Structure of the Human Erythrocyte Blood Group P_1 Glycosphingolipid†

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ABSTRACT: A glycosphingolipid with blood group P_1 activity was extracted from an acetone powder of human erythrocyte stroma with chloroform–methanol. It was purified by chromatography on columns of silicic acid and by preparative thin-layer chromatography of the fully acetylated and deacetylated glycolipid. The purified glycolipid contained galactose, *N*-acetylglucosamine, and glucose in a molar ratio of 3:1:1. Treatment of the P_1 glycolipid with fig α-galactosidase released a single galactosyl residue and destroyed the blood group activity, and the α-galactosidase product had the same chromatographic mobility as paragloboside. Substitution sites on the neutral sugars of the P_1 glycolipid and the α-galactosidase product were established by identification of methylated alditol acetates, and substitution on *N*-acetylglucosamine was determined by identification of methyl glycoside derivatives. The terminal nonreducing disaccharide of the P_1 glycolipid is Gal(α,1→4)Gal. *N*-Acetylglucosamine was identified as the next sugar in sequence by mass spectrometric analysis of the permethylated P_1 glycolipid. On the assumption that the glucose residue is linked to ceramide, we propose the following structure for the P_1 glycolipid: Gal(α,1→4)Gal(β,1→4)Glc-NAc(β,1→3)Gal(β,1→4)Glc-Cer.

The human P_1 blood group antigen (Race and Sanger, 1968) was first demonstrated on erythrocytes (Landsteiner and Levine, 1927) and was recently detected on skin fibroblasts and lymphocytes (Fellous et al., 1973). The first data on the chemical nature of the P_1 antigenic determinant were provided by studies of a cross-reacting glycoprotein isolated from sheep liver hydatid cyst fluid (Cameron and Staveley, 1957; Morgan and Watkins, 1964). They demonstrated that a terminal nonreducing α-galactosyl residue on the glycoprotein was essential for P_1 activity, and that human anti-P_1 sera could be inhibited by disaccharides containing a terminal nonreducing α-galactosyl residue (Watkins and Morgan, 1964). We previously extracted a glycosphingolipid with P_1 activity from human erythrocytes (Marcus, 1971) and we now present data on the purification and structure of this compound.

Materials and Methods

Glycosphingolipids and Glycoproteins (*Table I*). Ceramide monohexoside, ceramide trihexoside, and the *N*-acetylglucosamine-containing ganglioside were prepared from human erythrocytes (Yamakawa et al., 1965; Kawanami, 1967; Hakomori et al., 1971; Ando and Yamakawa, 1973; Li et al., 1973). Paragloboside (Ando et al., 1973; Wherrett, 1973; Siddiqui and Hakomori, 1973) was prepared by treatment of the ganglioside with neuraminidase (EC 3.2.1.18) from *Clostridium perfringens* (Sigma Chemical Co.) and isolation of the neutral glycolipid by chromatography on a column of DEAE-Sephadex or silicic acid. The preparation of other glycolipids has been described: ceramide trihexoside and globoside (Marcus and Janis, 1970), Forssman (Naiki et al., 1972), and ganglioside G_{M1} and asialo G_{M1} (Naiki et al., 1974).

A ceramide pentasaccharide with blood group B activity (Eto et al., 1968; Stellner et al., 1973b) isolated from rabbit erythrocytes was obtained from Dr. S. Hakomori.

A glycoprotein with P_1 and P^k antigenic activities was isolated from sheep liver hydatid cyst fluid (Morgan and Watkins, 1964), and the preparation and properties of glycoproteins with ABH activity have been described (Marcus and Grollman, 1966).

Antisera. Human anti-P_1, anti-A, and anti-B were obtained from individuals of P_2, B, and A_1 phenotypes, respectively. Eel serum, obtained from Dr. G. Springer, was used as an anti-H reagent. Hemagglutination inhibition tests were performed in a microtiter plate (Cooke Chemical Products). Four hemagglutinating units of serum in a volume of 0.025 ml were mixed with an equal volume of inhibitor and incubated at 4°C for 12–18 hr, 0.025 ml of a 2% (v/v) suspension of erythrocytes was added, and the hemagglutination patterns were read after an additional 2 hr at 4°C. The assays can be performed by incubating the inhibitor with the antibody for only 15–30 min, but the longer incubation period increases the sensitivity of the assay. Glycolipid dispersions were prepared for hemagglutination inhibition tests by evaporation of organic solvents under a stream of N_2 and dispersing the glycolipid in PBS[1] containing sodium taurocholate (Nutritional Biochemical Corp.) by ultrasonic irradiation in a Heat Systems-Ultrasonic water bath for 2 min at room temperature.

Chromatographic Materials and Solvents. Reagent

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[1] Abbreviations used are: Gal, D-galactose; Glc, D-glucose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; NAN, *N*-acetylneuraminic acid; ceramide (Cer), *N*-acylsphingosine; PBS, 0.02 *M* potassium phosphate (pH 7.3)–0.15 *M* NaCl; Me_3Si, trimethylsilyl; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; C-M, chloroform–methanol; C-M-W, chloroform–methanol–water; D-M-W, dichloroethane–methanol–water.

Table I: Structures of Glycosphingolipids.[a]

A. Glycosphingolipids of Human Erythrocytes	
Ceramide monohexoside	Glc-Cer
Ceramide dihexoside	Gal(β,1→4)Glc-Cer
Ceramide trihexoside	Gal(α,1→4)Gal(β,1→4)Glc-Cer
Globoside	GalNAc(β,1→3)Gal(α,1→4)Gal(β,1→4)Glc-Cer
Hematoside	NAN(α,2→3)Gal(β,1→4)Glc-Cer
GlcNAc-ganglioside	NAN(α,2→3)Gal(β,1→4)GlcNAc(β,1→3)Gal(β,1→4)Glc-Cer
Paragloboside	Gal(β,1→4)GlcNAc(β,1→3)Gal(β,1→4)Glc-Cer
P_1 antigen	Gal(α,1→4)Gal(β,1→4)GlcNAc(β,1→3)Gal(β,1→4)Glc-Cer
B. Glycosphingolipids Used as Chromatographic Standards	
Asialo G_{M1}	Gal(β,1→3)GalNAc(β,1→4)Gal(β,1→4)Glc-Cer
G_{M1}	Gal(β,1→3)GalNAc(β,1→4)Gal(β,1→4)Glc-Cer (α,2→3) NAN
Forssman	GalNAc(α,1→3)GalNAc(β,1→3)Gal(α,1→4)Gal(β,1→4)Glc-Cer

[a] Abbreviations used are given in footnote 1.

grade solvents obtained from Fisher Scientific Co., Merck Laboratory Chemicals, and Aldrich Chemical Co. were used for column chromatography, and were distilled for analytical and preparative TLC.[1] Materials used for column chromatography included DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Inc.), Unisil, 200–325 mesh silicic acid (Clarkson Chemical Co.), Florisil, 100 mesh (Fisher Scientific Co.), and silica gel 60 and silica gel H (Brinkmann Instruments, Inc.). Precoated plates for TLC, containing silica gel G or silica gel HR, were obtained from Analtech, Inc.

Gas Chromatography. GLC analyses were carried out on a Hewlett-Packard gas chromatograph Model No. 7610A equipped with a Model 3370 electronic integrator. Me_3Si derivatives of methyl glycosides were prepared and analyzed by the method of Vance and Sweeley (1967) as modified in this laboratory (Fong et al., 1975). The chief modifications were acetylation of the methyl glycosides produced by methanolysis with pyridine–acetic anhydride and removal of the *O*-acetyl groups with 0.2 *N* KOH in methanol for 2 hr prior to preparation of the Me_3Si derivatives. More reproducible recoveries of hexosamine were obtained by this method. Mannitol was used as an internal standard and the derivatized glycosides were analyzed under isothermal conditions (152°C) on a 6-ft glass column packed with 3% OV-1 on 80–100 mesh Chromosorb W (Supelco, Inc.). The ratio of hexose/hexosamine was determined empirically by reference to oligosaccharides or glycolipids of known composition that were analyzed simultaneously under the same conditions as the unknown samples. Globoside was used as a reference compound containing *N*-acetylgalactosamine, and lacto-*N*-difucohexaose, obtained from Dr. Adeline Gauhe, was used as a reference compound for *N*-acetylglucosamine.

Methylation. P_1 glycolipid and the tetraglycosylceramide resulting from the α-galactosidase reaction were permethylated according to the method of Hakomori (1964), as applied in this laboratory (Kundu et al., 1975a), and the products were purified by preparative TLC. Approximately 50 μg was subjected to hydrolysis and reduction according to the method of Bjorndal et al. (1970) as applied in this laboratory (Price et al., 1975). The mixture was passed through a small column of Dowex 50W (H^+) to remove hexosamine-derived products which gave poorly defined peaks on GLC; these sugars were analyzed separately (see below). The partially methylated neutral sugar alditols were acetylated and purified by chromatography on a 1-g column of Unisil (Clarkson Chemical Co.) by eluting impurities with 30 ml of benzene and eluting the sugars with 30 ml of chloroform. GLC analysis was performed isothermally at 175°C with a 6-ft OV-225 column, 3% on 100–120 mesh Supelcoport (Supelco Inc.). Similar resolution was obtained with an ECNSS-M column, 3% on 100/120 mesh Gas-Chrom Q (Applied Science Labs., Inc.). Standards were prepared from permethylated glycolipids of known structure.

Substitution on *N*-acetylglucosamine was determined by a recently described procedure (Kundu et al., 1975b) utilizing methyl glycosides of the substituted hexosamine. Approximately 20 μg of permethylated P_1 glycolipid was subjected to acid-catalyzed methanolysis followed by acetylation of the resulting free hydroxyl group. Products were identified by GLC on both OV-225 and OV-1.

Mass Spectrometry. Mass spectra (MS) were obtained with a Finnigan 3300 quadrupole mass spectrometer equipped with the Finnigan MS data system 6000. Operating conditions were: electron energy, 30 eV; electron multiplier voltage, 2000 V; preamplifier sensitivity, 10^{-8} A/V (high sensitivity); and probe temperature, 350–400°C. Approximately 10 μg of the permethylated glycolipid was deposited in a Pyrex sample tube and injected via direct inlet. Seventy or more scans were obtained with each injection. A limited mass search with the data system selected the best scan for the *m/e* 668 and 636 peaks. The assistance of Dr. Francis Hoffman and Ms. Vinca Parmakovich of Columbia University is gratefully acknowledged.

Enzymatic Hydrolysis of Glycolipids. Fig α-galactosidase (EC 3.2.1.22) and jack bean β-galactosidase (EC 3.2.2.23) were gifts from Drs. S.-C. and Y.-T. Li (Tulane University), and β-hexosaminidase from *Turbo cornatus* was a gift from Professor A. Kobata, Kobe University, Japan. The enzymatic activity of the galactosidases was measured by hydrolysis of *p*-nitrophenyl α- and β-galactosides (Sigma Chemical Co.) in 0.05 *M* citrate buffer (pH 4.0) (Li and Li, 1972a,b), and β-hexosaminidase activity was measured with *p*-nitrophenyl β-*N*-acetylglucosaminide in 0.02 *M* phosphate–citrate buffer (pH 4.4) (Kaback, 1972). The activity of the β-galactosidase against glycosphingolipids was demonstrated by the hydrolysis of the terminal galactose residue from paragloboside, and the β-hexosaminidase hydrolyzed the terminal *N*-acetylglucosamine residue from the ceramide trisaccharide created by removal

of galactose from paragloboside. Neither of these enzymes altered the immunological activity of the P_1 glycolipid.

Purified P_1 glycolipid (0.2 mg) was treated with 7 units of fig α-galactosidase in 0.2 ml of 0.05 *M* citrate buffer (pH 4.0) containing 0.5 mg of sodium taurocholate for 3 days at 37°C. TLC analysis of the reaction mixture revealed that most of the original P_1 glycolipid had disappeared and a new compound with the chromatographic mobility of paragloboside had appeared. The reaction mixture was concentrated by evaporation, dried, and acetylated in pyridine–acetic anhydride for 24 hr at 37°C. After removal of pyridine by repeated evaporation with toluene the reaction mixture was applied to a small Florisil column in dichloroethane to remove impurities (Saito and Hakomori, 1971), and the acetylated glycolipid was eluted with dichloroethane–acetone (1:1). This fraction was then applied to a silica gel G TLC plate that was developed with dichloroethane–methanol–water (95:7:0.3) three times, the major band detected with I_2 vapor was scraped off the plate, and the acetylated glycolipid was eluted with chloroform–methanol (2:1), deacetylated, and assayed for immunological activity and sugar content.

Purification of the P_1 Glycosphingolipid. Red cells were washed with 0.9% NaCl and lysed in a large volume of water containing 0.02% acetic acid. After standing at 4°C overnight the cell ghosts were collected by centrifugation and extracted with acetone at 4°C overnight. The acetone soluble material was removed by filtration and the residue was dried under vacuum and then extracted with 10 vol of chloroform–methanol (C–M)[1] (2:1, v/v) and the residue from this procedure was extracted twice with an equal volume of C–M (1:1, v/v). The pooled C–M extracts were evaporated to dryness, suspended in 0.2 *N* methanolic KOH for 2 hr at room temperature, partially evaporated, diluted with water, dialyzed against water for several days, and lyophilized.

The crude sphingolipid fraction was then chromatographed on a column of silica gel–silicic acid as described in detail in the legend to Figure 1. The active fraction from the first column was then applied to a second column. In a representative experiment, 262 mg of lipid was applied to a column consisting of 7.5 g each of silica gel H–Unisil and developed with 200 ml of each of the following solvents containing C–M–NH_4OH [80:20:3, 70:30:3, and 60:40:4] and C–M–W [50:50:5 and 30:70:7]. Ten-milliliter fractions were collected and 0.1-ml aliquots were analyzed by TLC. Sphingomyelin was eluted with the first and second solvents, hematoside was eluted with the second and third solvents, the *N*-acetylglucosamine ganglioside was eluted by the third and fourth solvents, and the P_1 glycolipid was eluted primarily with the fifth solvent system, along with blood group A and B glycolipids.

The P_1 active fraction obtained from the second column was dried over P_2O_5 and KOH, and then acetylated in 10 ml of pyridine–acetic anhydride at 37°C overnight. After removal of the acetylation solvents by repeated evaporation with toluene the residue was dried in vacuo and then applied to a column containing 5 g of Florisil. Colored impurities were removed by elution of the column with 50 ml of dichloroethane and the glycolipids were then eluted with 50 ml of acetone–dichloroethane (1:1). The acetylated glycolipids were then fractionated by preparative TLC, first on 0.5-mm silica gel H plates, using C–M–W (95:5:0.5, v/v) (Saito and Hakomori, 1971), three developments, and then on 0.25-mm silica gel H plates developed three times with

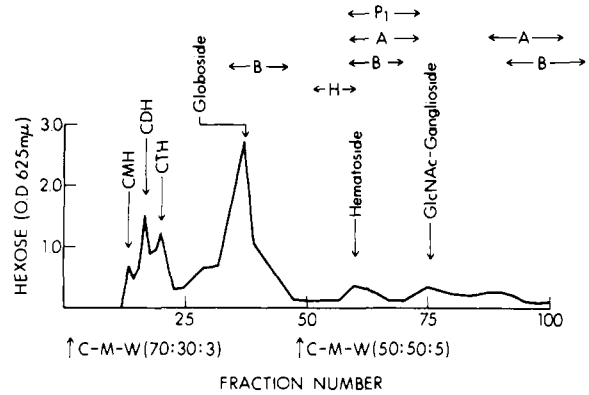


FIGURE 1: Five grams of a crude sphingolipid fraction was applied to a column composed of 75 g each of silica gel H and Unisil, 200–325 mesh. The column was first eluted with 500 ml of chloroform and then 1000 ml of each of the three following solvent systems: 60:30:3, 50:50:5, and 30:70:7 chloroform–methanol–water. Fractions of 20 ml were collected, the hexose content of 0.1-ml aliquots was measured by the anthrone reaction, and the blood group activity was measured on another 0.1-ml aliquot that was evaporated to dryness and dispersed in 0.5 ml of PBS.

dichloroethane–methanol–water (D–M–W, 95:7:0.3, v/v). Glycolipid bands were detected with iodine vapor and eluted from the silica gel with acetone–dichloroethane (1:1, v/v). The chromatographic mobility of the P_1 glycolipid was established initially by deacetylation of an aliquot of the acetylated glycolipid and assaying the fractions by hemagglutination inhibition. After it was established that two bands with P_1 activity had a chromatographic mobility intermediate between standards of acetylated globoside and Forssman glycolipids, they were identified by their chromatographic mobility alone.

The active fraction was deacetylated in 0.2 *N* KOH in methanol for 2–3 hr at room temperature, and the potassium was removed with IR-120 (H^+) resin. Final purification was achieved by preparative TLC on 0.25-mm silica gel H plates developed with C–M–W (60:35:8) and elution of the glycolipid with C–M–W (30:60:9).

Results and Discussion

Purification. The crude sphingolipid fraction prepared by mild alkaline hydrolysis was fractionated on a column containing a mixture of silica gel and silicic acid (Figure 1). Most of the neutral glycolipids were eluted by the first solvent, and the P_1 antigen was eluted by the second solvent after a peak of G_{M3}. The pooled fraction containing the P_1 antigen also contained sphingomyelin, G_{M3}, the *N*-acetylglucosamine-containing ganglioside, and glood group A and B glycolipids. This material was applied to another silica gel–silicic acid column which was developed with chloroform–methanol–ammonia eluents. This step separated most of the sphingomyelin and the two gangliosides from the P_1 glycolipid, but the active fraction still contained blood group A and B activities. Initially, we acetylated the glycolipids at this stage, but in our final purification procedure the remaining traces of gangliosides were removed by chromatography on a column of DEAE-Sephadex (Ledeen et al., 1973) prior to acetylation.

The acetylated lipids were passed through a column of Florisil to remove nonglycolipid impurities, and then fractionated by preparative TLC. The initial separation with a chloroform-containing solvent served to separate 3–4 bands with a rapid mobility, including the P_1 antigen, from several

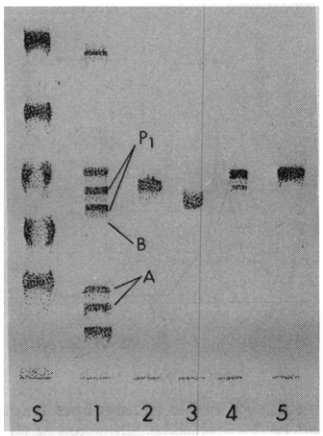


FIGURE 2: TLC of acetylated glycolipid (schematic representation). The TLC was carried out on silica gel G plates by three developments with the solvent dichloroethane–methanol–water (95:7:0.3), and the bands were visualized with the anthrone reagent: S, standard acetylated glycolipid mixture in order of decreasing R_f values: CTH, asialo, G_{M1}, globoside, Forssman, and G_{M1}; (1) the acetylated glycolipid fraction prior to preparative TLC; the blood group activities indicated were detected after preparative TLC and deacetylation; (2 and 3) the P_1-active upper and lower fractions obtained by preparative TLC; (4) the acetylated α-galactosidase-treated P_1 glycolipid; (5) acetylated paragloboside (schematic representation).

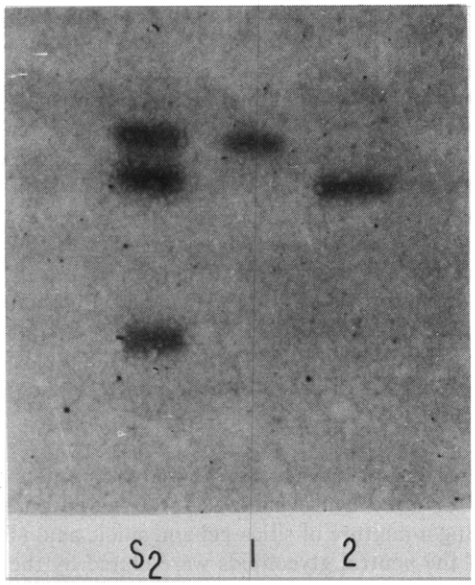


FIGURE 3: TLC of the purified P_1 glycolipid. TLC was carried out on silica gel G with chloroform–methanol–water (60:35:8), and the bands were visualized with the anthrone reagent: S_2, a standard glycolipid mixture containing Forssman glycolipid, asialo G_{M1}, and G_{M1} in order of decreasing R_f value; (1) an unknown glycolipid separated from the P_1-active upper fraction by preparative TLC after deacetylation; (2) the P_1 glycolipid obtained by preparative TLC from the P_1-active upper fraction after deacetylation.

slower compounds. Two acetylated glycolipids with P_1 activity were obtained in the second preparative TLC step (Figure 2), but neither compound was completely pure and the lower band contained contaminating blood group B activity. A homogeneous glycolipid was obtained from the upper P_1 band by deacetylation and preparative TLC with a solvent composed of C–M–W, 60:35:8 (Figure 3). The chromatographic mobility of the P_1 glycolipid was indistinguishable from that of a ceramide pentasaccharide with blood group B activity isolated from rabbit erythrocytes.

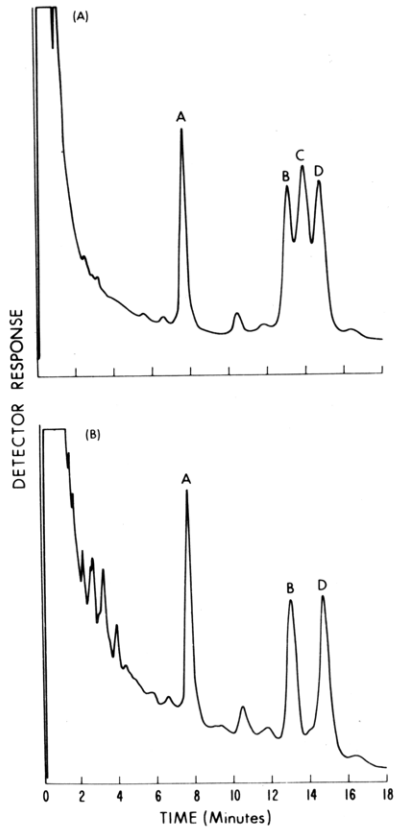


FIGURE 4: Gas–liquid chromatograms of partially methylated alditol acetates of P_1 glycolipid (A) and the α-galactosidase product (B). The analyses were performed on OV-225 columns operated isothermally at 175°C: (peak A) 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol; (peak B) 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol; (peak C) 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol; (peak D) 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.

The yield of pure P_1 glycolipid from 300–400 g of acetone powder was approximately 150–250 μg. Complete inhibition of hemagglutination was obtained with a concentration of 280 ng/ml of pure P_1 glycolipid or 12 ng/ml of glycolipid in the presence of 0.5 mg/ml of sodium taurocholate as an "auxiliary" lipid. No blood group A, B, or H activity was detected in this purified material.

Composition. The carbohydrate portion of the purified P_1 glycolipid consisted of glucose, galactose, and *N*-acetylglucosamine in a molar ratio of 1.0:2.8:1.2. Treatment of the P_1 glycolipid with fig α-galactosidase resulted in the appearance of a new glycolipid with a more rapid chromatographic mobility equivalent to paragloboside, and the acetylated α-galactosidase product had the same chromatographic mobility as acetylated paragloboside (Figure 2). The purified α-galactosidase-treated P_1 glycolipid had no detectable P_1 activity, and contained glucose and galactose in a molar ratio of 1.0:2.0; *N*-acetylglucosamine was detectable in this compound, but insufficient glycolipid was available for an accurate analysis. No alteration in immunological activity or chromatographic mobility was produced by treatment with jack bean β-galactosidase or *Turbo cornatus* β-hexosaminidase under conditions in which these enzymes hydrolyzed other glycolipids with appropriate terminal residues. These data indicate that the P_1 glycolipid contains a terminal nonreducing α-galactosyl residue.

Glycosidic Substitution Sites. The sites of substitution on the neutral sugars of the P_1 glycolipid were established by identifying the partially methylated alditol acetates on

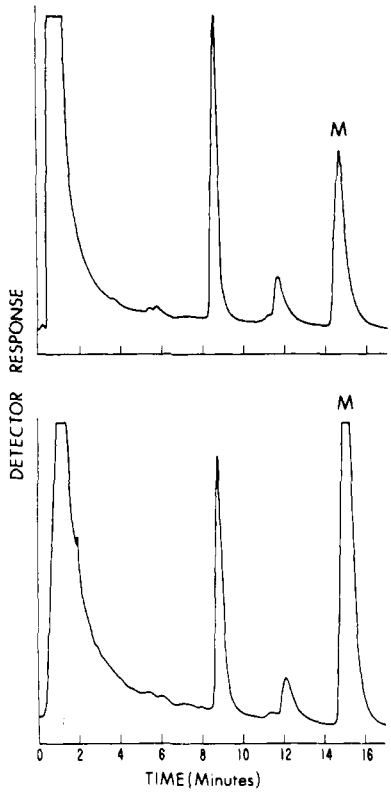


FIGURE 5: Gas–liquid chromatogram of methyl glycoside derivatives of hexosamines from paragloboside (upper tracing) and P_1 glycolipid (lower tracing). The acetylated samples were analyzed on an OV-1 column operated isothermally at 160°C. Paragloboside is known to contain a 4-substituted *N*-acetylglucosamine; M = mannitol hexaacetate.

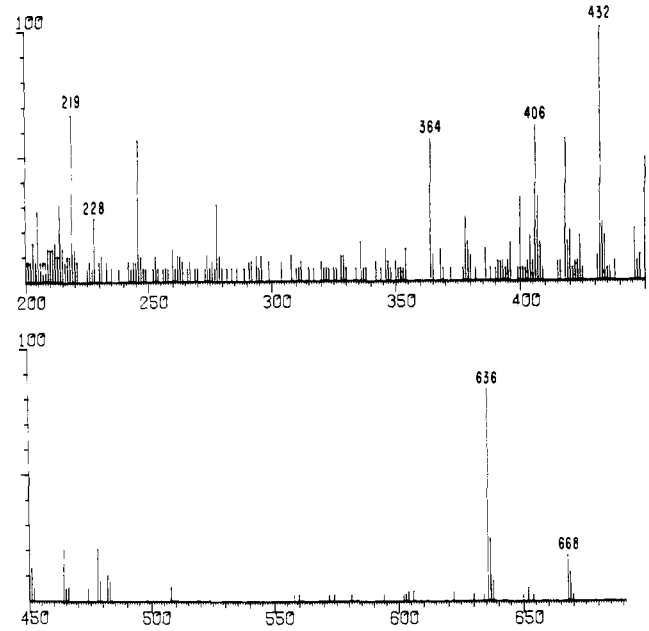


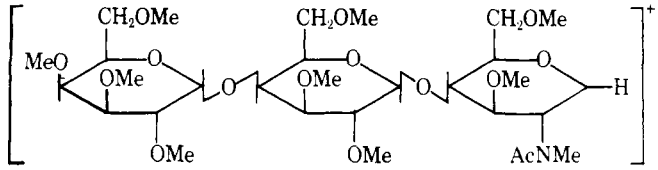
FIGURE 6: A portion of the mass spectrum of permethylated P_1 glycolipid recorded on a Finnigan 3300 quadrupole mass spectrometer. The spectrum was normalized by the data system with *m/e* 432 as the base peak.

both OV-225 (Figure 4A) and ECNSS-M columns (not shown). Galactose proved to be the terminal sugar of P_1 as well as the α-galactosidase product (Figure 4B). Both glycolipids contained a galactose unit substituted at the 3-hydroxyl, indicated by the presence of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, and also a 4-substituted glucose indicated by 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol. A peak corresponding to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, denoting a 4-substituted galactose, was present in the chromatogram of P_1 glycolipid (Figure 4A, peak C), but not that of the α-galactosidase product (Figure 4B). Peak identification by comparison with standards was aided by the previous observation (Lindberg, 1973) that the various isomers arising from monosubstituted galactose and glucose are resolved on ECNSS-M and OV-225.

Determining the substitution position on hexosamine has proved difficult by the usual alditol acetate procedure, but alternative methods have been successfully applied to a variety of glycolipids (Stellner et al., 1973a; Kundu et al., 1975a,b). Methanolysis of a permethylated glycolipid containing monosubstituted hexosamine generally gives rise to two products corresponding to the α- and β-methyl glycosides, and the various isomers are readily distinguished by their retention times on OV-225 and OV-1 columns. The two products from P_1 glycolipid were identical on GLC with those generated from paragloboside (Figure 5), a glycosphingolipid previously shown by Siddiqui and Hakomori (1973) to possess a 4-substituted *N*-acetylglucosamine. The 4-substituted *N*-acetylglucosamine was also identified by comparison with known standards (Kundu et al., 1975b). In addition, the methylated alditol acetates of the neutral sugars from paragloboside were identical with those from the α-galactosidase product of P_1 glycolipid, further suggesting identity of the two glycolipids.

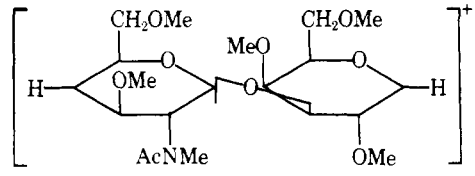
Mass Spectrometry. Formation of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol from both P_1 glycolipid and the α-galactosidase product indicated the presence of two galactose units at the nonreducing end. Identification of glucosamine as the next in sequence was accomplished by mass spectrometry of permethylated P_1 glycolipid, according to the procedure utilized for a variety of glycolipids (Ledeen et al., 1974).

The peak at *m/e* 668 (Figure 6) probably corresponds to the terminal trisaccharide unit, while the large peak at *m/e*



m/e 668

636 was derived from this by loss of 32 amu (668 − MeOH). As reported earlier, the presence of hexosamine has a stabilizing effect on such polyglycosyl fragments. The large peak at *m/e* 432 corresponds to a deprotonated form



m/e 433

of the nonterminal diglycosyl fragment. Similarly, the medium size peak at *m/e* 228 represents the internal hexosamine (Ledeen et al., 1974).

Prominent peaks at *m/e* 219 and 187 (not shown) correspond to terminal hexose, whereas a fragment with *m/e*

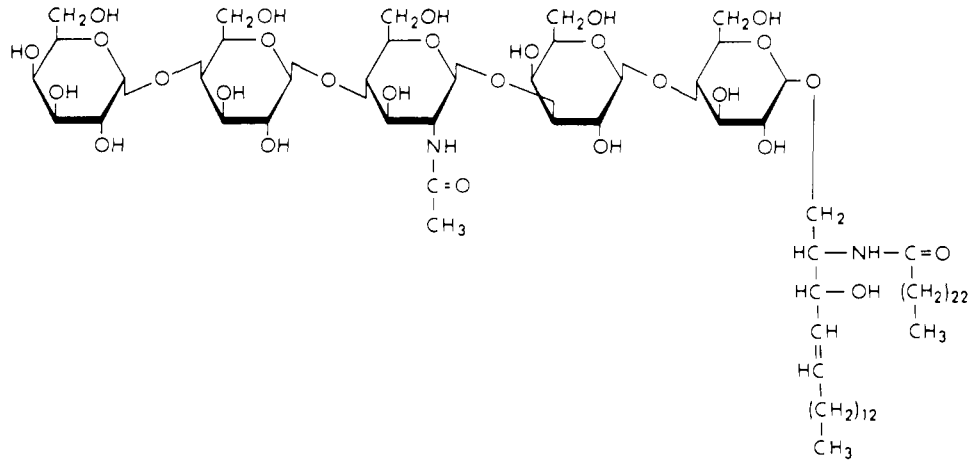
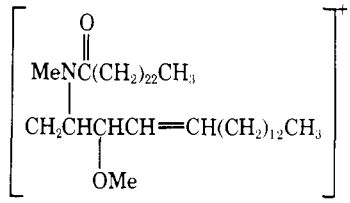


FIGURE 7: The proposed structure of the blood group P_1 glycosphingolipid isolated from human erythrocyte stroma.

423 representing the terminal digalactosyl moiety was very small in some scans and completely absent in others. The destabilizing influence of α-glycosyl groups was noted previously in the analysis of ceramide trihexoside (Ledeen et al., 1974). Peaks corresponding to the entire ceramide unit were not evident in this particular scan but in other scans a prominent peak was seen at m/e 660 corresponding to the lignocerate-containing ceramide.



m/e 660

The prominent peaks at m/e 364 and 406 are fragments characteristic of sphingosine and lignocerate, respectively (Ledeen et al., 1974). These data are consistent with preliminary GLC analyses which revealed lignocerate as the major fatty acid.

The location of the remaining galactose cannot be ascertained on the basis of these data alone, but it probably lies between *N*-acetylglucosamine and glucose since the latter sugar is joined to ceramide in all known glucose-containing glycolipids (Ledeen and Yu, 1973). We suggest that the α-galactosidase product has a carbohydrate structure identical with paragloboside, based on the identical chromatographic mobilities of the unmodified and acetylated glycolipids, and their identical glycosidic substitution sites. Our proposed structure for the P_1 glycolipid is presented in Figure 7. Cory et al. (1974) recently determined the structure of the terminal trisaccharide of the P_1 determinant of a glycoprotein obtained from sheep hydatid cyst fluid: Gal(α,1→4)Gal(β,1→4)GlcNac. This structure is identical with the terminal trisaccharide of the erythrocyte P_1 glycolipid.

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An Immunochemical Study of the Human Blood Group P_1, P, and P^k Glycosphingolipid Antigens†

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ABSTRACT: The erythrocyte P^k and P blood group antigens have been identified as ceramide trihexoside (CTH), Gal(α,1→4)Gal(β,1→4)Glc-Cer, and globoside, GalNAc(β,1→3)Gal(α,1→4)Gal(β,1→4)Glc-Cer, respectively, and the following structure has been proposed for the P_1 antigen: Gal(α,1→4)Gal(β,1→4)GlcNAc(β,1→3)Gal(β,-1→4)Glc-Cer. Although the P_1 and P^k determinants have identical terminal disaccharides, CTH did not inhibit anti-P_1. The P_1 glycolipid and hydatid cyst glycoprotein inhibited the agglutination of $P_1{}^k$ erythrocytes by anti-P_1 and unabsorbed anti-P_1PP^k sera, but neither antigen inhibited a specific anti-P^k serum. The P_1 and P^k glycolipids were equally effective in inhibiting the hemagglutinating activity of a lectin with α-galactosyl specificity obtained from ova of *Salmo trutta.* Anti-P sera were inhibited most effectively by human erythrocyte globoside, and to a lesser extent by Forssman glycolipid and rat kidney globoside. In the latter glycolipid the linkage between the internal galactosyl residues is α,1→3, rather than α,1→4, as in erythrocyte globoside. No cross-reactions between P and P_1 or P^k antigens were detected. New hypotheses are offered to explain the genetic regulation and biosynthesis of the P_1, P, and P^k antigens.

The human P blood group system consists of three antigens, P_1, P, and P^k (Table I) (Race and Sanger, 1968). These antigens were demonstrated originally on erythrocytes, but they have been detected also on skin fibroblasts and lymphocytes (Gurner and Coombs, 1958; Fellous et al., 1973, 1974). P^k is unusual among blood group antigens in not being expressed as a codominant character (Matson et al., 1959; Kortekangas et al., 1965), i.e. the P^k antigen is detectable only on the erythrocytes of homozygous P^kP^k individuals and not in P^k heterozygotes.

Immunochemical studies of a cross-reacting glycoprotein obtained from hydatid cyst fluid (Cameron and Staveley, 1957) demonstrated that both P_1 and P^k antigens have carbohydrate determinants with an immunodominant terminal nonreducing α-galactosyl residue (Watkins and Morgan, 1964; Voak et al., 1973). There is some cross-reactivity between the P_1 and P^k antigens (Voak et al., 1973) but neither antigen cross-reacts with P. The genetic and biosynthetic relationships among these antigens are unclear, but it was suggested recently (Fellous et al., 1974) that P is the precursor of the P^k antigen.

We recently reported the identification of the P and P^k antigens as the glycosphingolipids globoside and ceramide trihexoside (CTH), respectively (Naiki and Marcus, 1974). We now present more comprehensive data on the immunochemistry of the P_1, P, and P^k antigens, and offer a hypothesis concerning their genetic regulation and biosynthesis.

Materials and Methods

Glycosphingolipids (Table II). Globosides isolated from bovine adrenal glands (Kawanami, 1967) and rat kidney (Kawanami and Tsuji, 1968; Siddiqui et al., 1972) were obtained from Dr. J. Kawanami (Shionogi Research Lab., Japan). Asialo G_{M2} was prepared by mild acid hydrolysis of G_{M1} and chromatography on a column of silicic acid (Naiki et al., 1974). The sources of the other glycolipids were described in the preceding paper (Naiki et al., 1975).

Erythrocytes and Antisera. Group O,P_1, and O,P_2 cells were obtained from normal donors. Samples of O,p and $O,P_1{}^k$ erythrocytes were obtained from Mr. W. L. Marsh of the New York Blood Center. Anti-P_1 was obtained from an O,P_2 donor; anti-P sera from individuals of the P_1^k phenotype were gifts from Dr. P. Tippett (Blood Group Reference Laboratory, London), Dr. D. J. Anstee (South West Regional Blood Transfusion Centre, Bristol), and Mr. W. L. Marsh. Three anti-P_1PP^k sera from p individuals were gifts from Dr. P. Levine (Ortho Research Foundation, Raritan, N.J.). These antisera have a complex specificity and agglutinate erythrocytes of all phenotypes except p. Different as-

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