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On Neutral Fucoglycolipids Having Long, Branched Carbohydrate Chains: H-Active and I-Active Glycosphingolipids of Human Erythrocyte Membranes†

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ABSTRACT: H-Active ceramide heptasaccharide (H_2-glycolipid) and ceramide decasaccharide (H_3-glycolipid) were isolated from blood group O human erythrocyte membranes. Their structures have been determined by conventional methylation analysis, enzymatic degradation, and direct total mass spectrometry of the enzymatic degradation products after permethylation and reduction with sodium bis(2-methoxyethoxy)aluminum hydride. The branched sugar residue in the structure of H_3-glycolipid was unambiguously determined by a new method with the combination of enzymatic degradation and comparison of the total mass spectrogram of the reduced product of the enzyme-degraded compounds. The proposed structures are as follows:

H_2 component: α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→3)βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc–ceramide

H_3 component: α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→$^{3)}_{6)}$$\beta$Gal(1→4)$\beta$GlcNAc(1→3)$\beta$Gal(1→4)Glc–ceramide
α_L-Fuc(1→2)βGal(1→4)βGlcNAc(1→

The fourth component of H-active glycolipid (H_4-glycolipid) was also isolated in chromatographically heterogenous form, but chemical analysis and methylation study indicate heterogeneity of the fraction. Both H_3- and H_4-glycolipids inhibit I-hemagglutination, whereas H_1- and H_2-glycolipids do not inhibit I-hemagglutination.

Recent studies on blood group ABH glycolipid antigens of human erythrocyte membranes indicate the presence of multiple forms of glycolipids carrying A, B, and H determinants (see, for a review, Hakomori, 1974). Chemically all these are fucoglycosphingolipids with internal variance in the carbohydrate chain. Although H activity in the glycolipid fraction of human erythrocytes was previously claimed to be difficult to demonstrate (Koscielak et al., 1970), the presence of H-active glycolipids has been proven in recent studies (Stellner et al., 1973; Koscielak et al., 1973).

At least four types of H-active glycolipids have been distinguished according to their migration rates on thin-layer chromatography. The fastest migrating component (H_1-glycolipid) was characterized unambiguously as L-Fucα(1→2)Galβ(1→4)GlcNAcβ(1→3)Galβ(1→4)Glc→ ceramide, i.e. lacto-*N*-fucopentaosyl(IV)ceramide (Stellner et al., 1973). Koscielak et al. (1973) also reported the isolation of a ceramide pentasaccharide with a structure identical with "H_1-glycolipid" and of a ceramide heptasaccharide having a straight carbohydrate chain bearing the H-active determinant. Among a number of oligosaccharides liberated from a ganglioside mixture of human spleen by ozonolysis–alkaline degradation method, an H-active fucosylsialyl oligosaccharide was isolated whose structure was identified as αL-Fuc(1→2)Galβ(1→3)GalNAcβ(1→3)[NeuNG(2→3)]Galβ(1→4)Glc (Wiegandt, 1973). The presence of sialylhexaose was also noticed, whose desialylated residue was chromatographically indistinguishable from "lacto-*N*-neohexaose" (Wiegandt, 1973), the branched milk oligosaccharide previously reported by Kobata and Ginsburg (1972).

The presence of a third H-active component (H_3-component) with a slower migration rate on thin-layer chromatography was found in this laboratory, but further purification was necessary, and its chemical structure has been awaiting elucidation. The presence of a fourth H-active component was recently recognized and has been partially characterized.

Interestingly, H_3 and H_4 components clearly inhibit I-hemagglutination, in contrast to H_1 and H_2 components, which do not. The H_3 and H_4 components may represent a major recognition site on human erythrocyte membranes for anti-I ("Ma") of Feizi et al. (1971).

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Table I: Elution Schedule in DEAE-Cellulose of Human Erythrocytes "Upper Layer" Glycolipids.[a]

Solvent No.	Solvent Composition: Chloroform	Methanol	Water	Vol of Solvent for Elution (l.)	Substance Eluted
1	9	1	0.02	0.5	
2	8.75	1.25	0.02	1.0	CDH, CTH[b]
3	8.5	1.5	0.02	2.0	Globoside,[b] paragloboside[b,c]
4	8.0	2.0	0.02	3.0	H_1,[b] other cer. pentasac.[d]
5	7.75	2.25	0.03	3.0	A^{a}[e]
6	7.5	2.5	0.03	3.0	Unknown components
7	7.0	3.0	0.03	3.0	H_2, A^{b}[e]
8	6.75	3.25	0.04	2.0	
9	6.5	3.5	0.04	2.0	
10	6.0	4.0	0.04	2.0	H_3[f]
11	5.75	4.25	0.05	2.0	A^{c}[e]
12	5.5	4.5	0.05	2.0	
13	5.25	4.75	0.05	1.0	
14	5.0	5.0	0.05	1.0	H_4,[f] A^{d}[e]
15	0	1.0	0		
16	Chloroform–methanol–ammonium acetate–ammonia				Gangliosides

[a] Column size, 3 × 35 cm; fraction A or A′ loaded 500–600 mg. [b] Majority present in lower phase. [c] Lacto-*N*-neotetraosylceramide (Siddiqui and Hakomori, 1973). [d] Le^a glycolipid and galactosyllacto-*N*-neotetraosylceramide (Marcus and Cass, 1969; Stellner and Hakomori, 1974). [e] For A variants, see Hakomori et al. (1972). [f] These fractions contain unidentified glycolipids.

Table II: Relative Migration Rates of Various Components of Blood Group H as Compared to A Glycolipids.

		$R_{globoside}$ Value[a]		
As free glycolipid; solvent, chloroform–methanol–water (60:35:8)	H_1	0.85 (A^a 0.76)		
	H_2	0.65 (A^b 0.55)		
	H_3	0.33 (A^c 0.29)		
	H_4	0.15 (A^d 0.12)		
As acetylated compd; solvents, see footnotes *b–d*	H_2	0.80[b]	0.65[c]	0.51[d]
	H_3	0.60[b]	0.35[c]	0.07[d]

[a] Migration rate of globoside or its acetylated compound as 1.00; migration rates of H_1-, H_2-, H_3-, and H_4-glycolipids were compared respectively with their related A variants (A^a, A^b, A^c, and A^d) as shown in parentheses. [b] In butyl acetate–acetone–water (55:45:5). [c] In ethylene dichloride–acetone–water (55:45:5). [d] In butyl acetate–acetone (7:3).

Table III: Molar Ratio of Carbohydrate Composition in H-Active Glycolipid.[a]

	H_2-Glycolipid	H_3-Glycolipid	H_4-Glycolipid
Fucose	1.1	2.0	0.91
Galactose	2.8	4.1	1.90
Glucose	1.0	1.0	1.00
Glucosamine (*N*-acetylglycosamine)	2.1	2.9	1.37

[a] Molar ratio was calculated on basis of glucose as 1.0.

Experimental Procedure and Materials

Preparation and Purification of Glycolipids. The sphingolipid fraction of human O erythrocyte ghosts was prepared by extraction with 90% hot ethanol and precipitation at −20°, followed by fractionation with ether, acetone, and chloroform–methanol by the described method (Hakomori and Strycharz, 1968; Stellner et al., 1973; Siddiqui et al., 1972). The sphingolipid fraction obtained was dissolved in 500 parts (v/w) of chloroform–methanol (2:1); 1/6 vol of water was added and shaken. The mixture was allowed to stand to separate into upper and lower phases. The lower phase was partitioned twice with the "theoretical upper phase", consisting of chloroform–methanol–aqueous 0.1% NaCl solution (1:10:10, v/v/v). The combined upper phases were evaporated to a smaller volume, dialyzed against ice water for 3 days, and lyophilized (fraction A). A fluffy tan powder (600–700 mg) was obtained from 650–750 ml of packed membranes derived from 11–12 l. of sedimented human erythrocytes. To avoid possible loss of glycolipid which might occur during the procedure, the following alternate procedure was used. The upper phase glycolipid without dialysis was evaporated in a rotary evaporator to dryness; the residue was dissolved in chloroform–methanol (3:1) and freed from nonlipid impurities by fractionation on a column (1 × 25 cm) of LH-20 (Pharmacia Biochemical) prepared in chloroform–methanol (3:1), according to the method of Maxwell and Williams (1967). The glycolipid fraction was eluted with 0.56–0.93 column volumes of chloroform–methanol (3:1) (fraction A′) and separated clearly from nonlipid contaminants, which were eluted with 1.25–1.75 column volumes of chloroform–methanol (3:1). Either fraction A or fraction A′ was resolved into various components, including four kinds of H-active glycolipids by the following three steps of purification. (1) Column chromatography on DEAE-cellulose, eluted with 15 different solvents, enabled separation of about 12 kinds of glycolipids. Elution schedule and solvent composition for DEAE-cellulose chromatography are modified from a previous report (Stellner et al., 1973) and are shown in Table I. (2) Those glycolipid fractions containing H activity were separated by thin-layer chromatography and an apparently homogeneous H-active glycolipid was extracted from the silica gel bands. (3) Fractions containing H-active glycolipid were further purified as acetylated compounds on thin-layer chromatography developed with nonpolar solvents in order to separate them from nonactive components present as contaminants. The compounds were eluted, and the H-active glycolipids were recovered after deacetylation.

Preparation of H_2-, H_3-, and H_4-Glycolipids from O Erythrocytes. (A) H_1-glycolipid was eluted from a DEAE-cellulose column by chloroform–methanol–water (8:2:0.02) together with "paragloboside" (lacto-*N*-neotetraosylceramide (Siddiqui and Hakomori, 1973) and the tailing fraction of globoside as previously described (Stellner et al., 1973). One other type of ceramide pentasaccharide (β-galactosyllacto-*N*-neotetraosylceramide) also co-eluted (Stellner and Hakomori, 1974).

(B) H_2-Glycolipid was eluted from the column with 3 l. each of chloroform–methanol–water 7.5:2.5:0.03 (no. 6) and 7:3:0.03 (no. 7). Three glycolipid zones (or spots) having $R_{globoside}$ values of 0.66, 0.70, and 0.75, respectively, were separated by thin-layer chromatography on a silica gel G plate with chloroform–methanol–water (60:35:8). The slowest migrating band was the major component that carried H activity, and the two minor faster migrating bands were devoid of H activity. The H-active zone was extracted with chloroform–methanol–water (10:10:1) from the silica

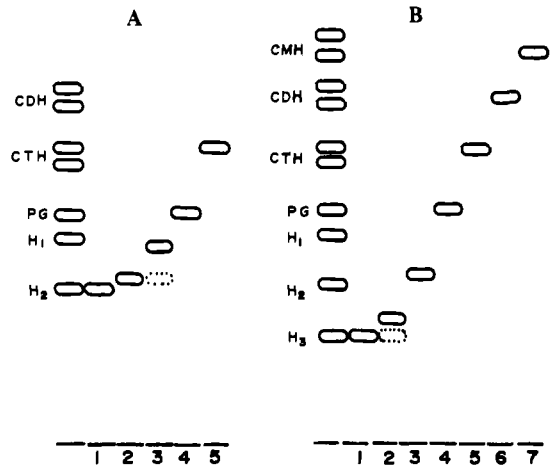


FIGURE 1: Location of spots of H-glycolipids and their degradation products on thin-layer chromatography: H_1, H_2, and H_3, each H_1-, H_2-, and H_3-glycolipid; PG, paragloboside, lacto-*N*-neotetraosylceramide; CTH, ceramide trihexoside, αGal(1→4)βGal(1→4)Glc→ceramide; CDH, ceramide dihexoside, β-Gal(1→4)Glc→ceramide; CMH, ceramide monohexoside, βGlc→ceramide. Double spots of CTH, CDH, and CMH are due to difference of ceramide; solvent, chloroform–methanol–water, 60:35:8. (A) Lane 1, intact H_2-glycolipid; lane 2, H_2-glycolipid incubated with α-L-fucosidase (*Charonia lampas*); lane 3, degradation product of H_2-glycolipid by α-L-fucosidase was further incubated with β-galactosidase (jack bean); lane 4, the lane 3 product was further degraded by incubation with β-*N*-acetylhexosaminidase (jack bean); lane 5, the lane 4 product was further degraded by incubation with β-galactosidase. (B) Lane 1, intact H_3-glycolipid; lane 2, H_3-glycolipid incubated with α-L-fucosidase (*Charonia lampas*); lane 3, the lane 2 product was incubated with β-galactosidase (jack bean); lane 4, the lane 3 product was degraded by incubation with β-*N*-acetylhexosaminidase (jack bean); lane 5, the lane 4 product (paragloboside-like glycolipid) was incubated with β-galactosidase (jack bean); lane 6, the lane 5 product was incubated with β-*N*-acetylhexosaminidase (jack bean); lane 7, the lane 6 product was incubated with β-galactosidase.

gel plate immediately after the plates were developed. Delay of extraction of zones after the thin-layer plate dries results in poor yield of glycolipid recoverable from the silica gel. The H-active zone with an $R_{globoside}$ value of 0.66 was then acetylated in pyridine–acetic anhydride (2:1), and the acetylated compound was fractionated on thin-layer chromatography with the solvent mixture ethylene dichloride–acetone–water (55:45:1). Two bands were separated; the lower band was H active while the upper band was inactive. These bands were eluted with chloroform–methanol (2:1) and then deacetylated with chloroform–methanol–0.5% sodium methoxide in methanol (2:1:0.5). The glycolipid was assigned as H_2-glycolipid, which was homogeneous on thin-layer chromatography as a free glycolipid and as the acetylated compound in various solvent systems. The yield of H_2-glycolipid was 3–4 mg from 500 mg of fraction A.

(C) H_3-Glycolipid, the third component of H-active glycolipid, was eluted from a DEAE-cellulose column with 2 l. each of solvent mixtures no. 9 (chloroform–methanol–water, 6.5:3.5:0.04), no. 10 (chloroform–methanol–water, 6:4:0.04), no. 11 (chloroform–methanol–water, 5.75:4.25:0.05), and no. 12 (chloroform–methanol–water, 5.5:4.5:0.05). The majority of H_3-glycolipid was eluted with solvent no. 11, but co-eluted partially with H_2-glycolipid and with three other components. These were separated on thin-layer chromatography. H_3-Glycolipid migrated on a thin-layer chromatogram in solvent system chloroform–methanol–water (60:35:8 or 56:38:10) with an $R_{globoside}$ value of 0.29. A slower migrating component ($R_{globoside}$ 0.25) and two faster migrating components ($R_{globoside}$ 0.35 and 0.40) were detected. The H_3 fraction was separated by preparative thin-layer chromatography on silica gel G and was extracted with chloroform–methanol–water, 1:1:0.1. The fraction was acetylated in pyridine–acetic anhydride (2:1), and the acetylated fraction was separated by thin-layer chromatography on silica gel G plates with ethylene dichloride–acetone–water (50:50:1). A major single band was separated from other minor components, and the band was extracted with chloroform–methanol (2:1). The glycolipid acetate, eluted from silica gel, was deacetylated in chloroform–methanol–0.1% sodium methoxide (2:1:0.5) at room temperature for 20 min. H_3-Glycolipid (4.5–5 mg) was obtained from 500 mg of fraction A or fraction A′.

Table IV: Partially O-Methylated Hexitol and Hexosaminitol Detected in the Hydrolysate of Permethylated H_2- and H_3-Glycolipids.[a]

	H_2-Glycolipid	H_3-Glycolipid	H_4-Glycolipid
2,3,4-Tri-*O*-methylfucositol (1,5-di-*O*-acetyl)	0.8	2.0	(0.5)
2,4,6-Tri-*O*-methylgalactitol (1,3,5-tri-*O*-acetyl)	2.5	2.6	6.5
3,4,6-Tri-*O*-methylgalactitol (1,2,5-Tri-*O*-acetyl)[b]	1.1	2.2	3.3
2,3,6-Tri-*O*-methylglucitol (1,4,5-tri-*O*-acetyl)[b]	1.0	1.3	3.3
2,3,4,6-Tetra-*O*-methylgalactitol (1,5-di-*O*-acetyl)	0.0	0.0	0.3
2,4-Di-*O*-methylgalactitol (1,3,5,6-tetra-*O*-acetyl)	0.0	1.0	2.0
3,6-Di-*O*-methylglucosaminitol (*N*-acetyl, *N*-methyl, 1,3,5-tri-*O*-acetyl)	2.0	2.8	6.5
4,6-Di-*O*-methylglucosaminitol (*N*-acetyl, *N*-methyl, 1,3,5-tri-*O*-acetyl)	0.0	0.0	0.0

[a] Numbers indicate molar ratio. [b] These two peaks separated on 3% ECNSS on Gas Chromosorb W column.

(D) The glycolipid fraction eluted from the DEAE-cellulose column with 1 l. each of solvent no. 13 (chloroform–methanol–water, 5.25:4.75:0.05), no. 14 (chloroform–methanol–water, 5:5:0.05), and solvent no. 15 (100% methanol) contained a very slow migrating H-active glycolipid (H_4-glycolipid; $R_{globoside}$ 0.09) with a tailing fraction of H_3-glycolipid. The H_4 fraction was separated into two bands on preparative thin-layer chromatography in chloroform–methanol–water (56:38:10). The slowest migrating glycolipid, the major component of this fraction, was eluted from silica gel by methanol–water (1:1) and was acetylated in pyridine–acetic anhydride (2:1). The product was purified by thin-layer chromatography on silica gel G (pre-scored "Uniplates", 250 μ, Analtech) with the solvent system ethylene dichloride–acetone–water (40:60:2). A band at R_f value 0.15 was eluted with chloroform–methanol (2:1) followed by deacetylation. This compound was designated as H_4-glycolipid.

Purity of glycolipid was tested in a fully acetylated state and in a free state on thin-layer chromatography with various solvent systems.

Analysis of Carbohydrate Composition. The carbohydrate composition of the glycolipids was analyzed by gas chromatography after hydrolysis with acetic acid–sulfuric acid, as previously described (Yang and Hakomori, 1971)

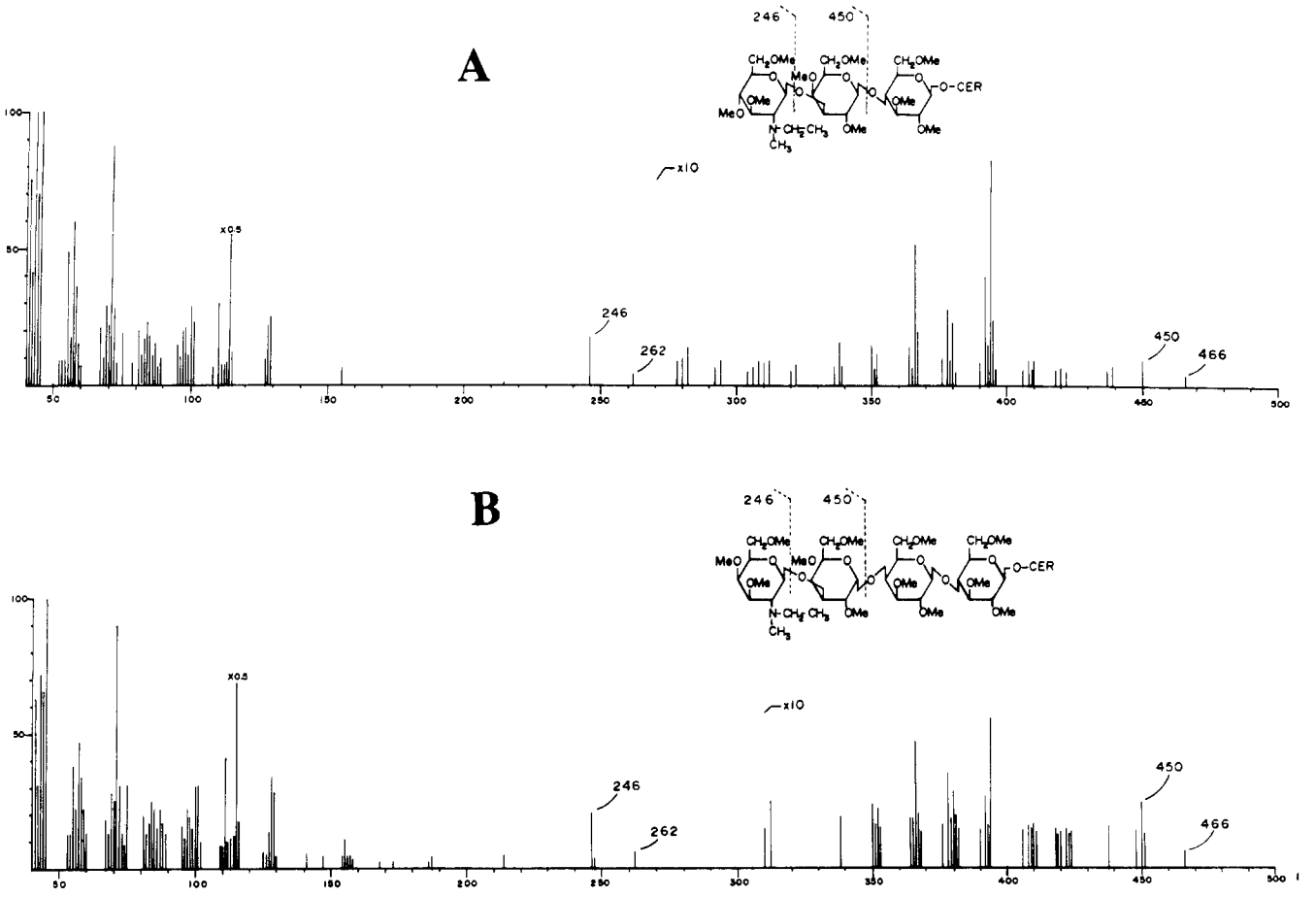


FIGURE 2: Mass spectra of permethylated and reduced product of some glycosphingolipids. Permethylation was according to Hakomori's method (1964) and reduction with sodium bis(2-ethoxymethoxy)aluminum hydride, as modified from Karlsson (1973, 1974): (A) GlcNAcβ(1→3)Gal-β(1→4)Glc→ceramide, degradation product from "paragloboside"; (B) globoside of human erythrocyte membranes; (C) degradation product from H_3-glycolipid by α-L-fucosidase; and by β-galactosidase (D) intact H_3-glycolipid. The condition for mass spectrometry is as follows: ion source pressure, 2×10^{-5} Torr; ionization current, 0.50 mA; electric energy, 70 eV, ion energy, 6.2 eV; sensitivity, 10^{-7} A/V; mass range, 0-500 amu. The mass peak 214 (246 minus 32 for methanol) was clearly seen in Figures 2B and 2C, but was barely detectable with the sensitivity recorded for Figure 2A. At higher sensitivity this peak was detected clearly. The intensive peaks between 350 and 400 due to fragments derived from ceramides (Karlsson et al., 1974).

and after methanolysis followed by N-acetylation according to the methods as reviewed by Laine et al. (1972).

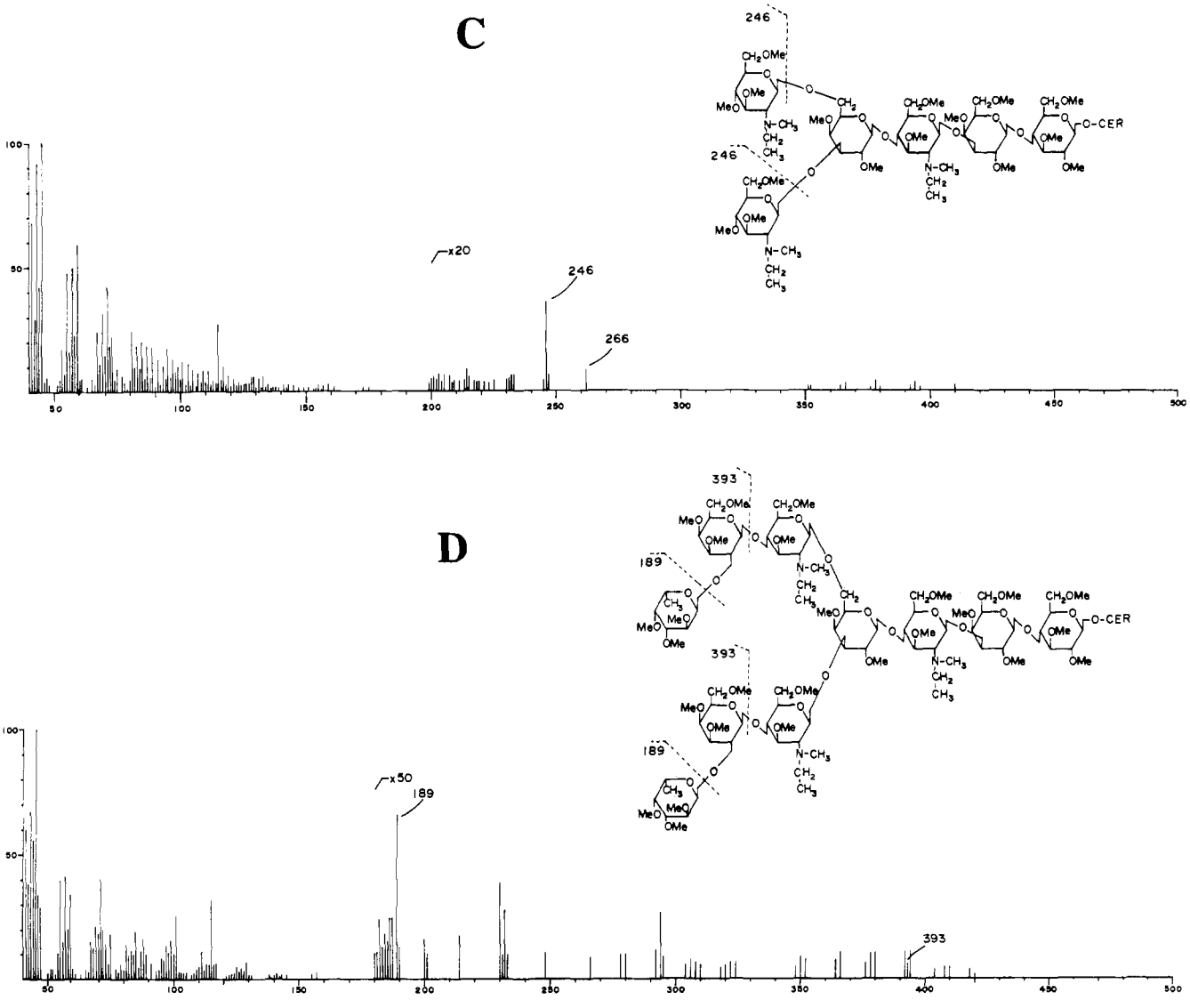
Fatty Acids and Sphingosine Analysis. Fatty acids and sphingosines were determined according to the methods of Gaver and Sweeley (1965), slightly modified as previously described (Yang and Hakomori, 1971). Methanolysis and separation of sphingosines and fatty acids were carried out by the method of Gaver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

Controlled Periodate Oxidation and Smith Degradation. These procedures were carried out according to the conditions described previously (Hakomori et al., 1971) under which conditions glucosyl residue directly attached to ceramide was not oxidized, but the peripheral moiety of the carbohydrate chain was degraded. The degradation product was examined by thin-layer chromatography.

Methylation Analysis. Methylation was carried out in dimethyl sulfoxide, with sodium methylsulfinyl carbanion, and methyl iodide (Hakomori, 1964). The methylated glycolipid was isolated by an LH-20 column and degraded in 95% acetic acid containing 0.5 *N* sulfuric acid at 80° for 18 hr, followed by preparation of partially O-methylated hexitol or hexosaminitol acetates, according to the method of Stellner et al. (1973). The partially O-methylated hexitol or hexosaminitol acetates were analyzed by gas chromatography–mass spectrometry using a glass column of 3% ECNSS-M on Gas-Chrom Q and connected to a Finnigan quadrupole mass spectrometer (Model 4000) with all-glass separator. For analysis of partially O-methylated hexitol acetates, the column temperature was set at 150–160° and for analysis of partially O-methylated 2-deoxy-2-*N*-methylacetamidohexitols, the column temperature was set at 190°.

Partially O-methylated hexitol acetates were identified according to the data of Björndal et al. (1967, 1970), and the partially O-methylated 2-deoxy-2-*N*-methylacetamidohexitol acetates were determined according to our data as described (Stellner et al., 1973).

Mass Spectrometric Identification of Permethylated Glycolipids. The total mass spectrometry of permethylated glycolipids was carried out according to the method of Karlsson (1973). However, the original method was modified in the purification of permethylated compound and in the use of sodium bis(2-methoxyethoxy)aluminum hydride for reduction. The glycolipids were permethylated in di-



methyl sulfoxide, methylsulfinyl carbanion, and methyl iodide (Hakomori, 1964); permethylated glycolipid was isolated by chromatography on an LH-20 column followed by thin-layer chromatography on silica gel G, developed with benzene–acetone (1:1, v/v). The permethylated glycolipid, eluted from the silica gel with acetone and evaporated under nitrogen in a Teflon-lined screw-capped Pyrex glass tube, was dissolved in 1 ml of benzene, and to it 0.5 ml of sodium bis(2-methoxyethoxy)aluminum hydride ("vitride", 70% in benzene, Eastman Kodak) was added. It was then heated at 80° for 2 hr. After cooling, 1 ml of water and 0.1 *N* sodium hydroxide was added, shaken well, and centrifuged. The upper benzene layer was separated, and the lower layer was partitioned twice with benzene. The first benzene layer and the benzene extract from the lower phase were combined and evaporated to dryness. The dried residue was dissolved in 1 ml of chloroform and shaken with 1 ml of water. The chloroform layer was evaporated to dryness in a glass "direct-probe tube" first under nitrogen stream and then in vacuo over phosphorus pentoxide. The residue was subjected to direct probe mass spectrometry in the Finnigan quadrupole instrument. For details of the conditions, see the caption to Figure 2.

Enzymatic Degradation of Glycolipids and Determination of Carbohydrate Sequence and Anomeric Structure. The following enzymes were used for sequential degradation of glycolipids: α-*N*-acetylgalactosaminidase, prepared from hog liver according to the method of Weissmann and Hinrichsen (1969); α-L-fucosidase of *Charonia lampas* (Iijima et al., 1971), donated by Dr. Takashi Okuyama of Seikagaku Kogyo Co., Ltd., Tokyo; α-galactosidase, of ficin (Li and Li, 1972b); β-galactosidase and β-*N*-acetylhexosaminidase, of jack bean (Li and Li, 1972a). Some of these preparations were donated by Dr. Y-T. Li of Tulane University, Department of Biochemistry. The hydrolyzability of glycolipids by *endo*-β-*N*-acetylglucosaminidase for hydrolysis of GlcNAc→GlcNAc (Tarentino et al., 1974) was kindly tested by Dr. F. Maley of New York State Laboratory. The enzymatic hydrolysis of glycolipids, followed by examination of degraded glycolipids with thin-layer chromatography, was carried out according to the conditions described previously (Hakomori et al., 1971).

Immunological Reactivity. H activity was determined by inhibition of hemagglutination caused by *Ulex europeus* and by eel serum. These reagents also gave a fairly distinctive and strong precipitin line with H-glycolipid in double diffusion agar. Anti-type XIV pneumococcal antisera was donated by Dr. Don M. Marcus of Albert Einstein College of Medicine. Eel serum was donated by Dr. Furukawa, Gumma University, Japan. Anti-I serum was donated by

Dr. Eloise Giblett of King County Central Blood Bank, Seattle, Wash. Inhibition of I-hemagglutination was tested at 4° with and without auxilliary lipids. One part of glycolipid was mixed with 5 parts of lecithin and cholesterol in a chloroform–methanol solution; the solvents were evaporated under nitrogen and sonicated with saline. For other conditions, refer to Table VI.

Results

Each glycolipid component with H activity was homogeneous on thin-layer chromatography as the free state and as acetate. Relative migration rates of these components are shown in Table II. The carbohydrate composition of H_2-, H_3-, and H_4-glycolipids is shown in Table III. The carbohydrate composition of both H_2- and H_3-glycolipids consists of fucose, galactose, glucose, and glucosamine in molar ratios of 1:3:1:2 and 2:4:1:3, respectively. H_4-Glycolipid did not show an integral molar ratio of constituent sugars, which may indicate that the H_4 fraction is still heterogeneous.

The change of chromatographic migration rate by successive enzymatic hydrolysis is shown in Figure 1, and the results of methylation analysis are shown in Table IV.

(1) *H_2-Glycolipid.* H_2-Glycolipid was hydrolyzed only by α-L-fucosidases to give a glycolipid showing a slightly higher migration rate than the original H_2 substance. The resulting glycolipids were cross-reactive with anti-type XIV pneumococcal polysaccharide antiserum. Only fucose and one of 3 mol of galactose were destroyed by controlled periodate oxidation and Smith degradation, as previously described (Hakomori et al., 1972). The glycolipid was not hydrolyzed by incubation with α- or β-galactosidase nor by α- or β-*N*-acetylhexosaminidase. The hydrolysis product of H_2-glycolipid after incubation with α-L-fucosidase was then hydrolyzed with β-galactosidase but not with other glycosylhydrolases. The product shows a migration rate between "paragloboside" and H_2-glycolipids, but slightly slower than H_1-glycolipid (lane 3 of Figure 1A). The compound was then hydrolyzed with β-*N*-acetylhexosaminidase to give a glycolipid showing an identical migration rate as that of "paragloboside" (Siddiqui and Hakomori, 1973) (lane 4 of Figure 1A). The material was indistinguishable from "paragloboside" (lacto-*N*-neotetraosylceramide) on thin-layer chromatography in many solvent systems, and also showed cross-reaction with anti-type XIV pneumococcal polysaccharide antiserum. This substance was further hydrolyzed by incubation with β-galactosidase to give a ceramide trisaccharide having *N*-acetylglucosamine at the terminal (lane 5, Figure 1A). The latter compound showed a characteristic reaction with wheat germ lectin. With these results it was concluded that H_2-glycolipids must have the carbohydrate sequence as follows: α-L-fucosyl-β-galactosyl-β-*N*-acetylglucosaminyl-β-galactosyl-β-*N*-acetylglucosaminyl-β-galactosyl-β-glucosylceramide. The results of sequential degradation with enzymes clearly indicate that any other alternative sequence of carbohydrates cannot be considered. The H_2-glycolipid was not hydrolyzed by *endo*-β-*N*-acetylglucosaminidase (Tarentino et al., 1974), excluding the possibility of such a linkage as R→GlcNAc→GlcNAc→R within the H_2 carbohydrate chain.

The position of carbohydrate linkages in H_2-glycolipid was determined by methylation study. The permethylated glycolipids gave 3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidoglucitol, but no trace amount of 4,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidoglucitol, indicating that both glucosamines were linked through the C-4 hydroxyl group. The presence of 2,3,4-tri-*O*-methylfucositol, 3,4,6-tri-*O*-methylgalactitol, 2,3,6-tri-*O*-methylglucitol, and 2,4,6-tri-*O*-methylgalactitol, and the absence of any trace amount of 2,3,4,6-tetra-*O*-methylgalactitol, di-*O*-methyl- or mono-*O*-methylhexitol, or mono-*O*-methyl-2-deoxy-2-*N*-methylacetamidohexitol supported the structure with a straight chain carbohydrate as seen in Figure 2. The presence of 2-hydroxyl-substituted galactosyl residue at the penultimate position was supported by the susceptibility of both terminal fucosyl and one galactosyl residues to periodate, and the degradation product was a ceramide pentasaccharide. The structure was also supported by the yield of a ceramide tetrasaccharide, whose structure was indistinguishable from "paragloboside" during the process of enzyme degradation.

(2) *H_3-Glycolipid.* Enzymatic hydrolysis of H_3-glycolipids is also shown in Figure 2. The first incubation with α-L-fucosidase of *Charonia lampas* turned the chromatographic migration rate of H_3-glycolipids to a substance with a slightly higher chromatographic rate than the original H_3-glycolipid (lane 2, Figure 1B). Neither β-galactosidase, α-galactosidase, nor β-*N*-acetylhexosaminidase altered the properties of H_3-glycolipid. The hydrolysis product of H_3-glycolipid was then hydrolyzed with jack bean β-galactosidase to a substance which had a slightly higher mobility than H_2-glycolipids (lane 3, Figure 1B). The compound was further degraded by β-hexosaminidase resulting in a degradation product identical in chromatographic and immunological properties with "paragloboside" (lane 4, Figure 1B). The "paragloboside"-like material was further identified through susceptibility to jack bean β-galactosidase and was converted to a ceramide trisaccharide (lane 5, Figure 1B) and by its reactivity to anti-type XIV pneumococcal polysaccharide antiserum. The ceramide trisaccharide was further hydrolyzed by β-*N*-acetylhexosaminidase to a ceramide lactoside. The resulting ceramide dihexoside was readily hydrolyzed by jack bean β-galactosidase to yield a ceramide glucoside which was distinguishable from ceramide galactoside by thin-layer chromatography on a borate-impregnated plate. H_3-Glycolipid was also not susceptible to *endo*-β-*N*-acetylglucosaminidase (Tarentino et al., 1974).

The results of successive degradation by hydrolases and the carbohydrate analysis of intact H_3-glycolipid clearly indicate the presence of 2 mol of fucose per glucoslyceramide. Both fucosyl residues and 2 of the 4 mol of galactosyl residues were readily destroyed by periodate oxidation under controlled oxidation conditions (Hakomori et al., 1971), suggesting the presence of two H-active carbohydrate chains linked to glycosylceramide by branching. The branched structure was further supported by methylation study, i.e. the presence of 2,4-di-*O*-methylgalactitol acetate was clearly demonstrated among other partially O-methylated sugar acetates, which indicates that branching occurred at this sugar residue with a 1→3(1→6)Gal structure. No trace amount of either 2,3,4,6-tetra-*O*-methylgalactitol or glucitol or 3,4,6-tri-*O*-methyl-2-deoxy-2-*N*-methylacetamidohexitol acetate was found, and only 2,3,4-tri-*O*-methylfucositol was demonstrated. This indicated that only the fucopyranosyl residue should be at the terminal. A large proportion of 2,4,6-tri-*O*-methylgalactitol and 3,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidoglucitol indicates that galactosyl and *N*-acetylglucosaminyl residues were substituted by 1→3 and 1→4 linkages, respectively. Two moles each of 3,4,6-tri-*O*-methylgalactitol and 2,3,4-tri-*O*-methylfucositol were yielded against 1 mol of 2,4-di-

O-methylgalactitol, which indicates the presence of two terminal Fucα(1→2)Gal structures per one carbohydrate chain branched at 1→3(1→6)galactose. This finding, together with enzyme degradation, strongly supports the structures shown below, either I or II.

$$\begin{array}{l}\alpha_L\text{-Fuc}(1\rightarrow2)\beta\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow{}^{3)}\\ \qquad\qquad\qquad\qquad\qquad\qquad\beta^{6)}\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow3)\beta\text{Gal}(1\rightarrow4)\text{Glc}\rightarrow\text{ceramide}\\ \alpha\text{-}_L\text{-Fuc}(1\rightarrow2)\beta\text{Gal}(1\rightarrow4)\beta\text{-GlcNAc}(1\rightarrow{}^{6)}\end{array}$$

I

$$\begin{array}{l}\alpha_L\text{-Fuc}(1\rightarrow2)\beta\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow3)\beta\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow{}^{3)}\\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\beta\text{ Gal}(1\rightarrow4)\text{Glc}\rightarrow\text{ceramide}\\ \alpha_L\text{-Fuc}(1\rightarrow2)\beta\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow{}^{6)}\end{array}$$

II

Whether the point of branching in an H-active carbohydrate chain is at the first galactosyl residue (proximal to ceramide) or at the second galactosyl residue (distal to ceramide) cannot be answered by combined studies with enzymatic hydrolysis and methylation.

By successive treatment of H_3-glycolipid with α-L-fucosidase and with β-galactosidase, H_3-glycolipid was converted to a glycolipid with two terminal *N*-acetylglucosamines, which were characterized by having a strong precipitin reaction with wheat germ lectin and by giving 3,4,6-tri-*O*-methyl-2-deoxy-2-*N*-methylglucitol after methylation analysis. This compound could have either of the following structures, III or IV.

$$\begin{array}{l}\beta\text{GlcNAc}(1\rightarrow{}^{3)}\\ \qquad\beta\text{Gal}(1\rightarrow4)\beta\text{GlcNAc}(1\rightarrow3)\beta\text{Gal}(1\rightarrow4)\text{Glc}\rightarrow\text{ceramide}\\ \beta\text{GlcNAc}(1\rightarrow{}^{6)}\end{array}$$

III

$$\begin{array}{l}\beta\text{GlcNAc}(1\rightarrow3)\beta\text{Gal}(1\rightarrow4)\text{GlcNAc}(1\rightarrow{}^{3)}\\ \qquad\qquad\qquad\qquad\beta\text{Gal}(1\rightarrow4)\text{Glc}\rightarrow\text{ceramide}\\ \qquad\qquad\beta\text{GlcNAc}(1\rightarrow{}^{6)}\end{array}$$

IV

Identification of either structure III or IV was of critical importance in determining whether the parent glycolipid could have the structure I or II. It was expected that the mass spectrum of the degraded glycolipid, structure III, would show a difference from that of structure IV, although mass spectra of compounds with structures I and II may be difficult to distinguish. An early work by Sweeley and Dawson (1969) showed some promise by this approach. A related, more recent method of Karlsson (1973) using the volatile permethylated and reduced derivatives of glycosphingolipids was employed. This gave 2-deoxy-2-*N*-methylethyl amino sugars which displayed an ion at *m/e* 246 when terminally located. An *N*-acetylhexosaminosylhexosyl residue at the terminal of the carbohydrate chain could be identified by the presence of *m/e* 450 and 246, as was exemplified in the mass spectra of permethylated and reduced GlcNAcβ(1→3)Galβ(1→4)glc→ceramide (see Figure 2A) and globoside (see Figure 2B). The *m/e* 466 is the ion with oxygen. A similar mass spectra should be given by compounds with structure IV but not by those with structure III.

As shown in Figure 2C, the enzyme-degraded compound derived from H_3-glycolipid gave only intensive mass with *m/e* 246 (and 266), but none at 450. This clearly indicates that the compound should have both GlcNAc terminals branched at the penultimate hexose; namely, the compound should have structure III rather than structure IV. The mass spectra of an intact permethylated and reduced H_3-glycolipid were characterized by the presence of *m/e* 189 and 393, which is derived from the terminal fucosyl and fucosylgalactosyl residue, respectively (see Figure 2D).

The composition of ceramide moiety is shown in Table V.

Discussion

The results of this study confirm clearly that the backbone structure of H_2-glycolipid is essentially the same as previously suggested by Stellner et al. (1973), Koscielak et al. (1973), and Hakomori and Andrews (1970), in which two repeating Galβ(1→4)GlcNAcβ(1→3)Gal residues are linked together thus forming a long, unbranched heptasaccharide chain. The sequence of carbohydrate and the position and anomeric properties of the linkages are clearly indicated by successive enzyme degradation and methylation studies. The location of 2-substituted β-Gal at the penultimate residue was supported by: (1) periodate susceptibility of the terminal fucose and one of the 3 mol of galactose under the condition which did not allow oxidation of the 4-substituted glucosyl residue directly linked to ceramide (Hakomori et al., 1971); and (2) yield of "paragloboside" by successive enzyme degradation in which no possibility of 2-substituted galactosyl residue is present.

The results of this study also show that the structure of H_3-glycolipid is assigned to structure I, rather than structure II, in which two H-active chains are linked by branching to a galactosyl residue of a ceramide tetrasaccharide which has the same structure as "paragloboside" of human erythrocytes (Siddiqui and Hakomori, 1973). In this structure, two H-active chains and the carbohydrate chain directly attached to ceramide are all "type II" chains (Painter et al., 1963; Watkins, 1966), which is similar to the structure of the side chain in glycoproteins carrying blood group determinants as described by Lloyd and Kabat (1968). However, only type II chain was found in this erythrocyte-derived blood group glycolipid in striking contrast to both type I and II chains being linked together in Lloyd and Kabat's glycoprotein formula (Lloyd and Kabat, 1968).

Although these ceramides with a long carbohydrate chain or those with branched carbohydrate chains occur in small quantities on the cell membrane, they could be nevertheless an extremely effective recognition site or strong antigen as compared to some of the membrane ceramides with shorter carbohydrate chains. If the extraction and purification scheme were quantitative, an estimated 5×10^5 H_2 molecules would occur on each erythrocyte.

The I-antigen of erythrocytes has been known to be a carbohydrate determinant (Marcus et al., 1963). The capa-

Table V: Fatty Acids and Sphingosines of H_3-Glycolipid.

Fatty Acid[a]	% Total	Sphingosines
C16:0	12.7	
C16:1	3.2	
C18:0	7.4	C18:0 0
C18:1	12.0	C18:1 + (over 95% 0)
C20:0	2.2	Phytosphingosine 0
C20:1	3.7	C20:0 0
C22:0	13.5	C20:1 0
C22:1	1.9	
C24:0	13.1	
C24:1	11.5	

[a] No α-hydroxy fatty acids were detected.

Table VI: Blood Group Activities of H-Glycolipids.

	H Activity				I Activity, Inhibition of I-Hemagglutination at Cold[c]
	Precipitin Reaction in Ouchterlony Agar Diffusion		Inhibition of H Hemagglutination[a,b]		
	Eel	*Ulex*	Eel	*Ulex*	
H_1	+	+	3	1	>200
H_2	+	+	1.5	1.5	>200
H_3	++	++	1.5	1.5	6
H_4	++	++	3	3	25

[a] Minimum doses of glycolipids in micrograms/0.1 ml which inhibit three hemagglutination doses of either eel serum or *Ulex europeus* extract. [b] The same range of activity (1.5 μg/0.1 ml) was demonstrated by H-active glycoprotein isolated from ovarial cyst mucin (sample obtained from Professor W. T. J. Morgan (Lister Institute, London) and Professor N. Hiyama (Hirosaki University, Hirosaki, Japan). [c] Minimum doses of glycolipids in micrograms/0.1 ml which inhibit three hemagglutination doses of anti-I-serum (Dr. Giblett); values determined with auxiliary lipids.

bility of H_3- and H_4-glycolipid to inhibit I-hemagglutination supports the results of Feizi et al. that the Gal-β(1→4)GlcNAcβ(1→6)Gal structure could be one of the I determinants (Feizi et al., 1971), the same structure found in H_3-glycolipid. Anstee and Tanner recently observed that the I-antigen of human erythrocyte membranes could be solubilized by aqueous butanol and recoverable exclusively in the aqueous phase when shaken with water (Anstee and Tanner, 1974a,b). The I activity of erythrocytes was, however, not found in glycoprotein, but was assumed to be associated with a complex glycolipid sufficiently hydrophilic to partition into aqueous phase (private communication from Dr. Anstee, National Blood Transfusion Service, Bristol, England). Gardas and Koscielak (1974) also described an association of I-antigen in the aqueous phase of butanol extract, but most probably not in glycoprotein. It probably is in an unusually complex glycosphingolipid having as many as 30–50 sugar residues ("megaloglycolipids"). H_3-Glycolipid was extensively purified by acetylation procedure, giving an integral molar ratio of sugars. Its structure was unambiguously established, as reported in this paper. Nevertheless, the compound obviously inhibits I-hemagglutination. An I-active component present in H_3-glycolipid as a contaminant can be ruled out. It is not inconceivable therefore that the "megaloglycolipid" of Gardas and Koscielak (1974) may comprise H_3 and H_4 components. As the heterogeneity of anti-I antisera is well known, the structure of H_3-glycolipid that contains the determinants as suggested by Feizi et al. (1971) could represent only one of the I structures. Further studies of immunological properties of glycolipids with different I antisera will be necessary to establish different I determinants.

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On the Relationship of Zinc Ion to the Structure and Function of the 7S Nerve Growth Factor Protein†

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ABSTRACT: The 7S nerve growth factor (7S NGF) is an oligomeric protein consisting of three distinct classes of subunits, α, β, and γ (A. P. Smith, S. Varon, and E. M. Shooter (1968), *Biochemistry 7,* 3259). The β subunit contains the growth promoting activity while γ is a potent esteropeptidase. The proteolytic activity of γ is virtually completely inhibited in the 7S NGF aggregate (L. A. Greene, E. M. Shooter, and S. Varon (1969), *Biochemistry 8,* 3735). In this paper, we report that divalent metal ion chelating agents effect a seven- to tenfold increase in the esteropeptidase activity of 7S NGF at pH 7.40. Plots of esteropeptidase activity vs. chelator concentration give saturation curves which are either sigmoidal (EDTA) or hyperbolic (*o*-phenanthroline) depending on the chemical structure of the chelator. A survey of common divalent metal ions shows that only zinc ion ($K_i = 8 \times 10^{-7}$ *M*) and, to a lesser extent, cadmium ion are effective, reversible inhibitors of both 7S NGF and the γ subunit esteropeptidase activities. We have found that during isolation of 7S NGF, Zn^{2+} is selectively associated with the oligomer in a ratio of approximately 1–2 g-atoms of zinc/mol of 7S NGF with an apparent affinity which is orders of magnitude tighter than is indicated by the K_i value for the γ subunit. Dialysis to pH 4.0 where 7S NGF is known to undergo a reversible dissociation (A. P. Smith, S. Varon, and E. M. Shooter (1968), *Biochemistry 7,* 3259) brings about a tenfold reduction in the zinc ion content of the protein. This reduction is reversed on dialysis back to pH 7.4. In contrast, the isolated subunits contain only trace amounts of zinc ion at pH 7.4. Preliminary metal ion exchange experiments indicate that, of the common metal ions known to substitute for zinc in other zinc-metalloproteins, only cadmium ion is effective in substituting for zinc ion in 7S NGF. The fact that zinc ion is specifically bound to native 7S NGF, and that the zinc ion content of the system is critically dependent on the subunit aggregation state strongly suggests that zinc ion is an integral structural component of native 7S NGF.

The in vivo growth and the differentiation of two neural crest derivatives, the noradrenergic neurons of the superior cervical ganglia and the sensory neurons of the dorsal root ganglia, are dependent upon, and are regulated by, the nerve growth factor (NGF)[1] protein (Schenkein, 1972). Two forms of NGF have been isolated from the adult male mouse submaxillary gland: a 25,000 mol wt species (2.5S NGF) composed of two identical polypeptide chains (Angeletti and Bradshaw, 1971), and a 140,000 mol wt/species (7S NGF) composed of three electrophoretically distinct classes of subunits, α, β, and γ. The 2.5S NGF is either identical with, or a close derivative of, the β subunit (Perez-Polo et al., 1972a). Both 7S and 2.5S NGF possess similar in vitro growth stimulating activities (Schenkein, 1972). Frazier et al. (1972) have suggested a common ancestral gene for 2.5S NGF and proinsulin on the basis of sequence similarities between the two. The three γ isozymes present in 7S NGF exhibit potent esteropeptidase activities with high specificity for the hydrolysis of α-*N*-acyl-L-arginine esters and amides (Greene et al., 1969). This activity is greatly diminished in the 7S NGF-subunit complex. No well-defined biological activity has been found for the α class of subunits.

There are many unanswered questions concerning the in vitro roles of 7S NGF vs. 2.5S NGF, or the β subunit. The fact that the esteropeptidase activity of the γ enzyme is virtually completely inactive in the 7S NGF oligomer implies that the 7S species, as isolated, is a precursor of the physiologically functional species. Since the 2.5S species is approximately as active as 7S NGF in promoting neurite outgrowth in the in vitro organ culture assay, the physiological role of 7S NGF is unclear. It has been shown, however, that the binding domains between 7S NGF subunits are highly specific, and that 7S NGF appears to be the molecular form

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[1] Abbreviations used are: NGF, nerve growth factor; BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide.