J., Behrens, N. H., Leloir, L. F., and Carminatti, H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3268–3272.

Quesada, L. A., Belocopitow, E., and Marechal, L. R. (1975), *Biochem. Biophys. Res. Commun. 66,* 1201–1208.

Robbins, P. W., Krag, S. S., and Liu, T. (1977), *J. Biol. Chem. 252,* 1780–1785.

Schwimmer, S., and Benvenue, A. (1956), *Science 123,* 543–544.

Simpson, D., Thorne, D. R., and Loh, H. H. (1976), *Biochemistry 15,* 5449–5457.

Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976a), *J. Biol. Chem. 251,* 6400–6408.

Spiro, M. J., Spiro, R. G., and Bhoyroo, V. D. (1976c), *J. Biol. Chem. 251,* 6420–6425.

Spiro, R. G., Spiro, M. J., and Bhoyroo, V. D. (1976b), *J. Biol. Chem. 251,* 6409–6419.

Steiner, S., and Lester, R. L. (1972), *J. Bacteriol. 109,* 81–88.

Van Nieuw Amerongen, A., van den Eijnden, D. H., Heijlman, I., and Roukema, P. A. (1972), *J. Neurochem. 19,* 2195–2205.

Waechter, C. J., and Harford, J. B. (1977), *Arch. Biochem. Biophys. 181,* 185–198.

Waechter, C. J., and Lennarz, W. J. (1976), *Annu. Rev. Biochem. 45,* 95–112.

Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973), *J. Biol. Chem. 248,* 7570–7579.

Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1974), *Biochem. Biophys. Res. Commun. 56,* 343–350.

Waechter, C. J., Kennedy, J. L., and Harford, J. B. (1976), *Arch. Biochem. Biophys. 174,* 726–737.

Wawszkiewicz, E. J. (1961), *Anal. Chem. 33,* 252–254.

Wedgewood, J. F., Strominger, J. L., and Warren, C. D. (1974), *J. Biol. Chem. 249,* 6316–6324.