Neuramin–Lactose, Neuramin–Lactose Sulfate, and Lactose Sulfate from Rat Mammary Glands. Isolation, Purification, and Permethylation Studies*

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ABSTRACT: A method for the isolation of acidic oligosaccharides from rat mammary gland extracts has been developed. This method involves fractionation of the extract on a column of Dowex 1-X8 formate which was eluted with a gradient of pyridinium formate. Six peaks were obtained containing neuramin–lactose, neuramin–lactose sulfate, lactose sulfate, *N*-acetylneuraminic acid (absent in fresh tissue), and two unidentified acidic sugars, respectively.

After purification by gel filtration on Sephadex G-15, neuramin–lactose, neuramin–lactose sulfate, and lactose sulfate were subjected to permethylation with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide solution. From the results obtained in the studies of these methylated sugars, the following structures were established: neuramin–lactose, *O*-α-D-*N*-acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose; neuramin–lactose sulfate, *O*-α-D-*N*-acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose; and lactose sulfate, *O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose.

Acidic oligosaccharides containing *N*-acetylneuraminic acid and/or sulfate monoester have been isolated from rat mammary gland extracts by ion-exchange chromatography (Carubelli *et al.*, 1961; Barra and Caputto, 1965). The strong acidity of the formic acid solutions utilized in the elution of the Dowex 1 formate columns posed serious problems during the purification stage, due to the acid lability of these compounds. The limitations of the previous methods have been eliminated from a new procedure based on the fractionation of the mammary gland extracts by ion-exchange chromatography under mild conditions, followed by purification of the isolated products by gel filtration.

Previous structural studies on the acidic oligosaccharides from rat mammary glands (Ryan *et al.*, 1965; Barra and Caputto, 1965) were hindered by the small amounts and, to some extent, by the relatively low purity of the products obtained.

The development of improved methods for the preparation of suitable amounts of these acidic oligosaccharides in a highly purified form and the availability of an efficient method of permethylation that permits working with minute amounts of material (Hakomori, 1964) made possible the structural studies that provided definitive proofs of the structures of neuramin–lactose, neuramin–lactose sulfate, and lactose sulfate.

Materials and Methods

Materials. All chemicals were reagent grade and were used as commercially obtained except in the cases specified below. Isoamyl alcohol was purified as described by Svennerholm (1957). Absolute ethanol was purified by distilling over barium oxide. Methyl iodide was redistilled at atmospheric pressure while dimethyl sulfoxide was treated with anhydrous $CaCl_2$ and then distilled *in vacuo*. Methylene chloride was purified by washing with 5% sodium carbonate followed by three washings with distilled water, dried over anhydrous $CaCl_2$, and distilled. Spectroquality CCl_4 was obtained from Matheson Coleman and Bell, East Rutherford, N. J.

Synthetic crystalline *N*-acetylneuraminic acid and bacterial neuraminidase (*Clostridium perfringens*, type V) were purchased from Sigma Chemical Co., St. Louis, Mo.

Methods. The thiobarbituric acid method of Warren (1959) was used for the estimation of free *N*-acetylneuraminic acid. The resorcinol method of Svennerholm (1957) was used for the determination of total free and combined *N*-acetylneuraminic acid. Lactose was determined by the anthrone method using a procedure previously described (Carubelli *et al.*, 1961) scaled down to a final reaction volume of 1.5 ml. The qualitative assay for sulfur was done by sodium fusion followed by detection of the resulting sulfide with nitroprusside and

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‡ Recipient of a career development award (1-K3-AM-38649) from the National Institute of Arthritis and Metabolic Diseases.

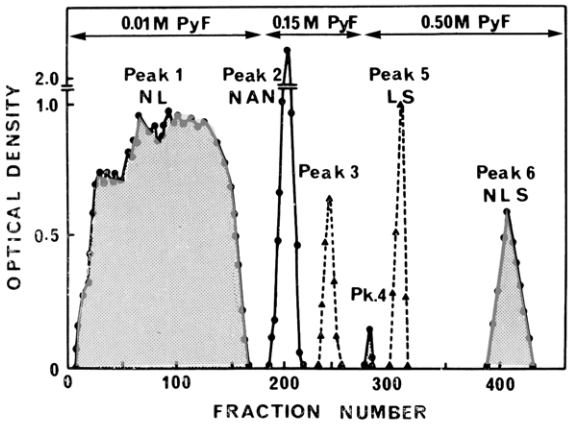

FIGURE 1: Fractionation of acidic sugars from an extract of 1 kg of rat mammary glands by ion-exchange chromatography on a column (8 × 27 cm) of Dowex 1-X8 formate. The tissue had been stored at −20° for 8–12 months prior to the extraction. (—●—●—) $OD_{580\,m\mu}$ for the resorcinol reaction (*N*-acetylneuraminic acid); (—▲—▲—) OD_{620} mμ for the anthrone reaction (hexoses). Shaded areas correspond to fractions that gave a positive reaction with both the resorcinol and the anthrone reagents. Py F, pyridinium formate; NL, neuramin–lactose; NAN, *N*-acetylneuraminic acid; LS, lactose sulfate; NLS, neuramin–lactose sulfate. Elution rate, 2.5 ml/min. Fraction volume, 100 ml.

lead acetate reagents (Cheronis, 1958). Sulfate ester content was determined spectrophotometrically by the method of Jones and Letham (1954) after mineralizing with nitric acid (Rees, 1961).

For analytical paper chromatography, approximately 0.5-μmole samples of sugars were spotted on Whatman No. 1 chromatography paper. The chromatograms were developed by descending irrigation with isopropyl alcohol–water (4:1) and the spots were detected with the aniline–diphenylamine reagent (Smith, 1958).

For analytical paper electrophoresis, approximately 0.5-μmole samples of sugars were spotted on Whatman No. 3MM chromatography paper. The electrophoretic analyses were run for 4–5 hr at 375 V in 0.1 M acetic acid–ammonium acetate buffer (pH 4.7). The instrument used was of the horizontal type with water cooling RSCO E-800-2 (Research Specialties Co., Berkeley, Calif.). The spots were detected with the benzidine spray reagent (Bacon and Edelman, 1951).

For preparative paper chromatography of methylated sugars, prewashed Whatman No. 3MM chromatography paper was used. The paper was washed successively with 0.1 N HCl, distilled water, and finally with the chromatographic solvent. The sample was applied as a band (*ca.* 1 μmole/cm) and the chromatogram was developed by ascending technique. The sugar spots were detected with *p*-anisidine hydrochloride reagent (Hough *et al.*, 1950) or with aniline–diphenylamine (Smith, 1958).

Silica gel G chromatoplates (250 μ in thickness) were used for thin-layer chromatography. Samples of 0.5–1 μg of sugar were applied and the plates were developed by ascending technique with one of the following systems: solvent 1, NH_4OH (*d* 0.9)–H_2O–methyl ethyl ketone (1:17:200) (Anderson and Rees, 1965); solvent 2, benzene–ethanol (20:5); solvent 3, NH_4OH (*d* 0.9)–H_2O–ethanol–benzene (1:15:47:200, upper phase) (Adams, 1955); solvent 4, H_2O–saturated butanol; solvent 5, ethyl acetate–ethanol–H_2O (4:4:1); and solvent 6, propanol–H_2O (85:15). Often the plates were subjected to two or more successive developments with the same solvent. The spots were detected with either *p*-anisidine hydrochloride spray reagent or by charring with H_2SO_4.

Infrared absorption spectra were recorded using a double-beam recording infrared spectrophotometer, Model 21, Perkin-Elmer Corp., Norwalk, Conn. Melting points were determined using a microscope heating stage (Melting Point Apparatus No. 4015) from the Nalge Co., Rochester, N. J. Optical rotations were measured at 589 mμ in a 0.5-dm cell with a Carl Zeiss polarimeter with circular scale reading to 0.01°.

Procedure and Results

Preparation of the Tissue Extract. Inguinal and pectoral mammary glands were obtained from lactating Sprague–Dawley albino rats sacrificed 1–6 days after delivery.

The procedure followed in the preparation of the extract was essentially as previously described (Carubelli *et al.*, 1961). In a typical experiment, 1 kg of frozen tissue was fragmented, ground, and finally extracted three times with hot distilled water. The final pooled extracts (6.67 l.) contained 4.80 mmoles of total *N*-acetylneuraminic acid.

Fractionation of the Extract. The extract was passed through a column (8 × 27 cm) of Dowex 1-X8 formate (200–400 mesh) in a cold room at 4°. After washing with distilled water, the column was eluted with three solutions of pyridinium formate (pH 4.4) of increasing concentration. The elution pattern (Figure 1) was followed by analyzing the fractions with the resorcinol reaction for *N*-acetylneuraminic acid containing compounds and with the anthrone reaction for hexoses. Fractions 9–167 (peak 1), eluted with 0.01 M pyridinium formate, gave both anthrone- and resorcinol-positive tests. The sugar present in this peak was identified as neuramin–lactose by its chemical composition and by comparison of its electrophoretic and chromatographic behavior with that of neuramin–lactose prepared according to our previous method (Carubelli *et al.*, 1961). The pool of these fractions (15.14 l.) contained 3.69 mmoles of neuramin–lactose.

Fractions 186–213 (peak 2) eluted with 0.15 M pyridinium formate gave anthrone-negative but resorcinol-positive tests. The material in this peak was identified as free *N*-acetylneuraminic acid; it gave a positive thiobarbituric acid assay, its electrophoretic mobility was identical with that of standard *N*-acetylneuraminic acid, and it gave a negative thiobarbituric acid test after treatment with sodium borohydride (Warren and Blacklow, 1962). The total volume of peak 2 was 2.94 l. and it contained 698 μmoles of *N*-acetylneuraminic acid. In fractionations of extracts of glands which had been frozen immediately after removal and extracted within 2 weeks, no free *N*-acetylneuraminic acid could be detected. Fractions 235–254 (peak 3) which were also eluted with 0.15

M pyridinium formate solution, gave anthrone-positive but resorcinol-negative tests. The sugar material in this peak migrates between neuramin–lactose sulfate and free *N*-acetylneuraminic acid on paper electrophoresis, is very unstable at room temperature, and has not been characterized yet.

Three peaks of acidic sugars were eluted by 0.50 M pyridinium formate solution. Fractions 281–284 (peak 4) gave both anthrone- and resorcinol-positive tests. The sugar material in this peak is present in minute amounts and has not been characterized yet. Fractions 302–314 (peak 5) gave anthrone-positive but resorcinol-negative tests. After lyophilization and purification by gel filtration, the sugar in this peak was identified as lactose sulfate by its chemical composition and by its electrophoretic and chromatographic mobilities which were identical with those of the lactose sulfate isolated from the hydrolysate of neuramin–lactose sulfate (Carubelli *et al.*, 1961). Fractions 389–432 (peak 6), eluted also with 0.50 M pyridinium formate, gave both anthrone- and resorcinol-positive tests. The sugar present in this peak was further characterized as neuramin–lactose sulfate by its chemical composition and by its electrophoretic and chromatographic mobilities which were identical with those of the neuramin–lactose sulfate prepared by our original procedure (Carubelli *et al.*, 1961). The pooled fractions (4.82 l.) contained 371 μmoles of neuramin–lactose sulfate. The recovery of *N*-acetylneuraminic acid and its derivatives from this column was 99.1%.

Fractionation on columns of Dowex 1 acetate with pyridinium acetate buffers (Schneir *et al.*, 1962) was also tried. This system was abandoned because lactose sulfate and neuramin–lactose sulfate were eluted together thus complicating the final purification of these compounds.

Preparation of the Potassium Salts of Neuramin–Lactose and Neuramin–Lactose Sulfate. The pyridinium salts of neuramin–lactose and neuramin–lactose sulfate, obtained by lyophilization of the column effluents, were converted into potassium salts which were much less hygroscopic and hence easier to handle. This was done by first converting the pyridinium salt into the acid form with Dowex 50W (H^+) followed by neutralization with 1 N KOH. These operations were conducted at 4°. The solution obtained was then lyophilized to yield a yellow, fluffy residue.

Purification by Organic Solvents. The purification of neuraminyl oligosaccharides can be accomplished by precipitation from methanolic solution on addition of ether, and then washing of the precipitate with ether, absolute ethanol, and anhydrous acetone. The details of this procedure have been described (Trucco and Caputto, 1954). When preparations of neuramin–lactose (potassium salt) were subjected to two consecutive treatments with organic solvents, a purity of 96% and an over-all recovery of 64% were obtained.

However, the maximum purity obtained for the potassium salt of neuramin–lactose sulfate by this method was 87.5% with only about 50% recovery after two treatments. Additional treatments failed to increase the purity or to remove the remaining ultraviolet-absorbing impurities.

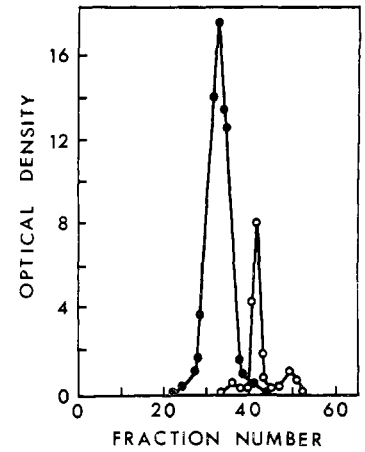

FIGURE 2: Chromatography of 354 μmoles of neuramin–lactose sulfate (potassium salt) on a column (2 × 175 cm) of Sephadex G-15. Elution with distilled water. Flow rate, 42 ml/hr. (—●—●—) $OD_{380\,m\mu}$ for the resorcinol reaction. (—○—○—) $OD_{260\,m\mu}$. Fraction volume, 7 ml.

Purification by Gel Filtration. Columns (2 × 175 cm) of Sephadex G-15 (20–80 mesh) were utilized. About 300 mg of material was dissolved in 2 ml of distilled water and applied to the column which was then eluted with distilled water. A typical elution pattern (Figure 2) shows the peak of neuramin–lactose sulfate eluted ahead of the ultraviolet-absorbing impurities. Chromatography on Sephadex G-10 and G-25 did not achieve a good separation between the oligosaccharides and the ultraviolet-absorbing impurities.

Sephadex G-15 chromatography was particularly useful in the purification of neuramin–lactose sulfate because in addition to the high purity achieved the quantitative recoveries obtained are essential in view of the minute amounts of this compound present in mammary tissue. As we saw above, fractionation of the extract prepared from 1 kg of rat mammary glands yielded 371 μmoles of neuramin–lactose sulfate. After conversion into the potassium salt and two successive chromatographies on Sephadex G-15, 340 μmoles of neuramin–lactose sulfate was recovered as a white powder (276 mg). The final purity of this preparation of neuramin–lactose sulfate was 97.3% and the over-all recovery during the whole purification was 92%. Analysis of neuramin–lactose sulfate showed equimolar amounts of *N*-acetylneuraminic acid and lactose, the average value for the molar ratio from three preparations was 1.03, and the sulfate analysis showed 1.06 moles/mole of neuramin–lactose sulfate. The specific rotation was $[\alpha]_D^{24}$ +23.8° (*c* 1.54, H_2O).

Structural Studies. The structures of neuramin–lactose, neuramin–lactose sulfate, and lactose sulfate were established by permethylation studies. The procedure followed for the preparation of permethylated oligosaccharides was essentially that described by Hakomori (1964) wherein the methylsulfinyl carbanion was used to generate the oligosaccharide alkoxide prior to addition of methyl iodide.

Permethylation of Neuramin–Lactose Sulfate. Sodium hydride (3.57 mmoles) was washed three times by stir-

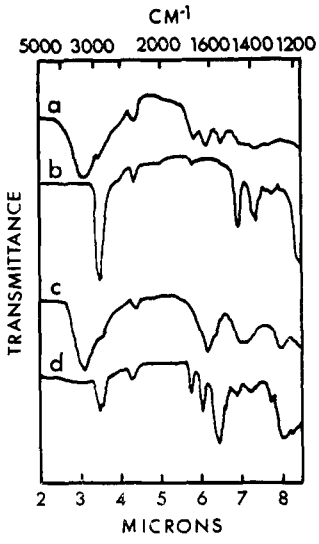

FIGURE 3: Infrared spectra of neuramin–lactose and neuramin–lactose sulfate before and after permethylation. (a) Neuramin–lactose in KBr pellet; (b) permethylated neuramin–lactose, CCl_4 solution; (c) neuramin–lactose sulfate in KBr pellet; (d) permethylated neuramin–lactose sulfate, CCl_4 solution.

ring with 8-ml portions of hexane in order to remove the mineral oil. The residual hexane was removed by flushing gently with a stream of nitrogen. After addition of 5 ml of dry dimethyl sulfoxide, the flask was fitted with a condenser and the contents were stirred at 65° until the solution had a clear green color and evolution of hydrogen had ceased (45 min); the solution was then cooled to room temperature. Special precautions must be taken because of the danger of explosions, especially when working with large amounts of methylsulfinyl carbanion (French, 1966; Olson, 1966).

A solution containing 100.6 mg (0.13 mmole) of neuramin–lactose sulfate (potassium salt) dissolved in 1 ml of dry dimethyl sulfoxide was added dropwise over a period of 1 min to the methylsulfinyl carbanion. After stirring at room temperature for 30 min, the reaction mixture was homogeneous and orange in color. The reaction mixture was left for 8 hr at room temperature with constant stirring and passing a stream of nitrogen.

For the methylation reaction, the oligosaccharide alkoxide solution was cooled to 20° in a cold water bath and 2 ml of methyl iodide (freshly distilled) was added to the stirred solution at a rate such that the temperature did not rise above 25° (5 min). The solution was stirred under nitrogen for 14 hr at room temperature, after which 10 ml of distilled water was added to destroy the excess methylsulfinyl carbanion. The yield of the permethylated neuramin–lactose sulfate after purification by gel filtration (see below) was 92.8%.

Permethylation of Neuraminidase Hydrolysate of Neuramin–Lactose Sulfate. A solution containing 9 mg of neuramin–lactose sulfate in 2 ml of distilled water was acidified to pH 5.2 with dilute HCl and 0.25 mg of bacterial neuraminidase was added. After a 24-hr incubation at 37° in presence of toluene, the tube was placed in a boiling-water bath for 5 min and the insoluble material was removed by centrifugation. Chromatographic and electrophoretic analyses of the hydrolysate showed that the hydrolysis was quantitative. The lyophilized hydrolysate (a mixture containing *ca.* 0.01 mmole each of *N*-acetylneuraminic acid and lactose sulfate) was methylated using 0.18 mmole of the carbanion in 2 ml of dimethyl sulfoxide solution and 2 ml of methyl iodide. The procedure was the same as described for neuramin–lactose sulfate.

Permethylation of Lactose Sulfate. Lactose sulfate (10.6 mg) was permethylated by the same procedure described for the neuraminidase hydrolysate of neuramin–lactose sulfate.

Permethylation of Neuramin–Lactose. A solution containing 1 g (1.49 mmoles) of neuramin–lactose (potassium salt) in 2 ml of dimethyl sulfoxide was added dropwise to a solution containing 31.2 mmoles of methylsulfinyl carbanion in 50 ml of dimethyl sulfoxide and the mixture was allowed to react at room temperature for 40 min. An excess of dry methyl iodide (12 ml) was added dropwise to the alkoxide solution, maintaining the reaction mixture at room temperature as described for neuramin–lactose sulfate.

Analysis of each permethylated oligosaccharide by thin-layer chromatography with solvents 1 and 4 showed only two closely migrating spots which correspond to the α- and β-methyl glycosides; no spot of the original sugar (which remains at the origin) was observed. The complete permethylation was confirmed by infrared spectral analyses of the final purified products in spectroquality methylene chloride and CCl_4. No appreciable absorption in the OH band region (3200–3700 cm^{-1}) was observed (Figure 3).

Purification of Permethylated Sugars. The permethylated neuramin–lactose was isolated from the reaction mixture by chloroform extraction (Kuhn and Brossmer, 1959). The washed chloroform extract was treated with anhydrous Na_2SO_4 and concentrated in a rotary evaporator to yield a yellow syrup. The residue was dried in an evacuated desiccator in the presence of phosphorus pentoxide to give a slightly yellow semicrystalline product. The yield was 92.5%.

The chloroform extraction method cannot be used for the isolation of permethylated sulfate-containing sugars due to their poor solubility in chloroform. These methylated sugars were isolated in a very pure form by gel filtration through a column (1.3 × 175 cm) of Sephadex G-15 which was eluted with distilled water. The methylated sugars were detected by the anthrone reaction. This gel filtration method was used for the purification of all the methylated oligosaccharides prepared with excellent results.

Hydrolysis of Permethylated Oligosaccharides. Dilute solutions (1–4%) of permethylated sugar in 0.5 N H_2SO_4 were refluxed for various lengths of time. The hydrolysates were cooled to room temperature, neutralized with saturated barium hydroxide, and decolorized with 0.1 g of activated charcoal (Nuchar C-190-N). After filtration through Whatman No. 5 filter paper, the barium sulfate precipitate and charcoal on the filter paper were washed four times with 5-ml portions of hot distilled water. The filtrate was evaporated in a rotary evaporator at 30°. Analyses of the hydrolysates by thin-layer chro-

TABLE I: Identification of Methylated Sugars by Thin-Layer Chromatography.[a]

Compound	$R_{2,3,4,6\text{-Tetra-}O\text{-methyl-D-glucose}}$		
	Solvent 1	Solvent 2	Solvent 3
Standard sugars			
2,4-Di-*O*-methyl-D-galactose	0.23	0.31	0.08
2,3,4-Tri-*O*-methyl-D-galactose[b]	0.50	0.56	0.27
2,4,6-Tri-*O*-methyl-D-galactose[b]	0.56	0.64	0.31
2,3,6-Tri-*O*-methyl-D-glucose	0.76	0.70	0.41
Hydrolysates of permethylated oligosaccharides			
Neuramin–lactose	0.56, 0.76	0.64, 0.70	0.31, 0.41
Neuramin-lactose sulfate	0.23, 0.76	0.31, 0.70	0.08, 0.41
Neuraminidase hydrolysate of neuramin–lactose sulfate	0.49, 0.76	0.57, 0.70	0.28, 0.41
Lactose sulfate	0.49, 0.76	0.57, 0.70	0.28, 0.41

[a] The composition of the solvents is described under Methods. [b] Mixtures of standard 2,3,4-tri-*O*-methyl-D-galactose and 2,4,6-tri-*O*-methyl-D-galactose were separated as two distinct spots by the three systems used.

matography with solvents 1, 2, and 3 showed that the methylated neuramin–lactose was completely hydrolyzed in 4 hr while the methylated sulfate-containing oligosaccharides were completely hydrolyzed in 7 hr. As shown in Table I, hydrolysis of permethylated neuramin–lactose yielded only 2,4,6-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. The hydrolysate of permethylated neuramin–lactose sulfate contained 2,4-di-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. The hydrolysates of both permethylated lactose sulfate and permethylated neuraminidase hydrolysate of neuramin–lactose sulfate contained only 2,3,4-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose.

Isolation of Methylated Monosaccharides. The hydrolysate of permethylated neuramin–lactose (194.9 mg) was fractionated by preparative paper chromatography using solvent 1. The sugars were eluted with distilled water and the light yellow eluates were decolorized with activated charcoal and lyophilized. Equimolar amounts of the white powdery products (45.8 mg of 2,3,6-tri-*O*-methyl-D-glucose (84.7% yield) and 45.7 mg of 2,4,6-tri-*O*-methyl-D-galactose (84.6% yield)) were obtained.

The hydrolysate of permethylated neuramin–lactose sulfate (45.1 mg) was fractionated by the same procedure described above. Upon lyophilization, 9.0 mg of 2,3,6-tri-*O*-methyl-D-glucose (80.2% yield) and 9.0 mg of 2,4-di-*O*-methyl-D-galactose (85.5% yield) were obtained. The final products were light yellow and the yields obtained represent a molar ratio of 1.00 to 1.07.

Crystallization of Methylated Monosaccharides. 2,4-DI-*O*-METHYL-D-GALACTOSE FROM PERMETHYLATED NEURAMIN–LACTOSE SULFATE. Approximately 2 mg of the methylated sugar was dissolved in 0.3 ml of hot ethyl acetate. The white needles obtained upon cooling were washed with 2 drops of a cold mixture of ethyl acetate and petroleum ether (bp 38.4–52.3°) and dried *in vacuo* yielding approximately 0.1 mg of crystals. The melting point range listed in Table II, agrees closely with values published by Baldwin and Bell (1938) and by Andrews *et al.* (1954).

2,3,6-TRI-*O*-METHYL-D-GLUCOSE FROM PERMETHYLATED NEURAMIN–LACTOSE SULFATE. Approximately 2 mg of the methylated sugar was dissolved in 0.1 ml of hot ethyl acetate. The crystals obtained upon cooling were dried over phosphorus pentoxide *in vacuo*, yielding about 0.2 mg of white needles. The melting point range and mixture melting point range (Table II) agree with each other and with the values published by Kuhn and Brossmer (1959).

2,3,6-TRI-*O*-METHYL-D-GLUCOSE FROM PERMETHYLATED NEURAMIN–LACTOSE. Approximately 10 mg of the methylated sugar was crystallized as described above. The melting point determinations (Table II) gave values identical with those found for the corresponding sugar isolated from permethylated neuramin–lactose sulfate.

2,4,6-TRI-*O*-METHYL-D-GALACTOSE FROM PERMETHYLATED NEURAMIN–LACTOSE. Approximately 3 mg of the methylated sugar was dissolved in 0.1 ml of boiling ethyl acetate and 0.05 ml of petroleum ether was added to the solution. The white elongated hexagonal plates were washed with a cold mixture of ethyl acetate and petroleum ether, and dried over phosphorus pentoxide *in vacuo*, yielding approximately 0.5 mg of crystals. The melting point range (Table II) agrees exactly with the mixture melting point range and with the values found by Hirano *et al.* (1961).

Specific Rotation of Methylated Monosaccharides. The experimental conditions under which the measurements were taken and the values obtained for the specific rotation of the various methylated sugars are shown in Table II. The value for 2,4-di-*O*-methyl-D-galactose is in close agreement with the values reported by Smith (1939) and by Baldwin and Bell (1938). The equilibrium values for 2,3,6-tri-*O*-methyl-D-glucose from permethyl-

TABLE II: Characterization of Methylated Sugars Isolated from the Hydrolysates of Permethylated Neuramin–Lactose and Neuramin–Lactose Sulfate.

Compound	Melting Point (°C)		$[\alpha]_D^{24}$ in H_2O (deg)		Mp of Anilide Derivatives (°C)	
	Found[a,b]	Lit.[e]	Found	Lit.[e]	Found[a,b]	Lit.[e]
2,4-Di-*O*-methyl-D-galactose[c]	101–103	100–103	+84.4 (*c* 0.224)	+85.6	217–218 (217–218)	216
2,3,6-Tri-*O*-methyl-D-glucose[c]	113–115 (113–115)	113–115	+69.7 (*c* 0.224)	+68.9	Liquid	
2,3,6-Tri-*O*-methyl-D-glucose[d]	114–116 (113–116)	113–115	+68.4 (*c* 0.805)	+68.9	Liquid	
2,4,6-Tri-*O*-methyl-D-galactose[d]	88–91 (88–91)	89–91	+91° (*c* 0.694)	+91	174–175°	175

[a] Uncorrected values. [b] Mixture melting points are shown in parentheses. [c] From permethylated neuramin–lactose sulfate. [d] From permethylated neuramin–lactose. [e] The references from which these values were obtained are mentioned in the text.

ated neuramin–lactose and neuramin–lactose sulfate are in close agreement among themselves and with the the value reported by Kuhn and Brossmer (1959). The equilibrium value obtained for 2,4,6-tri-*O*-methyl-D-galactose agrees with the value reported by Hirano *et al.* (1961).

Anilide Derivatives of the Methylated Monosaccharides. The preparation of the anilide derivatives was done using a method based on the techniques described by Hirano *et al.* (1961) and by Brown *et al.* (1949).

2,4-Di-*O*-methyl-*N*-phenyl-D-galactosylamine. Approximately 0.8 mg of 2,4-di-*O*-methyl-D-galactose from permethylated neuramin–lactose sulfate was refluxed with 0.01 ml of freshly redistilled aniline in 0.3 ml of absolute ethanol for 30 min. The solution was then concentrated to about 0.15 ml and refrigerated overnight. The white needles were washed once with cold absolute ethanol and dried *in vacuo*, yielding approximately 1 mg of crystals. The melting point range (Table II) agrees exactly with the mixture melting point range and closely with the values published by Baldwin and Bell (1938), White (1942), and Wolfrom *et al.* (1952).

2,4,6-Tri-*O*-methyl-*N*-phenyl-D-galactosylamine. Approximately 1.5 mg of 2,4,6-tri-*O*-methyl-D-galactose from permethylated neuramin–lactose was converted into the crystalline anilide derivative as described above. This material was then recrystallized from ethyl acetate–petroleum ether (bp 38.4–59.3°) and dried *in vacuo;* the yield being approximately 1.3 mg. The melting point (Table II) agrees closely with the value reported by Jones (1949).

2,3,6-Tri-*O*-methyl-*N*-phenyl-D-glucosylamine. The 2,3,6-tri-*O*-methyl-D-glucose, obtained from the acid hydrolysates of both permethylated neuramin–lactose and permethylated neuramin–lactose sulfate, were subjected to the same treatments described above, but no crystalline derivative was obtained.

Demethylation of Methylated Monosaccharides. The methylated monosaccharides were dried thoroughly, dissolved in anhydrous dichloromethane, and treated with cold boron tribromide as described by Bonner *et al.* (1960). Precautions must be taken during the addition of the boron tribromide because of its explosive reactivity in moist air. After removal of the solvent and reagent the final products were examined by thin-layer chromatography with solvents 4, 5, and 6. Only free glucose or free galactose were obtained from the respective methylated derivatives.

Discussion

Two neuraminyl trisaccharides, neuramin–lactose and neuramin–lactose sulfate, have been found in rat mammary gland extracts (Carubelli *et al.*, 1961). Fractionation on columns of Dowex 1 formate eluted with acidic formate solutions gave good separations and satisfactory results for the isolation of pure neuramin–lactose. Elution of neuramin–lactose sulfate, however, required a solution of strong acidity (0.2 M ammonium formate in 4 M formic acid) and the subsequent removal of this reagent was cumbersome and hazardous (ether extraction); in addition, the yield and final purity were relatively low.

In the work reported here, the Dowex 1 formate columns were eluted using dilute solutions of pyridinium formate. These solutions are only mildly acidic and, furthermore, most of the pyridinium formate can be removed by lyophilization of the column effluents. The elution pattern (Figure 1) shows well-defined peaks containing a single sugar each.

On the average, the mammary glands utilized contained 2.12 mg of neuramin–lactose and 0.23 mg of neuramin–lactose sulfate per g of wet tissue.

Purification of neuramin–lactose and neuramin–lactose sulfate by gel filtration on columns of the Sephadex G-15 gave almost quantitative recoveries of essentially 100% pure compounds.

A *N*-acetylneuraminic acid free sulfur-containing oli-

gosaccharide isolated in the course of these studies was found to be lactose sulfate. The presence of this sugar in rat mammary gland extracts was recently reported by Barra and Caputto (1965).

Structural studies by permethylation of neuramin–lactose, neuramin–lactose sulfate, and lactose sulfate were then undertaken. In the case of neuramin–lactose sulfate the composition, properties, and some knowledge of the structure of this compound were available from our previous work. On the basis of chemical and enzymic studies (Carubelli *et al.*, 1961; Ryan *et al.*, 1965), the structure *O*-α-D-*N*-acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose was postulated. The assignment of the sulfate monoester to the position 6 of the galactosyl moiety was based on the isolation of D-galactose 6-*O*-sulfate from the hydrolysate of neuramin–lactose sulfate; however, due to the possibility of ester migration during the acid hydrolysis it was recognized that final proof for this structure would require permethylation studies (Ryan *et al.*, 1965). The main difficulties anticipated in these studies were the very small amounts of neuramin–lactose sulfate available and the recognized fact that the presence of of the ionic group makes the methylation of sugar sulfates a difficult process (Turvey, 1965). The method of methylation developed by Hakomori (1964) which is very efficient and can be used in a small scale proved to be a very valuable tool for our studies.

If the proposed structure of neuramin–lactose sulfate (Figure 4) were correct, treatment of permethylated neuramin–lactose sulfate with 0.5 N H_2SO_4 at 100° should cause the hydrolysis of all glycosidic, ketosidic, and sulfate monoester linkages as well as the subsequent degradation of the liberated *N*-acetylneuraminic acid moiety, and the final hydrolysate should contain 2,3,6-tri-*O*-methyl-D-glucose and 2,4-di-*O*-methyl-D-galactose.

The identification of 2,4-di-*O*-methyl-D-galactose in the hydrolysate of fully methylated neuramin–lactose sulfate points out that the hydroxyl groups at C-3 and C-6 of the galactosyl moiety of neuramin–lactose sulfate were substituted. Since it had been previously established that the glucose moiety of neuramin–lactose sulfate occupies the reducing terminal position (Carubelli *et al.*, 1961; Ryan *et al.*, 1965), the C-3 and C-6 of galactose are blocked by *N*-acetylneuraminic acid and by the sulfate monoester. This leads to two possible structures: the sulfate group could be linked to the position 6 and the *N*-acetylneuraminic acid to the position 3 of galactosyl moiety or *vice versa*. Distinction between these two alternatives can be accomplished by removing either one of the two groups involved, and repeating the permethylation studies. Owing to the extreme lability of the ketosidic linkage of *N*-acetylneuraminic acid (Svennerholm, 1958), selective removal of the sulfate monoester by acid hydrolysis is impossible and the enzymic removal of the sulfate group of neuramin–lactose sulfate has not been investigated yet because of difficulties encountered in trying to secure a glycosulfatase for these studies. The removal of *N*-acetylneuraminic acid, on the other hand, could be easily done by treatment with neuraminidase. The enzymic hydrolysate of neuramin–lactose sulfate was permethylated and

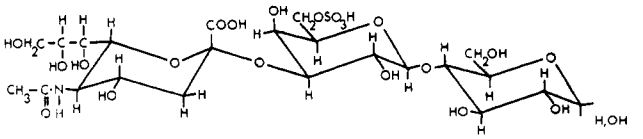

FIGURE 4: The structure of neuramin–lactose sulfate. *O*-α-D-*N*-Acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose.

subjected to acid hydrolysis. Identification of 2,3,4 tri-*O*-methyl-D-galactose in the hydrolysate proved that the position 6 is occupied by the sulfate monoester group, while the position 3 of the galactosyl moiety of neuramin–lactose sulfate is the point of attachment of *N*-acetylneuraminic acid, which, due to its susceptibility to neuraminidase, can be assigned the α-anomeric configuration (Gottschalk, 1958). Identification of 2,3,6-tri-*O*-methyl-D-glucose in the hydrolysate of permethylated neuramin–lactose sulfate together with the conclusions reached above clearly indicate that the hydroxyl group at position 4 of the glucose moiety is the point of attachment of the galactosyl moiety of neuramin–lactose sulfate. The presence of a lactose type of linkage (β 1→4) between galactose and glucose is further supported by the actual isolation of small amounts of lactose from the products of partial hydrolysis of neuramin–lactose sulfate (Carubelli, *et al.*, 1961; Ryan *et al.*, 1965). The experimental findings described here proved that the structure of neuramin–lactose sulfate corresponds to *O*-α-D-*N*-acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose (Figure 4). Agreement of these results with our previous findings (Ryan *et al.*, 1965) points out that the sulfate monoester of D-galactose 6-*O*-sulfate does not migrate during the hydrolysis of neuramin–lactose sulfate, thus adding support to the suggestion of Turvey (1965) that unlike certain sugar phosphates, ester migration does not occur in sugar sulfates under acid conditions.

Similar studies were also conducted on neuramin–lactose. Chromatographic analyses of the products of acid hydrolysis of permethylated neuramin–lactose showed only two methylated monoses. Equimolar amounts of these two sugars were obtained in high yields and they were identified as 2,4,6-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. Since neuramin–lactose is split by neuraminidase and the products of hydrolysis were identified as *N*-acetylneuraminic acid and lactose (Gottschalk, 1957; Carubelli *et al.*, 1962), the results of the permethylation studies described here indicate that the structure of the neuramin–lactose isolated from rat mammary glands is *O*-α-D-*N*-acetylneuraminyl-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose, which is identical with the neuramin–lactose from cow colostrum described by Kuhn and Brossmer (1959).

Permethylation of lactose sulfate followed by acid hydrolysis produced 2,3,4-tri-*O*-methyl-D-galactose and 2,3,6-tri-*O*-methyl-D-glucose. Since lactose has been identified among the products of partial acid hydrolysis of lactose sulfate (Barra and Caputto, 1965), the permethylation studies indicate that the structure of lac-

tose sulfate is *O*-β-D-galactopyranosyl 6-*O*-sulfate-(1→4)-D-glucopyranose. These results confirm the structure proposed by Barra and Caputto (1965) and establish the identity between free lactose sulfate and the lactose sulfate moiety of neuramin–lactose sulfate.

Acknowledgments

The authors are indebted to Drs. Karl Meyer and V. P. Bhavanandan of Yeshiva University for their generous gift of standard methylated sugars, and to Dr. Paul B. McCay for helpful suggestions during the preparation of this manuscript.

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