Isolation and Characterization of Glycosphingolipids with Blood Group H Specificity from Membranes of Human Erythrocytes†

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ABSTRACT: Three forms of blood group H-glycolipid (H_1, H_2, H_3 variants) were isolated from lipid extract of blood group O human erythrocyte membrane. The structure of one form of H-glycolipid (H_1-glycolipid) was determined as L-fucopyranosyl-α-(1→2)-galactopyranosyl-β-(1→4)-*N*-acetylglucosaminosyl-β-(1→3)-galactopyranosyl-β-(1→4)-glucopyranosyl-β-(1→1)-ceramide, *i.e.*, "type 2" H-chain (Painter, T. J., Watkins, W. M., and Morgan, W. T. J. (1963), *Nature (London) 199*, 282; Watkins, W. M. (1966), *Science 152*, 172) is linked to lipid. No "type 1" H chain was found as lipid bound in erythrocyte membrane. The H activity was greatly diminished by mixing with globoside, and practically no H activity was detected when a pure H_1-glycolipid was mixed with 20 times (w/w) of globoside. This is probably the basis for the difficulty of demonstrating H activity, as the H_1-glycolipid was coeluted with globoside. The second and third H-active components (H_2- and H_3-glycolpid) were possibly ceramide octasaccharide and ceramide decasaccharide carrying the H-active terminal, L-fucopyranosyl-α-(1→2)-galactopyranosyl.

Blood group A, B, H, Le^a, and Le^b glycosphingolipids of human erythrocyte membranes have been isolated and partially characterized as having a common carbohydrate skeleton that contains fucose, glucosamine, galactose, and glucose (Yamakawa *et al.*, 1965; Hakomori and Strycharz, 1968; Hakomori, 1970; Koscielak *et al.*, 1970). Lewis-active glycolipids and their positional isomer of human adenocarcinoma have been characterized as lacto-*N*-fucopentaosyl(II)ceramide (Hakomori and Jeanloz, 1970), lacto-*N*-fucopentaosyl(III)-ceramide (Yang and Hakomori, 1971), and lacto-*N*-difucohexosyl(I)ceramide (Hakomori and Andrews, 1970). Lewis-active glycolipid of human serum was partially characterized, and the uptake of Le^a- and Le^b-active glycolipids by erythrocytes was demonstrated (Marcus and Cass, 1969). The backbone structure of all these glycolipids was assigned as having galactosyl(*N*-acetyl)glucosaminylgalactosylglucosylceramide, based on carbohydrate composition and degradation studies (Hakomori, 1970; Iseki, 1970). However, no further evidence for this structure or for positional and anomeric linkages has yet been furnished.

This paper reports separation of three H-active glycolipids of type O human erythrocyte and structure of the simplest major H-active component (H_1-glycolipid).

Materials and Methods

Glycolipids and Oligosaccharides. The following glycolipids, used as references in analysis, were prepared in our laboratory: glucosylceramide from human spleen, lactosylceramide (Galβ1→4Glc→ceramide), trihexosylceramide (Galα1→4Galβ1→4Glc→ceramide), globoside (GalNAcβ1→3Galα1→4Galβ1→4Glc→ceramide) from human erythrocytes (Yamakawa *et al.*, 1960), *N*-acetylhematoside (*N*-acetylneuraminosyl2→3Galα1→4Glc→ceramide) from human spleen (Svennerholm, 1963), *N*-glycolylhematoside (*N*-glycolylneuraminosyl2→3Galβ1→4Glc→ceramide) from horse spleen (Yamakawa *et al.*, 1960); Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide (Siddiqui and Hakomori, in preparation), lacto-*N*-fucopentaose I (L-Fucα1→2Galβ1→3GlcNAcβ1→3Galβ1→4Glc) (Kuhn *et al.*, 1956), lacto-*N*-fucopentaose II, Galβ1→3(L-Fucα1→4)GlcNAcβ1→3Galα1→4Glc (Kuhn *et al.*, 1958), and lacto-*N*-tetraose (Kuhn and Baer, 1956) were donated by Dr. Victor Ginsburg (National Institute of Health) and Dr. Akira Kobata (Kobe University, Japan). Partially methylated glucitol acetates and galactitol acetates were prepared by incomplete Purdie methylation of methyl α-glucoside or methyl α-galactoside (Siddiqui *et al.*, 1972). Partially methylated 2-deoxy-2-*N*-methylacetamidohexitols were prepared by methylation of a partially *O*-(1-methoxy)ethyl-substituted methyl glucoside of *N*-acetylhexosamine (Stellner *et al.*, 1973). Both partially methylated hexitol acetates and hexosaminitol acetates derived from oligosaccharides with firmly established structures were also referred.

Preparation of Glycolipid Fraction from Human Erythrocyte Ghost. Sedimented erythrocytes were lysed in tap water containing acetic acid (2 ml of acetic acid/l.), and the ghosts were collected in a Sharpless centrifuge. The packed ghosts were stored frozen until use. Extraction of glycolipids with hot ethanol and subsequent precipitation of glycolipids (at −20°), followed by fractionation with various solvents, were carried out according to the procedure described previously (Siddiqui *et al.*, 1972). The sphingolipid fraction thus obtained was dissolved in 500 parts (w/v) of chloroform–methanol (2:1); one-sixth volume of water was added and shaken, and then separated into upper and lower phases. The lower phase was partitioned with the same volume of "theoretical upper phase" (chloroform–methanol–0.1% NaCl, 1:10:10). The second lower phase was again partitioned with the theoretical upper phase. The upper phases were dialyzed, evaporated, and further fractionated by DEAE-cellulose chromatography, and the

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lower phase was fractionated into components by chromatography on Anasil S (Magnesia-Silica gel, Analab Co., North Haven, Conn. 06473).

The ratio of Anasil S and the amount of total lipid loaded on the column was 1:200. Elution of the solvent composition is shown in Figure 1. At least two column volumes of the solvent were used for eluting glycolipids, and about 45-ml aliquots were taken by a fraction collector. Every third fraction was analyzed by thin-layer chromatography. DEAE-cellulose chromatography was carried out according to the method of Rouser *et al.* (1963). Solvent composition and volumes used for elution are also shown in Figure 1.

Further purification of glycolipids on thin-layer chromatography was carried out as acetylated compounds. The glycolipids were acetylated with pyridine and acetic anhydride (2:1) at room temperature overnight, followed by evaporation of the solvents in a rotary evaporator with a large excess of toluene. The residue was applied on a thin-layer plate and developed three times with1,2-dichloroethane–methanol–water (93:7:0.1). Development was carried out three times with intermediate drying. Two zones were separated; the zones indicated by iodine stain were extracted with chloroform–methanol (2:1) and then evaporated to dryness. This method was satisfactory for separation of substances which migrated closely, for example, H-active and Le^a-active glycolipids. The fast-migrating zone was H active, and the second zone was Le^a active. Each glycolipid was deacetylated in 0.1% sodium methoxide in chloroform–methanol (2:1).

The second and third H components obtained by DEAE-cellulose chromatography or by Anasil S chromatography were used without further purification.

Analytical Method. The purity of the substance was determined by thin-layer chromatography as fully acetylated form on silica gel H and G plates in 1,2-dichloroethane–methanol–water (97:3:0.1), developed three times with intermediate drying. Free deacetylated glycolipids were separated on thin-layer precoated Unisil plate with chloroform–methanol–water (60:35:8) and chloroform–methanol–water (65:30:8, lower phase).

Carbohydrate composition was analyzed by gas chromatography according to the method described previously (Yang and Hakomori, 1971), and by the method of Sweeley and Walker (1964). Oligosaccharide was released from a lipid moiety by osmium tetroxide–periodate–alkali degradation (Hakomori, 1966), and the oligosaccharide was then compared by paper chromatography (ethyl acetate–pyridine–water, 12:5:4) with authentic lacto-*N*-fucopentaose I and II.

Fatty acid and sphingosines were determined as described previously (Yang and Hakomori, 1971). Methanolysis and separation of sphingosines and fatty acids were carried out by the methods of Gaver and Sweeley (1965). Sphingosine was analyzed as the form of *N*-acetyl-*O*-trimethylsilyl derivative by gas chromatography on 3% SE-30, 3% ECNSS-M (cyanoethylsilicone–ethylene glycol succinate), and 15% ethylene glycol succinate on Gas Chrom Q column with or without trimethylsilylation of α-hydroxy group, if any was present.

Methylation Analysis. Methylation was carried out in dimethyl sulfoxide, sodium hydride, and methyl iodide (Hakomori, 1964). The permethylated glycolipid (0.5–1 mg) was degraded by either of the following two methods: (1) in 90% formic acid, followed by 0.5 N sulfuric acid according to the method of Björndal *et al.* (1967), and (2) in 0.2 ml of 95% acetic acid containing 0.5 N sulfuric acid at 80° for 18 hr, then added 0.2 ml of water, heated at 80° for 5 hr. For amino sugar analysis the second method is necessary, while both methods can be applied to analysis of neutral sugars. The sulfate ion in the hydrolysate of either method was eliminated by passage through a small column of 200 mg of AG3-X8 (Bio-Rad, Richmond, Calif.). The eluate and washings were added with 20 mg of sodium borohydride and reduced at room temperature overnight. The mixture was acidified by addition of a drop of glacial acetic acid. The residue was finally dried *in vacuo* over phosphorus pentoxide. The resulting solid (mainly anhydrous sodium acetate) was mixed with acetic anhydride and heated at 100° for 2 hr. The reaction mixture was evaporated in a rotary evaporator to dryness after addition of toluene. The residue was dissolved in chloroform and the insoluble salt was shaken with water. Sugar acetates remained in the chloroform phase, and after evaporation under nitrogen, the material was analyzed by gas chromatography–mass spectrometry on a glass 3% ECNSS-M column connected to a Finnigan mass spectrometer (Model 4000) with all-glass quadrupole separator.

For analysis of the partially O-methylated neutral sugars, column temperature was set at 150–160°, and for that of partially methylated amino sugars, the column temperature was set at 190°. The substances were identified by retention time on gas chromatography and by mass spectra. The mass spectra of partially methylated alditol acetate were determined according to published data (Björndal *et al.*, 1967, 1970), and that of 2-deoxy-2-*N*-methylacetamidohexitol were determined according to our data (Stellner *et al.*, 1973).

Graded Hydrolysis with Weak Acid and by Enzymes. Fucosyl residue of H-glycolipid was hydrolyzed in 0.1 *N* trichloroacetic acid at 100° for 2 hr.[1] The hydrolysate was shaken with six volumes of chloroform–methanol (2:1) and centrifuged. The degraded glycolipid was recovered from the lower phase. α-L-Fucosidases were donated by Professor Toshiaki Osawa, Tokyo University, and by Dr. Takashi Okuyama, Seikugaku Kogyo Co., Tokyo. The enzymes were purified by gel filtration on Sephadex G-200 (Iijima *et al.*, 1971). Hydrolysis with fucosidases[2] was carried out in acetate buffer (pH 4.0), containing 0.1% sodium deoxycholate at 37° for 18 hr. β-Galactosidase and β-*N*-acetylhexosaminidase were donated by Dr. Li, Tulane University, Delta Regional Primate Center. The enzymatic hydrolysis of glycolipids followed by examination with thin-layer chromatography was carried out according to the conditions described previously (Li and Li, 1970; Hakomori *et al.*, 1971).

Immunological Reactivity. H activity was determined by inhibition of hemagglutination caused by *Ulex europeus* and by eel serum. The extract of *U. europeus* also gave a fairly distinctive, strong precipitin line with H-glycolipid in double-diffusion agar.

Results

Three H-active glycolipids have been distinguished: one component (H_1-glycolipid) was eluted with solvent 7 and the second component (H_2-glycolipid) with solvent 9 from the Anasil S column (see Figure 1). The third component (H_3-glycolipid) was eluted from the Anasil S column, which was

[1] Fucosyl residue linked to oligosaccharides was easily hydrolyzed by 0.05 N sulfuric acid–2 N acetic acid at 100°, whereas fucosyl residue attached to glycolipid was not easily hydrolyzed. The best result was obtained under this condition.

[2] A purified preparation of α-fucosidase freed from other enzyme activities was donated by Dr. Takashi Okuyama, Seikagaku Kogyo Institute Co., Ltd., Kurashiki, Tokyo 189, Japan.

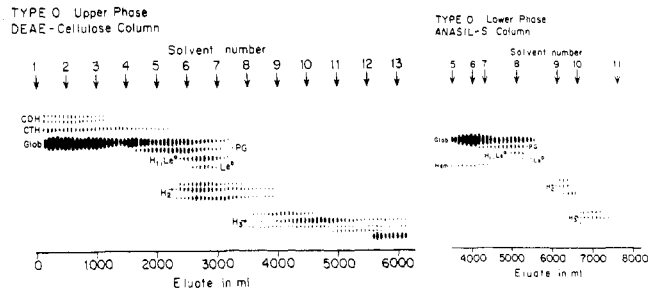

FIGURE 1: Separation pattern of glycolipids from lipid extract of human blood group O erythrocyte membrane. The pattern on the left (type O, upper phase) was obtained by combination of chromatographies on DEAE-cellulose and thin-layer silica gel H. Column size 2.5 × 30 cm. Solvent numbers indicated by chloroform–methanol–water mixtures of the following proportions: 1, 9:1:0.02; 2, 8.75:1.25:0.02; 3, 8.5:1.5:0.02; 4, 8:2:0.02; 5, 7.5:2.5:0.02; 6, 7:3:0.02; 7, 6.75:2.25:0.04; 8, 6.5:3.5:0.04; 9, 6:4:0.05; 10, 5.75:4.25:0.05; 11, 5:4:0.05; 12, 5.25:4.75:0.05; 13, 5:5:0.05.

The pattern on the right (type O, lower phase) was obtained by combination of chromatographies on Anasil S and thin layer of silica gel H. Column size 2.5 × 30 cm. Solvent numbers indicated by chloroform–methanol–water–acetone mixtures of the following proportions: 5, 60:30:3.0; 6, 60:30:4.0; 7, 60:20:5:20; 8, 60:25:5:25; 9, 60:30:10:30; 10, 50:30:10:30; 11, 50:40:10:20.

Each 60-ml fraction eluted from DEAE-cellulose and each 45-ml fraction eluted from Anasil S column were concentrated to dryness, and the residue was dissolved in 0.5–1 ml of chloroform–methanol, spotted on silica gel H plate, and developed with chloroform–methanol–water (60:35:8). Spots were revealed with 0.2% orcinol in 2 M sulfuric acid. Thin-layer chromatography pattern of every fraction from DEAE-cellulose chromatography and of every third fraction from Anasil S chromatography are shown in this figure. CDH, lactosylceramide; CTH, Galα1→4Galβ1→4Glc→Cer; Glob, globoside (GalNAcβ1→4Galα1→4Galβ1→4Glc→Cer); PG, paragloboside (Galβ1→4GlcNAcα1→3 Galβ1→4Glc→Cer, B. Siddiqui and S. Hakomori, unpublished).

partially overlapped with ganglioside. In contrast, the neutral glycolipids in the upper phase of Folch's partition were separated from gangliosides by DEAE-cellulose chromatography, and the recovery of the second and third H-active glycolipids was much higher. The pattern of separation is shown in Figure 1. These components equally well inhibit H-specific hemagglutination caused by *Ulex europeus* and neutralized anti-H eel serum agglutination. The minimum doses of H_1-, H_2-, and H_3-glycolipid to inhibit three to four hemagglutination doses of *Ulex* or eel agglutinin were found to be 3–6 μg for H_1 and 1.5–3 μg for H_2- and H_3-glycolipid, respectively. These components were also precipitated by *U. europeus* extracts. This precipitin line was intensified when H-glycolipid was mixed with 5–10 times lecithin and cholesterol, and the emulsion was sonicated for 3 min in a sonic cleaning bath. The H_1-glycolipid was purified as a homogeneous form freed from Le^a or Le^b activities. Le^a and Le^b glycolipids were separated from H-active glycolipids.

The first H-active component (H_1-glycolipid) had a slightly lower R_F value than globoside ($R_{globoside}$ value 0.82–0.85) and migrated together with ceramide pentasaccharides such as Forssman glycolipid and Le^a glycolipid, indicating that H_1-glycolipid could be a ceramide pentasaccharide. Carbohydrate analysis of H_1-glycolipid ("first component") showed that it contained fucose, galactose, glucose, and glucosamine in an approximate ratio 1:2:1:1. The second H-active component (H_2-glycolipid) had an $R_{globoside}$ value of 0.55, and thus could be a ceramide hepta- or octasaccharide. The carbohydrate composition of this component was found to have an approximate molar ratio of glucose:galactose:glucosamine:fucose 1:3:2:1. The third H-active component (H_3-glycolipid) had an $R_{globoside}$ value of 0.32, indicating a ceramide decasaccharide. These glycolipids with longer carbohydrate chains (H_2- and H_3-glycolipids) could be still a mixture of glycolipids having similar carbohydrate residues (see Discussion).

TABLE 1: Fatty Acids and Sphingosines in Blood Group H_1 Glycolipid.

Fatty Acids	% Total	Sphingosine	
C16:0	9.9		
C16:1	3.3		
C18:0	11.5	C18:0	0
C18:1	9.5	C18:1	+ (over 90%)
C22:0	14.0	Phytospingosine	0
C22:1	7.5	C20:0	0
C24:0	44.3	C20:1	0

The structure of the first component was identified as L-fucosyl-α-(1→2)-galactosyl-β-(1→4)-*N*-acetylglucosaminyl-β-(1→3)-galactosyl-β-(1→4)-glucosyl-(1→1)-ceramide (see Figure 4), on the following basis. (1) Methylated glycolipids, after hydrolysis in 0.5 N H_2SO_4 in 95% acetic acid followed by reduction and acetylation, gave four peaks of hexitol acetates and one peak of hexosaminitol acetate with retention time corresponding respectively to acetates of 2,3,4-tri-*O*-methylfucositol, 2,4,6-tri-*O*-methylgalactitol, 3,4,6-tri-*O*-methylgalactitol, 2,3,6-tri-*O*-methylglucitol, and 3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidoglucitol. Absence of 2,3,4,6-tetra-*O*-methylgalactitol, 2,3,4,6-tetra-*O*-methylglucitol and 3,4,6-tri-*O*-methyl-2-deoxy-2-(*N*-methyl)acetamidoglucitol excludes the possibility of Gal, Glc, and GlcNAc as the nonreducing terminal. Absence of 4,6-di-*O*-methyl-2-deoxy-2-(*N*-methyl)acetamidoglucitol was particularly noticeable as this excludes the 1→3GlcNAc linkage. Each peak was identified by mass spectra (see Figures 2B and 3). (2) Weak acid hydrolysis with 0.1 N trichloroacetic acid at 100° for 2 hr^{-1} hydrolyzed the fucosyl residue of H_1-glycolipid and converted it to a ceramide tetrasaccharide having the same migration rate as "lacto-*N*-neo-tetraosylceramide" whose $R_{globoside}$ value was 0.9 (B. Siddiqui and S. Hakomori, in preparation). (3) Partially purified α-fucosidase of *Haliotis* (abalone) liver and of *Charonia lampas* (triton) liver hydrolyzed the terminal fucosyl residue of H_1-glycolipid and further degraded into ceramide tri- and disaccharides because of the presence of β-galactosidase and *N*-acetylhexosaminidase.[2] The H_1-glycolipid was, however, not hydrolyzed by β-galactosidase and *N*-acetylhexosaminidase. (4) The ceramide tetrasaccharide found in either the trichloroacetic acid hydrolysate or in the α-fucosidase hydrolysate was hydrolyzed by Jack bean β-galactosidase. The product had an identical R_F value as GlcNAcβ1→4Galβ1→4Glc→ceramide. This ceramide trisaccharide was further hydrolyzed by Jack bean β-*N*-acetylhexosaminidase and was converted to lactosylceramide. (5) The oligosaccharide released from H_1-glycolipid was chromatographically similar to lacto-*N*-fucopentaose I, but showed distinctively slower migration rate than lacto-*N*-fucopentaose II or III. (6) The major fatty acid was C24:0, and the long-chain base was exclusively C18:1 sphingosine (octadecasphingenine) (see Table I).

The second and third components of H-active glycolipid, which had migration rates as shown in Figure 1 ($R_{globoside}$

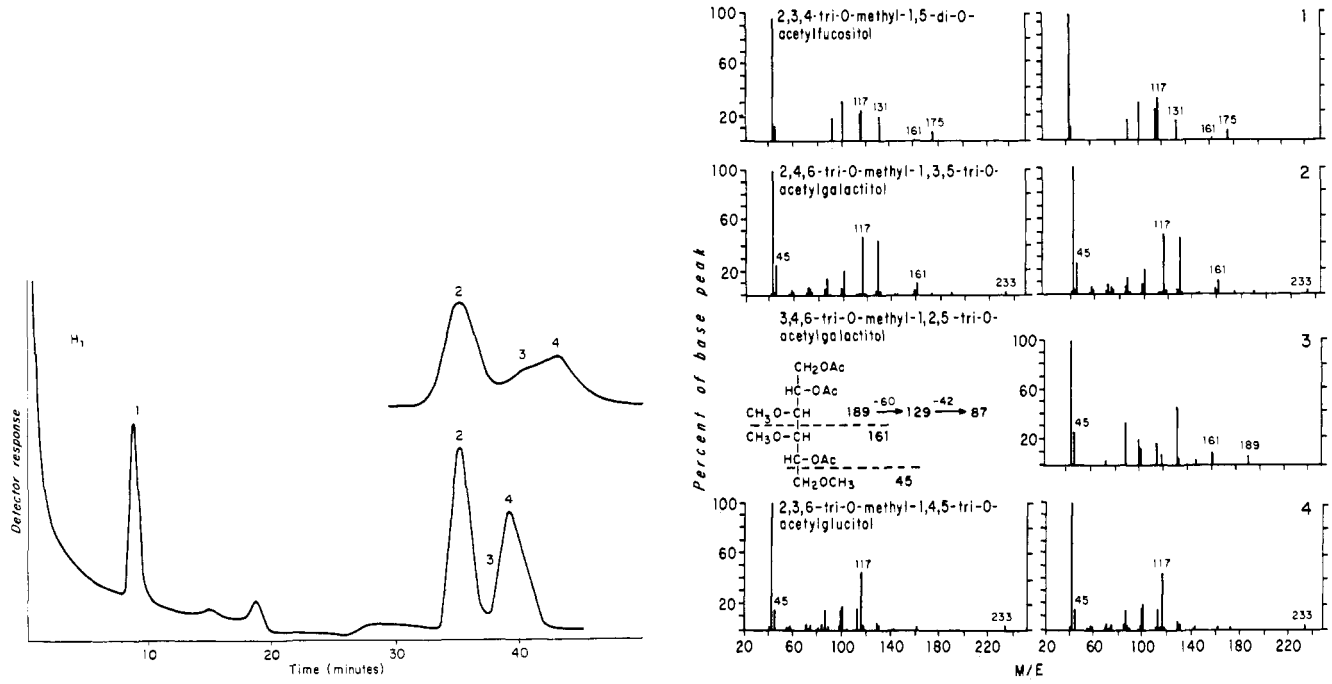

FIGURE 2: (A, left) Gas–liquid chromatogram and total ion gas chromatogram of partially O-methylated hexitol acetates found in the hydrolysate of permethylated H_1-glycolipid. Gas–liquid chromatogram with flame ionization detector is shown in the lower pattern, and total ion gas chromatogram is shown in the upper pattern, in which peaks 3 and 4 were partially separated. (The reason for this better resolution is unknown.) Permethylated glycolipid was degraded by formolysis in 90% formic acid, followed by hydrolysis with 0.5 N sulfuric acid. The procedure was carried out according to Björndal *et al.* (1967). The quantity of hexitol acetates did not agree with the monosaccharide composition, which was analyzed by the method of Yang and Hakomori (1971). The molar ratio of partially methylated sugars in the hydrolysates of permethylated complex carbohydrates does not always agree with the quantitative composition of monosaccharides. This is caused by partial volatilization occurring during derivatization of methylated sugars and incomplete hydrolysis due to mild conditions of hydrolysis which are necessary in order to avoid demethylation. (B, right) Mass spectra of peaks 1, 2, 3, and 4 in gas chromatogram (A) and that of reference compound. Peak 3 in total ion gas chromatogram was identified as 3,4,6-tri-O-methyl-1,2,5-tri-*O*-acetylgalactitol according to the mass spectra described by Björndal *et al.* (1970).

value for H_2-glycolipid 0.55, and for H_3-glycolipid 0.35), were still impure. Because of the small quantity, only the second component (H_2-glycolipid) was analyzed; it contained 1 mol of fucose and glucose, 2 mol of glucosamine, and 3 mol of galactose. However, the permethylated product of this glycolipid did not give any trace amount of di-*O*-methylgalactose. The permethylated H_2-glycolipid gave qualitatively identical permethylated sugars as found in the hydrolysate of permethylated H_1-glycolipid, but the proportions of the acetates of 2,3,4-trimethylfucositol, 2,4,6-trimethylgalactitol, 3,4,6-trimethylgalactitol, and 2,3,6-trimethylglucitol were approximately 1 : 2.5 : 1 : 1.

As the yield of the third component (H_3-glycolipid) was extremely low and was still grossly contaminated with other components, further study was not possible.

Discussion

The presence of more than two forms ("variants") of glycolipids carrying the same A and B specificities has been demonstrated (Yamakawa *et al.*, 1960; Hakomori and Strycharz, 1968; Hakomori *et al.*, 1972).

The presence of H-active glycolipid in erythrocytes and in pancreas has been demonstrated (Hakomori and Strycharz, 1968; Hakomori, 1970), contradicting the repeated statements of Koscielak, who was unable to find H-active glycolipid in erythrocyte membrane (Koscielak, 1963; Koscielak *et al.*, 1970; Gardas and Koscielak, 1971). In a recent brief note, however, Koscielak also found the presence of H-active glycolipids in agreement with our previous finding (Gorniak and Koscielak, 1972). Nevertheless, H-active glycolipid isolated from human erythrocyte was not separated from Le^b activity, whereby the possibility of H-activity due to a cross reaction with Le^b structure was left open (Hakomori and Strycharz, 1968). We have therefore tried to establish the presence of H-active glycolipid.

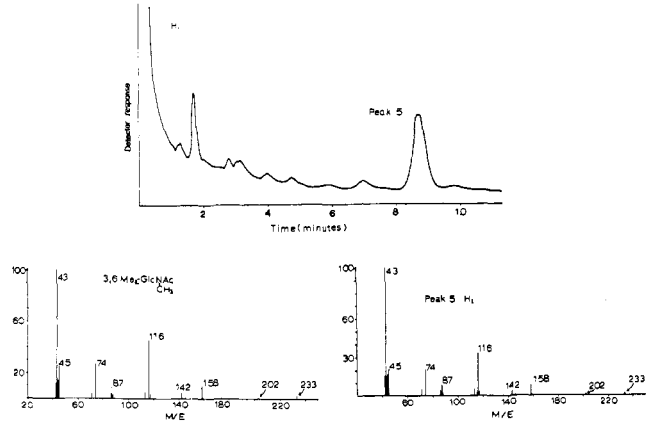

FIGURE 3: Total ion gas chromatogram–mass spectrum of partially O-methylated 2-deoxy-2-*N*-methylacetamidohexitol found in the hydrolysate of permethylated H_1-glycolipid. Permethylated glycolipid was acetolyzed in 95% acetic acid containing 0.5 N sulfuric acid in order to obtain a higher yield of *N*-methylacetamidohexose (see text) which was identified by mass spectrum. The first peak is a noncarbohydrate contaminant. The yield of *N*-methylacetamido hexoses by acetolysis followed by hydrolysis is nearly theoretical.

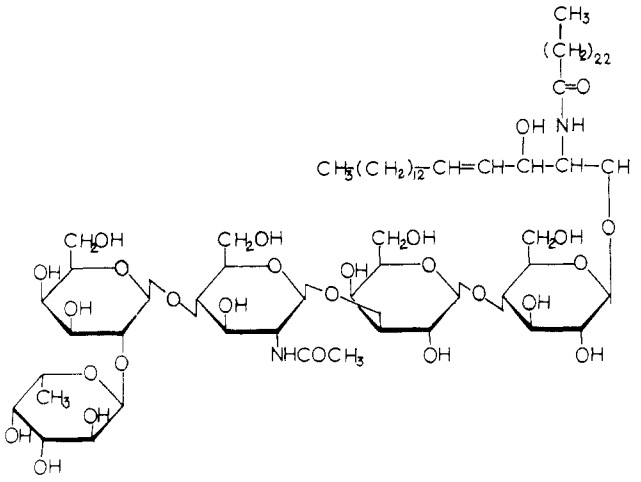

FIGURE 4: Structure of H_1-glycolipid.

At least three H-active variants have been isolated by two methods. The first method for separation of H-active glycolipid was found to be satisfactory for isolation of the major H-active glycolipids that have shorter carbohydrate chains (H_1 variant), but was not satisfactory for isolating the two other minor H-active components with longer carbohydrate chains (H_2 and H_3 variants). The second method using the upper phase of the Folch partition followed by chromatography on DEAE-cellulose column was found to be satisfactory for separation of the H_2 and H_3 variants. Both H_1 and H_2 variants have a straight-chain carbohydrate, and no branching structure was detected. This is compatible with our previous finding of two Le^b-active components with long and short carbohydrate chains (Hakomori and Andrews, 1970). However, a branching structure, GlcNAcl→6(GlcNAcl→3)Gal, has been detected in some "variants" of blood group A active glycolipid (Hakomori *et al.*, 1972). It is highly possible that H_3-glycolipid could have a similar branching structure.

The yield of H_2 and H_3 variants was less than 1 mg, while that of H_1 was 2–3 mg from 500 g of wet cell membranes. It is extremely difficult to determine the purity of these slow migrating glycolipids; the longer the carbohydrate chain in glycolipid, the more difficult it is to separate, and a number of different glycolipids with longer carbohydrate chains show the same migration rate on thin-layer chromatography, so that separation into components is impossible. As a matter of fact, the H_3 variant, after removal of fucosyl residue by weak acid hydrolysis, shows an identical migration rate as before hydrolysis. It is highly possible, therefore, that H_2 and H_3 components are mixtures of multiple components, although they appear essentially homogeneous on thin-layer chromatography.

The structure of H_1-glycolipid is now established fucosylα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glc→ceramide (Figure 4). The carbohydrate moiety is the "H-active type 2" chain" (Painter *et al.*, 1963; Watkins, 1966), but there was no "type 1" chain detected. This is in striking contrast to the fact that type 1 chain is more abundant in secreted oligosacchrides and in blood group B glycolipid of glandular tissue (Wherrett and Hakomori, unpublished). This finding agreed with the previous immunological data (Hakomori and Strycharz, 1968; experiment performed by Dr. Donald Marcus of Albert Einstein College of Medicine) that H-glycolipid of human erythrocytes did not react to the anti-hog H antiserum having a narrow specificity directed to the structure Fucα1→2Galβ1→3GlcNAc (Marcus and Cass, 1967), although it did react with anti-human H serum having a wider specificity directed to both Fucα1→2Gal1→3GlcNAc→R and Fucα1→2Gal1→4GlcNAc→R. The oligosaccharide as seen in Figure 4 has not been isolated, although it is predicted to be present. The oligosaccharide of H_1-glycolipid can be called "lacto-*N*-fucopentaose IV," a new fucopentaose.

TABLE II: Effect on H_1-Glycolipid H Activity by Addition of Globoside.

Sample	Min Amt of H_1-Glycolipid Reqd for H-Hemagglutination Inhibn (μg)	Precipitation (Ouchterlony)
H_1-glycolipid	1	++
H_1-glycolipid–globoside, weight ratio 1:20	>50	–

The H activity of H_1 component was greatly reduced by addition of globoside and the activity was abolished by addition of a large excess of globoside (globoside–H_1-glycolipid, 20:1, see Table II). It was impossible to detect H activity unless the H-glycolipid was purified. The failure to detect H activity in glycolipid fraction described in earlier works of Koscielak (1963; Koscielak *et al.*, 1970) could be due to contamination with globoside, as H-glycolipid was often coeluted with globoside from the silica gel chromatography column.

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Added in Proof

Separation between "peak 3 and 4" in Figure 2 was found to be better by 3% OV225 coated on Supelcoport (Supelco Inc., Bellfonte, Pa. 16823) at 165°.

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Complete Amino Acid Sequence of a Nonneurotoxic Hemolytic Protein from the Venom of *Haemachatus haemachates* (African Ringhals Cobra)†

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ABSTRACT: The complete amino acid sequence of the nonneurotoxic, hemolytic, basic protein 12B from the venom of *Haemachatus haemachates* is described. The protein consists of 61 amino acids, cross-linked by four disulfide bridges, molecular weight 6841 from amino acid composition. The cyanogen bromide fragments and chymotryptic and tryptic peptides were separated by gel filtration on Sephadex G-50 or G-25 and zone electrophoresis on a cellulose column. The sequence was determined by Edman degradation using the direct phenylthiohydantoin method, and carboxypeptidase A, and is shown to be homologous to other types of proteins, cardiotoxins, and neurotoxins, in the same size range from Elapid venoms.

Elapid venoms contain neurotoxins of the post-synaptic curariform type and in most cases other proteins of similar molecular weight which have cardiotoxic or lytic activity. The purification of the hemolytic protein 12B from the venom of *Haemachatus haemachates* has already been described by Porath (1966) but the separation has been improved by the use of ammonium acetate buffers. The protein 12B, which is very basic, has been shown to directly lyse red blood cells and to have cardiotoxic and hypotensive activity, but is not neurotoxic (Cheymol, 1972[1]). The primary structure was determined in order to investigate possible homology between nonneurotoxic proteins and neurotoxins. Simultaneously with the completion of this, Narita and Lee (1970) described the sequence of a cardiotoxin from the venom of the Formosan cobra, *Naja naja atra*, which is shown to be homologous to the neurotoxin from the same venom, and is also very similar to the lytic protein 12B.

Experimental Procedure

Purification of the Protein 12B. Haemachatus haemachates venom (HHIF) was obtained from Miami Serpentarium, Miami, Fla. One gram of crude venom was dissolved in 10.0 ml of 0.2 M ammonium acetate, clarified by centrifugation for 10 min at 20,000*g*, and then run on a Sephadex G-75 column, 3.2 × 93 cm, in 0.2 M ammonium acetate. The gel-filtration fraction containing the lytic activity was further separated by

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