Isolation and Characterization of a Glycosphingolipid Having a New Sialic Acid*

Sen-itiroh Hakomori and Terunobu Saito†

ABSTRACT: A glycosphingolipid, having a carbohydrate structure similar to that of hematoside but with a higher migration rate on thin-layer chromatography, was isolated from equine erythrocyte membranes. The sialosyl residue of this glycolipid was not hydrolyzed by neuraminidase. The sialic acid, isolated from the acid hydrolysate, was identified as a new sialic acid, *O*-acetyl(*N*-glycolyl)neuraminic acid. After removal of *O*-acetyl group, the sialic acid of this glycolipid was hydrolyzable by neuraminidase and its migration rate on thin-layer chromatography was similar to that of known hematoside. The neuraminidase resistant property of the *O*-acetyl-(*N*-glycolyl)neuraminosyl was discussed.

During the course of investigation on immunologically active glycolipids of erythrocytes and some other mammalian cells (Hakomori and Strycharz, 1968), the presence of a hitherto uncharacterized glycosphingolipid in erythrocyte membranes was observed. Although the glycolipid has been characterized by having a chemical composition similar to "hematoside" (Yamakawa and Suzuki, 1951; Klenk and Padberg, 1962; Handa and Yamakawa, 1964; Svennerholm, 1963), nevertheless, the glycolipid differed from hematoside because the sialosyl residue was not hydrolyzed by sialidase under the conditions suitable for hydrolysis of sialosyl residues of ordinary hematoside or ganglioside.[1] Also this glycolipid had a much higher migration rate on thin-layer chromatography compared with hematosides or gangliosides. This paper describes isolation and structure of this glycolipid from equine erythrocyte membranes.

Experimental Procedure

Isolation of the Glycolipid. The membrane fraction of equine erythrocytes was prepared in hypotonic phosphate of pH 7.4 (Dodge *et al.*, 1962). The sphingolipid fraction was precipitated at −10° from the ethanol extract of membrane and treated with acetone and ether (Koscielak, 1963; Hakomori and Strycharz, 1968). The material (1 g) was dissolved in chloroform–methanol (9:1) and applied on a column of silicic acid (Bio-Sil A, Bio-Rad Co., Calif., 100–200 mesh, dimension 2 × 30 cm) eluted with each 900 ml of chloroform–methanol (9:1, 8:2, and 7:3). Aliquots of 300 ml were fractionated and examined by thin-layer chromatography. The fractions containing hematoside and the unknown glycolipid were pooled and evaporated to dryness (fraction 1, Fr. 1). Fraction 1 was subjected to rechromatography in order to separate the unknown glycolipid from the ordinary hematoside. The column of Bio-Sil A (dimensions 1 × 30 cm) was prepared in chloroform–methanol (9:1). Fraction 1 (100 mg) dissolved in 2 ml of the same solvent was applied onto the column. The column was eluted sequentionally with 100 ml each of chloroform–methanol (9:1, 8:2, 75:25, and 7:3), respectively. Fractions of 5 ml were collected, and a few drops from each fraction were applied on thin-layer plates and analyzed by thin-layer chromatography. The unknown substance was eluted with chloroform–methanol (8:2 and 75:25). Hematoside was eluted with chloroform–methanol (7:3). The unknown glycolipid (36 mg) and hematoside (42 mg) were obtained from 100 mg of fraction 1. Although the unknown glycolipid was essentially homogeneous, a small quantity of contaminants was detected on thin-layer plates. Further purification was carried out by chromatography on silica gel H thin-layer plates. An approximate 2% solution of the substance in chloroform–methanol (2:1) was applied on an activated silica gel H plate with the Radin streaker (Applied Science, Inc., State College, Pa.) and developed with chloroform–methanol–water (60:35:8). When the plate was sprayed with water, the glycolipid appeared as a white zone on a translucent background. The zone was scraped, and extracted with chloroform–methanol–water (10:10:1), evaporated, and finally precipitated from methanol at cold.

Methods of Analysis. The homogeneity of the glycolipid was examined by thin-layer chromatography on activated silica gel H plates. The following solvents were used: chloroform–methanol–water (65:30:8, lower phase, solvent 1) and chloroform–methanol–water (60:35:8, solvent 2). Spots were detected by iodine vapor, orcinol–sulfuric acid, and resorcinol–hydrochloric acid–copper sulfate (Svennerholm, 1957). Molybdenum blue spray (Zinzadze, 1935) for phosphate was used in order to detect contamination by phospholipids. Infrared spectra of the glycoplipids and of sialic acid were determined on "Irtran" (zinc sulfide) plate.

Sugar components were studied by conventional paper chromatography and by gas chromatography. Two methods were used for gas chromatography: the method of Sweeley and Walker (1964) using the F & M Model 402 with a 6-ft SE-52 glass column (3% SE-52 glass column on "Gas Chrom Q")

* From the Department of Preventive Medicine, University of Washington School of Medicine, Seattle, Washington 98105. *Received June 27, 1969.* Supported by National Cancer Institute Grant (No. CA-10909), U. S. Public Health Service, and by the American Cancer Society Grant (T-475).

† Present address: Institute of Balneology, Tohoku University, Miyagi-Ken, Japan.

[1] The term "hematoside" implies a sialosyl sphingolipid without hexosamine in contrast to "ganglioside" which contains both sialic acid and hexosamine (Yamakawa, 1966).

and the method of Björndal *et al.* (1967a) in a 6-ft glass column packed with 5% "ECNSS" on "Gas Chrom Q." Fatty acids in methanolysate were analyzed by gas chromatography after they were separated and classified into "normal" and "α-hydroxy" fraction according to the method of Kishimoto and Radin (1960).

Oligosaccharides were released from the glycolipid by osmium-catalyzed periodate oxidation followed by alkaline treatment (Hakomori, 1966) and run on paper chromatograms with methyl ethyl ketone–acetic acid–water (7:3:1) and ethyl acetate–pyridine–water (12:5:4).

The sphingosine base fraction was analyzed on thin-layer chromatography according to the method described by Sambasivarao and McCluer (1963).

Methylation Analysis. In most cases the glycolipid was methylated in dimethyl sulfoxide with methylsulfinyl carbanion base and methyl iodide (Hakomori, 1964). In some cases methylation was performed in dimethylformamide, methyl iodide, and silver oxide (Kuhn *et al.*, 1955). The permethylated glycolipid was degraded by formolysis in 90% formic acid, followed by hydrolysis with 0.25 N sulfuric acid (Björndal *et al.*, 1967b). After removal of sulfate ion by anion exchanger "AG 3 × 8," the partially methylated sugars in the hydrolysate were reduced in sodium borohydride and acetylated in acetic anhydride and pyridine. The resulting partially methylated alditol acetate was identified by gas chromatography (Björndal *et al.*, 1967a). The substances separated by F & M 402 Model gas chromatography apparatus were analyzed by mass spectrometry in the Hitachi Perkin-Elmer apparatus. The mass spectra of partially methylated alditol-acetate were compared with the data of Björndal *et al.* (1967b).

Procedure for Locating Alkali-Labile Acyl Residue. The methylation procedure described above resulted in partial or complete deacylation of base-sensitive acyl residues. Therefore, all free hydroxyl groups of the glycolipid were protected with alkali-stable acetal groups obtained by acetalization of the glycolipid with methylvinyl ether and a trace amount of *p*-toluenesulfonic acid (De Belder and Norrman, 1968). Acetalization was repeated until all the infrared absorption due to hydroxyl group disappeared. The fully acetalized glycolipid was then subjected to deacylation with sodium methoxide followed by methylation with silver oxide and methyl iodide (Kuhn *et al.*, 1955), or deacylative methylation (Hakomori, 1964). The resulting substance, which should have methyl groups at positions where alkali-labile acyl groups were originally located, was examined by different methods appropriate to identification of residues. HEXOSES. The product was degraded by formolysis followed by hydrolysis with 0.25 N sulfuric acid. The hydrolysate was examined by paper chromatography, and a part of the hydrolysate was reduced, acetylated, and analyzed by gas chromatography on "ECNSS" column (see above). The substances in questionable peaks was taken by "splitter" and examined by Hitachi Perkin-Elmer mass spectrometer. SIALIC ACID. The product of acetalization, followed by deacylation and Kuhn's methylation, was hydrolyzed by 0.1 N hydrochloric acid in aqueous methanol (1:1, v/v) in a sealed tube for 2 hr. The solubilized part of hydrolysate was separated by centrifugation and passed through a small column (0.5 × 7 cm) of an anion exchanger "AG 3 × 8," washed with water, and the sialic acid and carboxylic acid were eluted from the resin by 1 N ammonia. The ammonium salt obtained after evaporation was examined by paper and thin-layer chromatography. *O*-METHOXYGLYCOLIC ACID. The product was methanolyzed with 2 N methanolic hydrochloric acid for 4 hr, both *O*- and *N*-acyl group was liberated as methyl ester, which was converted into acyl hydroxamate by reaction with hydroxylamine and potassium hydroxide in methanol and examined by paper (Whatman No. 3MM, solvent, butan-2-ol–formic acid–water, 6:1:2) and by thin-layer chromatography (borate-impregnated silica gel H; solvent, chloroform–methanol–ammonia, 1:1:0.1). Hydroxamates of acetic acid, glycolic acid, and *O*-methoxyglycolic acid were run as references. *O*-Methoxyglycolyl hydroxamate was prepared by methylation of glycolic acid, followed by treatment with diazomethane and reacting with hydroxylamine and alkali in methanol. METHYL KETOSIDE METHYL ESTER OF NEURAMINIC ACID. The methanolysate of the acetalized, methylated glycolipid was analyzed by the "Procedure B" of Sweeley and Walker (1964).

Identification of Fatty Acid and Carboxylic Acid. The fatty acid or carboxylic acid methyl ester in the methanolysate was analyzed by gas chromatography. For analysis of short-chain volatile acid, the methanolysate was briefly treated with a small quantity of "AG 3" to eliminate hydrochloric acid in the methanolysate and analyzed on 6-ft column of "Porapak Q" (ethylvinylbenzene–divinylbenzene polymer; Applied Science Laboratories) (Hollis and Hayes, 1966). The *O*-acyl groups were liberated as methyl ester by treatment with sodium methoxide in methanol–chloroform. The reaction mixture was treated with Dowex 50 (H^+ form) for a very short period (a few seconds) and analyzed by gas chromatography on "Porapak Q" column.

Identification of an O-Acyl Residue. An ester-linked *O*-acyl residue was identified as hydroxamate (Inouye and Noda, 1950). The glycolipid was dissolved in chloroform–methanol (2:1), added with an equal volume of 1 N hydroxylamine in methanol and 1.1 N potassium hydroxide in methanol, heated for 2 min followed by room temperature for 1 hr. The ester *O*-acyl group of sialic acid was also detected as hydroxamate; the lyophilized sialic acid was added with a few drops of methanol and treated in the same way. The reaction mixture either from whole glycolipid or from the sialic acid was separated on washed paper (Whatman No. 3MM), irrigated with butan-2-ol–formic acid–water (6:1:2). The hydroxamate zone as indicated by guide strip was cut out and eluted by water. The eluate was run by paper chromatography with solvents, butan-2-ol–formic acid–water (6:1:2) and water-saturated 2-butanone–formic acid (10:1), and by thin-layer chromatography on borate-impregnated silica gel H (Kean, 1966) irrigated with chloroform–methanol–concentrated ammonia (1:1:0.1). Hydroxamates of the following acids were prepared for references: formic, glycolic, acetic, α-hydroxypropionic, β-hydroxypropionic, propionic, butyric, α-hydroxybutyric, β-hydroxybutyric, γ-hydroxybutyric, α-hydroxyisobutyric, α-hydroxyisovaleric, pyruvic, and acetoacetic. Further identification was made by mass spectrometry of a peak obtained on gas chromatography of the Lossen-rearranged isocyanate (Vagelos *et al.*, 1961). Separation was carried out on 5% "ECNSS" column on "Gas Chrom Q" at 100° programmed to 150°. In some experiments, the reaction mixture containing sodium methoxide was directly distilled according to the method of Ludowieg and Dorfman (1960). The methyl ester distilled was converted into hydroxamate and analyzed as above.

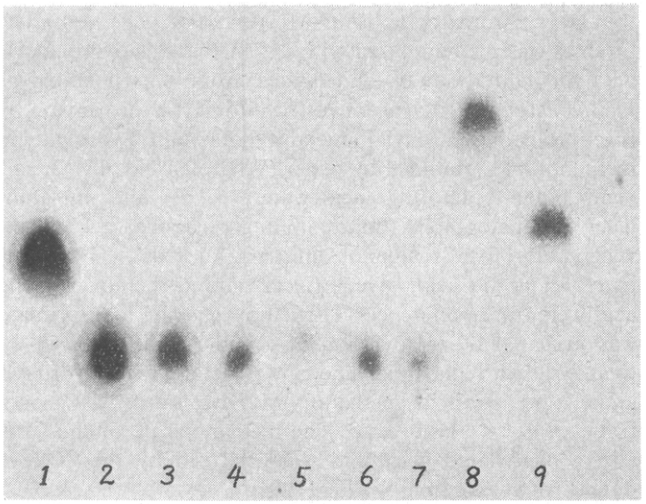

FIGURE 1: Thin-layer chromatography of hematosides on silica gel H plate, solvent chloroform–methanol–water (65:30:8, lower phase). (1) The unknown hematoside; (2) the unknown hematoside, treated with 0.1% sodium methoxide at room temperature for 30 min; (3) hematoside with *N*-glycolylneuraminic acid; (4) hematoside with *N*-glycolylneuraminic acid, treated with 0.1% sodium methoxide; (5) hematoside with *N*-acetylneuraminic acid; (8) lactosylceramide; and (9) galactosylgalactosylglucosylceramide

Procedure for Isolation of O-Acetyl-(N-glycolyl)neuraminic Acid from the Glycolipid and Examination of the Sialic Acid. Glycolipid (100 mg) was dissolved in 18 ml of distilled water, heated until completely dissolved, and 2 ml of 1 N hydrochloric acid was added. The mixture was heated at 80° over a hot plate and magnetic stirrer for 1 hr. The hydrolysate was neutralized with 0.5 N ammonium hydroxide and lyophilized. The residue was dissolved in chloroform–methanol–water (9:1:0.1), put onto a column of cellulose powder (M-N 300) and Hyflo supercel (2:1, w/w) which had been washed well with water, methanol, and chloroform–methanol (2:1), and equilibrated with chloroform–methanol–water (9:1:0.1). The column was eluted with this solvent, followed by chloroform–methanol–water of various ratios (see Figure 3). Fractions (5 ml) of the eluate were collected, and 0.2-ml aliquots were analyzed by the Warren (1959) procedure.

The substance represented by peak X (see Figure 3) was found to be a sialic acid carrying an *O*-acyl and *N*-glycolyl group.[2] The migration rate of this sialic acid on paper chromatography was compared with *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, and *N,O*-diacetylneuraminic acid of bovine submaxillary mucin (Sigma Biochemicals) in various solvents. Gas chromatography of this sialic acid was attempted using the trimethylsilyl derivative (trimethylsilation was carried out in pyridine and bistrimethylsilyltrifluoroacetamide) and the methyl ester of trimethylsilyl derivative (suggested by Dr. C. C. Sweeley).

Infrared spectra were taken as a film on "Irtran" plate in comparison with *N*-glycolylneuraminic acid. An *O*-acyl residue was detected as methyl ester and as hydroxamate according to the procedure described above.

The color yields by different color reactions were compared with *N*-glycolylneuraminic acid. The assays by Warren (1959) and by Aminoff (1959) and Svennerholm's (1957) reaction were used.

Methods for Degradation of Glycolipids. DEGRADATION WITH SODIUM METHOXIDE. A few milligrams of glycolipid dissolved in a few milliliters of chloroform–methanol was added with one-fourth volume of 0.5% sodium methoxide and left at room temperature for 1 hr. The reaction mixture was treated with Dowex 50 (H^+ form) in methanol, and the degraded glycolipid was examined by thin-layer chromatography, by infrared spectrometry, and by hydrolysis with neuraminidase. The liberated carboxylic acid methyl ester was examined by gas chromatography directly on a "Porapak" column, or converted into hydroxamate.

HYDROLYSIS WITH NEURAMINIDASE. A neuraminidase preparation from *Vibrio cholerae* ("Calbiochem") was used which had an activity of 100 units/1.0 ml when α_1-glycoprotein was used as substrate. Hydrolysis experiments were carried out with or without presence of detergent (Triton X-100) according to the condition described by Handa and Yamakawa (1964) and by Leibovitz and Gatt (1968) (see footnote of Table IV). The sialic acid liberated at different times was measured by thiobarbituric acid assay. The glycolipid, yielded upon degradation, was determined by thin-layer chromatography.

HYDROLYSIS WITH ACID. Hydrochloric acid (0.1 N) at 75°, 1 N acetic acid at 100°, and 10 N acetic acid at 100° were used as varying hydrolysis conditions with several micrograms of glycolipid in sealed tubes. The sugars liberated or the glycolipids yielded by degradation were determined by thin-layer chromatography in comparison with ordinary hematoside.

Periodate Oxidation and Determination of Periodate-Oxidized Product. Glycolipids weighing 3–5 mg were dissolved in 0.2–0.4 ml of chloroform–methanol (2:1) and to this was added 20–40 μl of 0.1 M sodium metaperiodate dissolved in 70% methanol. A 1–2-μl aliquot was injected into gas chromatography apparatus (Porapak column, 50° programmed to 150°) to detect formic acid. An aliquot of the reaction mixture was analyzed for formaldehyde by the chromotropic acid reaction (Frisell *et al.*, 1954). The rest of reaction mixture was subjected for iodometric microtitration of formic acid (Smith and Montgomery, 1955). The oxidized glycolipid was recovered after dialysis and examined for sugar components which survived by oxidation.

Results

The glycolipid was isolated in homogeneous form on thin-layer chromatography and its migration rate was about the same as that of a ceramide trihexoside (galactosylgalactosylglucosylceramide), much faster than that of any known sialosylsphingolipid, namely, hematosides and gangliosides (see Figure 1). Chemical analyses by conventional color reactions and by gas chromatography indicated that this glycolipid was composed of 1 mole each of sialic acid, galactose, glucose, and ceramide, very similar to the chemical composition of hematoside. The fatty acids with the carbon chain length longer than C-20 were the majority as detected by gas chromatography. The pattern of sphingosine base which was sep-

[2] The best recorded yield was about 7 mg of sialic acid from 100 mg of hematoside which is about 28% of the theoretical; the material was amorphous at the writing time. Because of the presence of an *O*-acetyl group which tends to be hydrolyzed by even a mild hydrolysis, the yield was variable even under careful control of hydrolytic conditions.

TABLE I: Hydrolysis of Sialosyl Group of Hematosides.

	With Triton X-100[a]	Without Triton X-100[a]
Unknown hematoside	10	0
Do., treated with sodium methoxide	265	52
Hematoside with *N*-glycolylneuraminic acid	155	114
Do., treated with sodium methoxide	75	
Hematoside with *N*-acetylneuraminic acid	180	120
Do., treated with sodium methoxide	150	

[a] In millimicromoles per milligram per hour. The quantity of the liberated sialic acid in mμmoles from 1 mg of substrate (glycolipid) per hr which was catalyzed by 20 units of neuraminidase. One incubation mixture consists of 1 mg of the substrate dissolved in 0.15 ml of water, 0.15 ml of an aqueous solution containing 1.5 mg of Triton X, 0.2 ml of 0.35 M acetate buffer (pH 4.5), and 0.2 ml of the neuraminidase solution which had 100 units/ml when assayed α_1-glycoprotein as substrate. Measured the liberated sialic acid on 0.2-ml aliquots by the Warren procedure at 30 and 60 min.

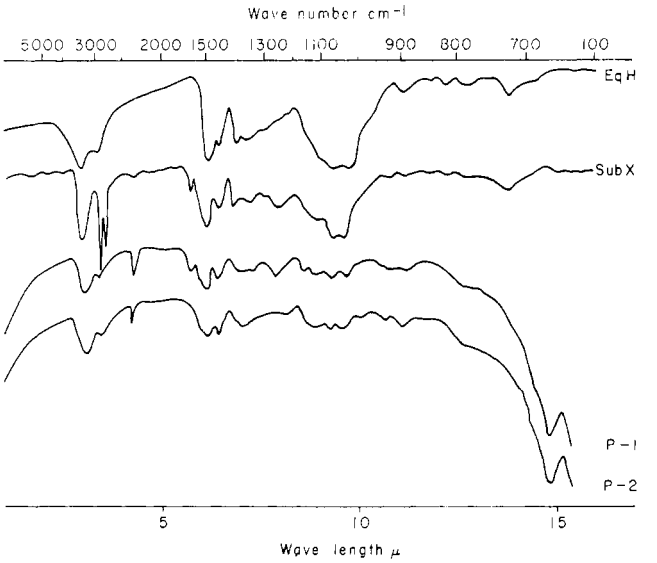

FIGURE 2: Infrared spectra of hematosides and sialic acids; Eq H: hematoside with *N*-glycolylneuraminic acid, Sub X: the unknown glycolipid, P-1: the unknown sialic acid separated from the hydrolysate of the unknown glycolipid, P-2: *N*-glycolylneuraminic acid, isolated from the hydrolysate of unknown glycolipid.

arated on thin-layer chromatography was identical with that of the ordinary sphingolipid of animal sources; five spots were demonstrated, each corresponded to dihydrosphingosine, sphingosine (two spots, each *erythro*- and *threo*-), 3-*O*-methylsphingosine, and a fast-migrating spot which could be *N*,*O*-dimethylsphingosine (the last two spots were the by-product which occurred during methanolysis). Further detail of the analysis of fatty moiety, in comparison with ordinary hematosides, will be reported elsewhere.

Permethylation of this glycolipid followed by hydrolysis, reduction, and acetylation gave, on gas chromatography, only two equal-size peaks of methylated alditol acetates which were identified as 1,3,5-trimethyl-2,4,6-triacetylgalactitol and 1,4,5-trimethyl-2,3,6-triacetylglucitol by *T* value on gas chromatography and mass spectrometry (numbering of substituents on alditol according to IUPAC nomenclature). Methylation analysis of hematoside showed the same results. These results were further supported by the fact that degradation of this glycolipid by osmium periodate and alkaline released an oligosaccharide which had identical migration rate as that of the trisaccaride obtained from horse hematoside, namely, having a structure *N*-glycolylneuraminosyl(2→3)galactosyl-(1→4)glucose.

This glycolipid differed from a known hematoside in that it had an ester linkage as evidenced by infrared absorption at 1750 cm^{-1} (ester carbonyl) which was absent in hematoside and in other sphingolipid (see Figure 2). Consequently, the glycolipid was readily converted into ordinary hematoside by treatment of this glycolipid in 0.1% sodium methoxide at room temperature (see Figure 1) resulting in the release of a carboxylic acid as was demonstrated by gas chromatography. Treatment of a hematoside under the same conditions did not liberate any appreciable quantity of carboxylic acids. The acyl residue was identified as acetyl hydroxamate on paper, thin-layer, and gas–liquid partition chromatography[3] and mass spectrometry of a peak obtained on gas chromatography.

An attempt to locate the acetyl residue on any hydroxyl group of galactose, glucose, or sphingosine failed. Deacylative methylation of the fully acetalized glycolipid followed by formolysis and hydrolysis did not give partially methylated galactose, or 3-*O*-methylsphingosine, but gave galactose, glucose, and sphingosine as in the original glycolipid. A small quantity of a material which had approximately the same R_F value as 6-*O*-methylgalactose was found in the hydrolysate; however, its mass spectrum differed from any methylated sugar. This material, although not identified, was considered to be a degradation product of sialic acid.

The most unique characteristic for this glycolipid was found, however, in its sialosyl residue; the sialosyl residue of this glycolipid was not hydrolyzed by neuraminidase under the same condition under which the sialosyl residues of hematosides were hydrolyzed (see Table I). In addition, it was found that the sialosyl residue of this glycolipid was susceptible to sialidase upon removal of an *O*-acetyl residue (see Table I), and *N*-glycolylneuraminic acid was released.

[3] Identification of hydroxamates on paper chromatography was difficult, since the following hydroxamates showed almost the same R_F values on paper chromatography with various solvents: acetyl hydroxamate, α-hydroxybutyryl hydroxamate, β-hydroxyisobutyryl hydroxamate, and other isomeric hydroxybutyryl hydroxamates had close migration rate. These hydroxamates were well separated by gas chromatography of the Lossen-rearranged isocyanate.

TABLE II: R_F Values of a New Sialic Acid As Compared with the Known Sialic Acids on Paper Chromatography.

Solvent No.[a]	*N*-Glycolyl		*N*-Acetyl		*N,O*-Diacetyl		New Sialic Acid	
	R_F	$R_{N\text{-acetyl}}$	R_F	$R_{N\text{-acetyl}}$	R_F	$R_{N\text{-acetyl}}$	R_F	$R_{N\text{-acetyl}}$
1	11	85	13	100	30	230	16	123
2	32	89	36	100	52	161	48	134
3	37	73	51	100	15[b]	358[b]	59	116
4	8	67	12	100			17	142
5	4	57	7	100	25[b]	310[b]	10	143

[a] Solvent 1, butanol–acetic acid–water (4:1:5); 2, ethyl acetate–pyridine–water (2:1:2); 3, ethyl acetate–acetic acid–water (3:1:3); 4, chloroform–methanol–water (60:35:8); 5, ethyl acetate–pyridine–water (12:5:4). Paper, Whatman No. 3MM. [b] In a separate ascending run with *N*-acetylneuraminic acid which gave R_F values of 8 and 4 by solvents 3 and 5, respectively. R_F values are described as 100×; $R_{N\text{-acetyl}}$ signifies rate of migration as compared with the migration rate of *N*-acetylneuraminic acid.

Rate of hydrolysis of sialosyl residue, even by weak acid, was found to be also more sluggish than that of usual hematoside. Hydrolysis in 0.1 N hydrochloric acid at 80° for 1 hr gave a fast-migrating sialic acid on paper as well as on column chromatography.

The fast-moving sialic acid (X in Figure 3) disappeared on prolonged hydrolysis with simultaneous appearance and increase of *N*-glycolylneuraminic acid. About the same color was yielded by Warren's or Aminoff's procedure; 1 mole each of formic acid and formaldehyde per approximately 1 mole of hematoside was yielded by periodate oxidation. The infrared absorption of this sialic acid possessed two remarkable absorptions at 1750 and 1250 cm^{-1} which were not present in either *N*-acetyl- or *N*-glycolylsialic acid (Figure 2), indicating the presence of an *O*-acetyl ester group. An acyl hydroxamate was liberated from the sialic acid which was identified as acetyl hydroxamate.

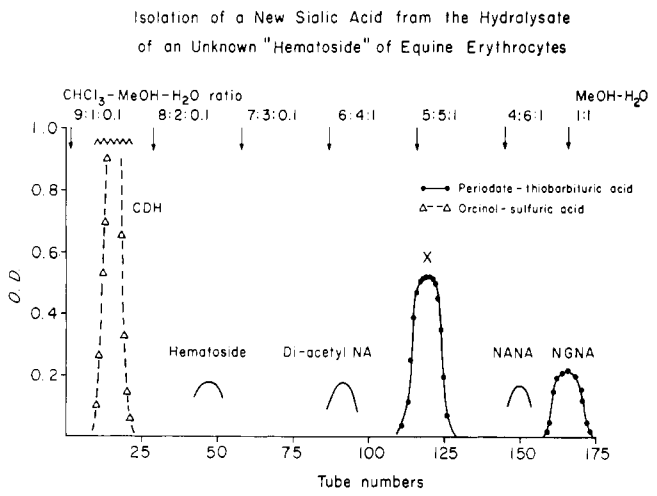

FIGURE 3: Column chromatography on cellulose Hyflo supercel (for a detailed condition, see the text). The position of hematoside, *N,O*-diacetylneuraminic acid (diacetyl-NA), and *N*-acetylneuraminic acid (NANA) on the same chromatography on a separate run was marked. The peaks found in the hydrolysate of the unknown glycolipid were ceramide dihexoside (CDH), the unknown sialic acid (X), and *N*-glycolylneuraminic acid (NGNA), and a small quantity of unhydrolyzed hematoside. The proportion of these four peaks depends upon the hydrolytic condition. On prolonged hydrolysis, the peak "X" disappears, and the peak "NGNA" increases.

R_F values of this new sialic acid were found to be faster than *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid, but slower than *N,O*-diacetylneuraminic acid (see Figure 3 and Table II).

O-Methoxyglycolylic acid (or its hydroxamate) was found in the hydrolysate (or its derivatives) of an acetalized, deacylated, and methylated product of glycolipids. Ordinary methyl ketoside methyl ester of neuraminic acid was recovered in the methanolysate of the glycolipid which had been acetalized, deacylated, and methylated.

Discussion

The results establish that the glycolipid has an identical structure to hematoside except that the sialic acid carries both an *O*-acetyl ester group and *N*-glycolylamide group, namely, *O*-acetyl-(*N*-glycolyl)neuraminosyl(2→3)galactosyl(1→4)glucosylceramide. The position of *O*-acetyl group at neuraminosyl residue has not been conclusively determined, however, the possibility of locating this ester group at the C-7, -8, or -9 hydroxyl group of neuraminic acid could be excluded by: (1) formic acid and formaldehyde were produced by oxidation with periodate and (2) periodate–thiobarbituric acid reaction of the Aminoff procedure (Aminoff, 1959) gave as much color yield as that of the Warren procedure (Warren, 1959), and 7-*O*-acetyl-(*N*-acetyl)neuraminic acid (*O*-sialic acid) was known to have failed to react by the Aminoff procedure (Aminoff, 1961).

The location of *O*-acetyl group therefore is considered to be either on the hydroxyl group of glycolic acid as the form of *N*-acetoglycolyl group (Figure 4-I) or on the C-4 hydroxyl group of neuraminic acid (Figure 4-III). To settle this question, the glycolipid was fully acetalized by methylvinyl ether in dimethyl sulfoxide and a trace amount of *p*-toluenesulfonic acid, followed by deacetylation (with sodium methoxide) and methylation (Kuhn *et al.*, 1955). The methanolysate of this product examined by gas chromatography or by paper chromatography gave only a neuraminic acid methyl ketoside methyl ester. The *O*-methoxyneuraminate methyl ketoside methyl ester was not detected, which should give a faster migrating spot on paper chromatography as compared with the methyl

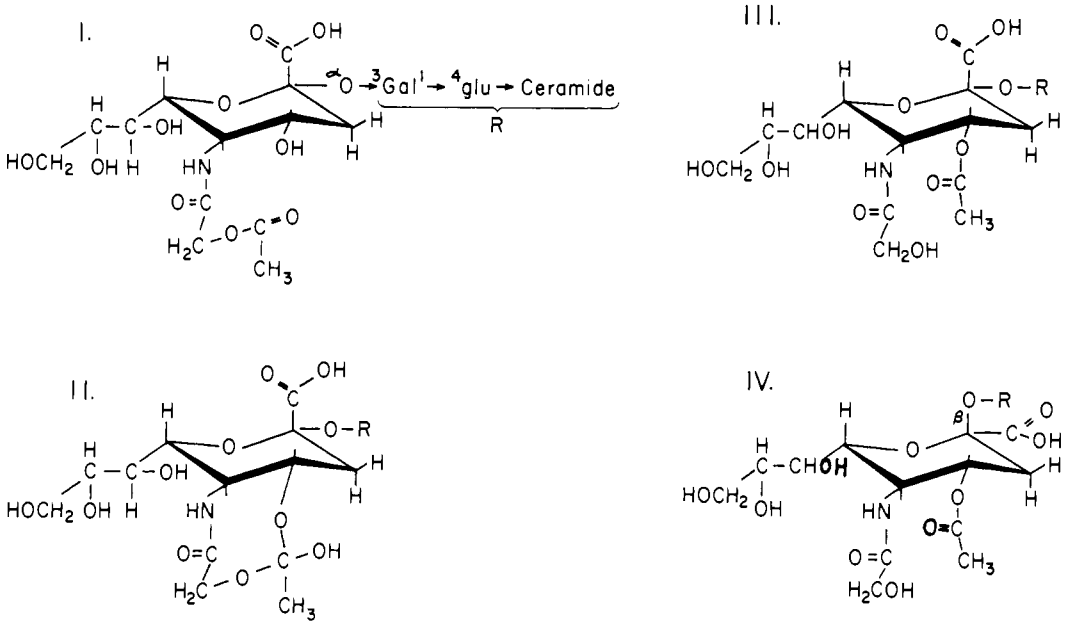

FIGURE 4: The possible structure of the unknown glycolipid. The conformation of the pyranosidic ring of the sialic acid was described as "1C" conformation according to Yu and Ledeen (1969). The acetyl group of a possible *N*-acetoglycolylamide group at the C-5 position (in "I") can migrate easily to the C-4 position (in III) through an intermediate (II) since both are equatorial.

ketoside methyl ester of neuraminic acid. On gas chromatography, analyzed as trimethylsilyl derivatives, the *O*-methoxy derivative of methyl ketoside methyl ester neuraminic acid should give a slower migrating peak than the methyl ketoside methyl ester of neuraminic acid. These experimental findings might support the possibility for the *N*-acetoglycolyl group rather than for the 4-*O*-acetyl group. However, the possibility of deacetylation or acetyl migration from C-4 to the glycolyl hydroxyl (Figure 4-I, II, III) cannot be excluded in this experiment, since the procedure of acetalization includes a long (several hours) exposure of the glycolipid in dimethyl sulfoxide and *p*-toluenesulfonic acid. The methanolysate of the fully acetalized, deacetylated, and methylated compound gave, on treatment with hydroxylamine and alkali, two hydroxamates, each corresponding to glycolyl hydroxamate and *O*-methoxyglycolyl hydroxamate. The product obtained by direct methylation gave, on methanolysis followed by treatment with hydroxylamine and alkali, three hydroxamates, each corresponding to acetyl, glycolyl, and *O*-methoxyglycolyl hydroxamates. These results again indicate the possibility for migration of acetyl group (see Figure 4-I ⇆ III) and also indicate the possibility for the presence of an *N*-acetoglycolyl group (Figure 4-I).

Unless a suitable method can be devised which permits the locating of an easily migrating acetyl group, the problem will not be solved unequivocally through acetalization by methylvinyl ether (DeBelder and Norman, 1968) is the most advanced procedure, protective to an *O*-acetyl group, that is available at the present time. The earlier findings that 4-*O*-acetyl-(*N*-acetyl)-neuraminic acid gave a positive periodate thiobarbituric acid reaction whereas 7-*O*-acetylneuraminic acid gave the negative reaction (Aminoff, 1961) is of great interest, since this recorded reactivity is the reverse of that expected from the accepted formula unless the acetyl group specifically migrates during the reaction.

The neuraminidase susceptibility of ketosides of the neuraminosyl residue has not been studied in detail until recently. Although both *N*,*O*-diacetyl- and *N*-acetyl-*O*-diacetylneuraminosyl residues have been described to be hydrolyzed by neuraminidase (see Faillard, 1959; Gottschalk, 1960; Neuberger and Marshall, 1966), a recent study by Schauer and Faillard (1968) has indicated that the 4-*O*-acetyl-(*N*-acetyl)neuraminosyl residue of equine submaxillary mucine was resistant to neuraminidase despite the fact that triacetylneuraminosyl residue (which should contain a 4-*O*-acetyl group) of bovine submaxillary mucine was hydrolyzed by neuraminidase. On the other hand, it was clearly shown that the synthetic ketoside of sialic acid with a bulky *N*-acyl group such as *N*-butyryl or *N*-benzoyl was totally resistant to neuraminidase (Meindl and Tuppy, 1966).

The β-ketosidic linkage in the C-1 conformation (Reeves, 1951), that is "axial" (see Figure 4-IV), is known to be resistant to neuraminidase (Yu and Ledeen, 1969). The increased susceptibility to neuraminidase by removal of an acetyl group cannot be explained by the β-ketosidic model. The ketoside of neuraminic acid ester was found to be resistant to neuraminidase (Karkas and Chargaff, 1964). However, a neuraminic acid ester should give a neuraminyl hydroxamate when the glycolipid was reacted with hydroxylamine. This trial, however, did not yield neuraminyl hydroxamate.

The failure of neuraminidase to hydrolyze the sialosyl residue of this glycolipid and its ability to do so following the removal of an acetyl group, therefore, are explained most feasibly by steric hindrance of the enzymic reaction by a bulky *N*-(acetoxyglycolyl)amide group at the C-5 position. Alternatively, the neuraminidase resistance could be explained by the neighboring steric effects to the ketosidic linkage at the C-4 acetoxy group if the finding of Schauer and Faillard could be extended to this case. This possibility was, however, less favored by chemical studies if migration of the C-4 acetyl to the hydroxyl

group of *N*-glycolyl occurred only a minimal extent. The sialic acid has not been crystallized, and further chemical structures and physical properties are therefore yet to be elucidated.

Two bands or spots of hematoside were often demonstrated on thin-layer chromatography similar to the two spots observed with cerebroside or in lactosylceramide. Possibly these differ in fatty acid profile and most probably each spot represents hematosides with normal and or α-hydroxy fatty acids (S. Hakomori and T. Saito, unpublished data). Hematosides having *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid gave two bands, respectively. Lipid samples which contained two hematosides, one possessing *N*-glycolylneuraminic acid and the other containing *N*-acetylneuraminic acid (like those from dog erythrocytes (Handa and Yamakawa, 1964) and mouse fibroblasts (Hakomori *et al.*, 1968)), gave four hematoside spots on borate-impregnated silicic acid plate (two were given by each hematoside). These hematoside bands were stable and did not change following treatment with alkali or ammonia. In contrast to these known hematosides, the glycolipid under discussion was characterized by having a much higher migration rate on thin-layer chromatography than those of the known hematosides, and by greater lability in alkali or ammonia. In ammonia-containing solvent, the glycolipid was partially degraded to normal hematoside, and the degree of degradation depended upon the normality of ammonia used.

It has been known that two peaks of equine hematosides were demonstrated by silicic acid chromatography ("Hematoside I and II;" Yamakawa *et al.*, 1961; Handa and Yamakawa, 1964). More recently McCluer and Siddiqui reported the presence of a fast-migrating hematoside in adrenal cortex (presented at the International Symposium on Sphingolipids, May 1969). Although the chemical nature of these fast-migrating hematosides reported by Yamakawa and McCluer was not known, they may be related to our material.

The distribution and function of this neuraminidase-resistant sialosyl glycolipid in membrane of mammalian cells compared with normal hematoside is of great interest, and studies using the immunochemical approach are underway. The presence of an *O*-acetyl group on sialosyl residue of the glycolipid may greatly change the biological function (interacting properties) of the sialosyl group of the cell membrane.

Acknowledgment

The authors wish to thank Dr. Harley Bovee and Mr. Lee Monteith of this department for their help in mass spectrometric analysis.

References

Aminoff, D. (1959), *Virology 7*, 355.
Aminoff, D. (1961), *Biochem. J. 81*, 384.
Björndal, H., Lindberg, B., and Svensson, S. (1967a), *Acta Chem. Scand. 21*, 1801.
Björndal, H., Lindberg, B., and Svensson, S. (1967b), *Carbohydrate Res. 5*, 433.
DeBelder, A. N., and Norman, B. (1968), *Carbohydrate Res. 8*, 1.
Dodge, J. T., Mitchell, C., and Hanahan, D. (1962), *Arch. Biochem. Biophys. 100*, 119.
Faillard, H. (1959), *Z. Physiol. Chem. 317*, 257.
Frisell, W. R., Meech, L. A., and Mackenzie, C. G. (1954), *J. Biol. Chem. 207*, 709.
Gottschalk, A. (1960), The Chemistry and Biology of Sialic Acids and Related Substances, Cambridge, Cambridge University, p 100.
Hakomori, S. (1964), *J. Biochem. (Tokyo) 55*, 205.
Hakomori, S. (1966), *J. Lipid Res. 7*, 789.
Hakomori, S., and Strycharz, G. D. (1968), *Biochemistry 7*, 1279.
Hakomori, S., Teather, C., and Andrews, H. (1968), *Biochem. Biophys. Res. Commun. 33*, 563.
Handa, S., and Yamakawa, T. (1964), *Japan J. Exptl. Med. 34*, 293.
Hollis, O. L., and Hayes, W. V. (1966), *J. Gas Chromatog. 4*, 235.
Inonye, Y., and Noda, M. (1950), *J. Agr. Chem. Soc. Japan 23*, 294, 368.
Karkas, J. D., and Chargaff, E. J. (1964), *J. Biol. Chem. 239*, 949.
Kean, E. L. (1966), *J. Lipid Res. 7*, 449.
Kishimoto, Y., and Radin, N. (1960), *J. Lipid Res. 1*, 72.
Klenk, E., and Padberg, G. (1962), *Z. Physiol. Chem. 327*, 249.
Koscielak, J. (1963), *Biochim. Biophys. Acta 78*, 313.
Kuhn, R., Trischmann, H., and Löw, H. (1955), *Angew. Chem. 67*, 32.
Leibovitz, Z., and Gatt, S. (1968), *Biochim. Biophys. Acta 152*, 136.
Ludowieg, J., and Dorfman, A. (1960), *Biochim. Biophys. Acta 38*, 212.
Meindl, P., and Tuppy, H. (1966), *Monatsch. Chem. 97*, 1628.
Neuberger, A., and Marshall, R. D. (1966), *in* Gottschalk, O., Glycoproteins, Amsterdam, Elsevier, p 264.
Reeves, R. E. (1951), *Advan. Carbohydrate Chem. 6*, 107.
Sambasivarao, K., and McCluer, R. H. (1963), *J. Lipid Res. 4*, 106.
Schauer, R., and Faillard, H. (1968), *Z. Physiol. Chem. 349*, 961.
Smith, F., and Montgomery, R. (1955), *Methods Biochem. Anal. 3*, 195.
Svennerholm, L. (1957), *Biochim. Biophys. Acta 24*, 604.
Svennerholm, L. (1963), *Acta Chem. Scand. 17*, 860.
Sweeley, C. C., and Walker, B. (1964), *Anal. Chem. 36*, 1461.
Vagelos, P. R., Van den Heuvel, W. J. A., and Horing, M. G. (1961), *Anal. Biochem. 2*, 50.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Yamakawa, T. (1966), *in* Lipoide, 16 Colloqium der Gesellschaft Physiologische Chemie Mosbach Baden, Schütte, E., Ed., Berlin, Springer, p 87.
Yamakawa, T., Irie, R., and Iwanaga, M. (1961), *J. Biochem. (Tokyo) 48*, 490.
Yamakawa, T., and Suzuki, S. (1951), *J. Biochem. (Tokyo) 38*, 199.
Yu, R. K., and Ledeen, R. (1969), *J. Biol. Chem. 244*, 1306.
Zinzadze, C. (1935), *Ind. Eng. Chem. 7*, 227.