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STUDIES ON NEURAMINYL-OLIGOSACCHARIDES

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To my

Wife

and

Little one

Susan

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CHAPTER I

INTRODUCTION

Neuraminic acid is a 9 carbon amino sugar acid having the structure 5-amino-3,5-di-deoxy-D-glycero-D-galacto-nonulonic acid.

This acid is found in nature as its acylated derivatives (N-acetylated or N-glycolylated, or N-acetylated-O-acetylated). The type and number of acyl groups vary with animal species, and often within one species depending on the tissue or biological fluid under consideration. Sialic acid is the group name adopted by convention to designate all acylated neuraminic acid derivatives (1).

The sialic acids are found widely distributed in nature, generally, as a constituent of an oligosaccharide or polysaccharide which in turn forms the prosthetic group of a glycoprotein. There are a few exceptions such as the milk neuraminyl-oligosaccharides which are found in the free form, and colominic acid which is a homopolysaccharide of bacterial origin composed exclusively of N-acetylneuraminic acid. The wide distribution of sialo-glycoproteins in animal secretions and excretions would indicate a protective rather than a structural function, while in bacteria its main function is believed to be structural (cell walls, etc.).

In all sialic acid-containing compounds so far investigated, sialic acid invariably occupies a non-reducing terminal position. So far, only galactose, galactose sulfate, N-acetylgalactosamine and N-acetylneuraminic acid itself have been found as natural partners in the ketosidic linkage.

In 1936, Blix discovered that upon heating aqueous solutions of glycoproteins from bovine submaxillary gland, an acidic compound was liberated (2). The acid, isolated in crystalline form, had reducing power, contained two acetyl groups, one of them being an N-acetyl group, and on titration reacted as a monocarboxylic acid. Elementary analysis suggested $C_{14}H_{25}O_{11}N$ as its most probably formula. Since it was isolated from salivary materials Blix called this compound "Sialic Acid".

In the course of studies on the chemical composition of glycolipids of human brain, Klenk in 1941 isolated a crystalline acidic compound by treating gangliosides with 5% methanolic hydrogen chloride. The substance had the most probable formula, $C_{11}H_{21}O_9N$, and contained a carboxyl group, a free amino group and did not have reducing power (3). Since it was isolated from nervous tissue Klenk called this compound "Neuraminic Acid". It was later recognized that this compound contained a methoxyl group introduced during methanolysis of the glycolipid and in the modern nomenclature this compound is called methoxyneuraminic acid (4).

The demonstration of the key role played by the sialic acid components of a number of glycoproteins in their relationship to the influenza virus particle and probably to the mechanism of influenza virus infection had a great impact on sialic acid chemistry (5-11).

N-Acetylneuraminic acid (NANA), the most abundant natural form of sialic acid, was first isolated from animal sources by Klenk and Faillard in 1954 (12).

The basic structure of NANA as an aldol condensate between pyruvic acid and N-acetylhexosamine was first proposed by Gottschalk (13). Proof of the correctness of the structure was first provided by the fragmentation of NANA to N-acetylglucosamine and pyruvic acid by heating in the presence of pyridine and nickelous acetate (14); an aldolase from Vibrio cholerae was found to catalyze a similar fragmentation (15). The chemical synthesis of N-acetylneuraminic acid from N-acetylglucosamine and oxaloacetate at alkaline pH was achieved later (16). The actual hexosamine component of NANA, however, proved to be N-acetyl-D-mannosamine. N-Acetylglucosamine was found to be an artifact produced by the epimerization of N-acetylmannosamine at high pH values. The finding that N-acetylmannosamine is part of the molecule of NANA helped to establish its steric configurations at C-5, C-6, C-7 and C-8. This was based on the observation that an enzyme, aldolase from Clostridium perfringens, catalyzes the reaction:

N-acetylneuraminic acid $\rightleftharpoons$ N-acetyl-D-mannosamine + pyruvate

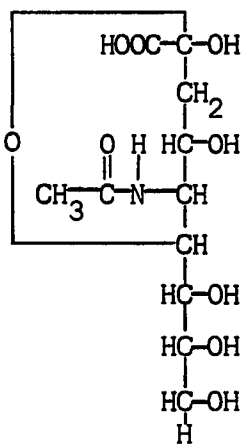
The enzyme utilizes N-acetylmannosamine but not N-acetylglucosamine nor N-acetylgalactosamine (17, 18). The steric configuration at C-4 was elucidated by Kuhn and Brossmer (19) as having the D-configuration (it is directed to the right in a Fischer projection formula). They converted NANA to R(-)-pentane-1,4,5-triol, the absolute configuration of which was ascertained by the preparation of the latter substance, with identical optical properties, from L-glutamic acid and also from D-glyceraldehyde,

two compounds of known absolute configuration. It should be noted that earlier Kuhn and Brossmer (20) suggested that the hydroxyl at C-4 had the L-configuration. This assignment was made on the basis of Hudson's γ-lactone rule after they found that the diethylmercaptal of N-acetyl-neuraminolactone and the corresponding dithiolactone were strongly levo-rotatory. It is now evident that neuraminolactone is another exception to the Hudson's lactone rule. The absolute configuration at the anomeric atom (C-2) of NANA is not known. The structure is shown in Figure 1.

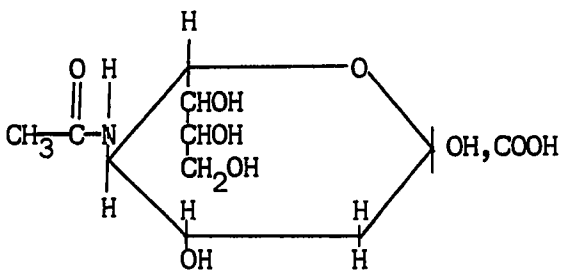
The preferred conformation of the pyranose form of NANA is believed to be the 1C conformation of the chair form as shown in Figure 2. This is based on molecular models which indicate that the number of large axial substituents is at a minimum in the 1C conformation, but the crystalline form of D-sialic acid is yet to be subjected to conformational analyses such as x-ray methods to prove this point.

N-Acetylneuraminic acid (NANA), being a deoxysugar, is very sensitive even to dilute mineral acids and weak alkali, and has a very fast tautomeric mobility. 2-Deoxysugars have a much greater tendency to change from the cyclic forms to the open-chain form than have ordinary sugars; and this change causes fast mutarotation and initiates sugar degradation by acid and alkali (22).

In dilute mineral acid, NANA is decomposed quickly to a dark humin-material with the liberation of about 25% of its nitrogen as ammonia. A mechanism for this degradation involving enolization, dehydration, rearrangement of the double bond, hydrolytic release of acetamide, cyclization, formation of furan derivatives and polymerization has been proposed (23).



a. Fischer projection form.



b. Haworth form.

Figure 1. Structure of N-acetylneuraminic acid.

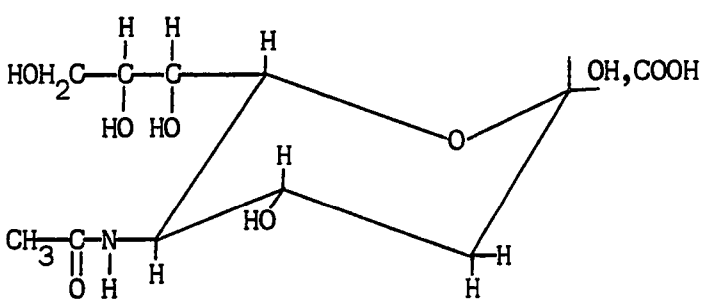


Figure 2. The preferred conformation (1C) of the pyranose form of N-acetylneuraminic acid (21).

In weak alkali, NANA undergoes a characteristic transformation: ring closure of the deacylated molecule takes place with the loss of 2 moles of water and with a retro-aldolization between C-5 and C-6 resulting in the formation of pyrrole-2-carboxylic acid (24).

When studied in aqueous solution, NANA does not exhibit mutarotation; $[\alpha]_D^{22} = -32^o$. However, when crystalline NANA is dissolved in dimethyl sulfoxide, mutarotation is observed; $[\alpha]_D^{23} = -115^o$ (7 min) $\rightarrow$ -37^o (23 min) $\rightarrow$ -24^o (final value) (19). The ascending mutarotation of NANA in the non-aqueous solvent strongly suggests that the crystalline form is the β-anomer. The mutarotation in water proceeds so quickly that it is nearly complete by the time the first reading can be taken.

Neuramin-lactose (NL), also called sialyl-lactose, was first isolated by Trucco and Caputto in 1952 from lactating rat mammary glands. On the basis of chemical, spectrophotometric and chromatographic analyses this compound proved to be a trisaccharide consisting of NANA and lactose (25, 26).

Treatment of NL with neuraminidase is accompanied by the release of free NANA (9, 14) and lactose. β-Galactosidase on the other hand is inactive on this trisaccharide (27) suggesting that the neuraminic acid is linked to the galactose moiety of the molecule. Conclusive evidence for the structure of NL from cow colostrum was obtained by permethylation studies (28). Upon hydrolysis of the exhaustively permethylated trisaccharide, 2,3,6-Tri-O-methyl-D-glucose and 2,4,6-Tri-O-methyl-D-galactose were obtained showing that NANA is linked to C-3 of the galactose moiety of lactose and the galactose is in turn glycosidically linked to C-4 of the glucose moiety.

From the values for the specific rotation of N-acetylneuramin-lactose ($[\alpha]_D^{21}$ = +16°; C = 2; water; equilibrium value), lactose ($[\alpha]_D^{20}$ = +55.4°; C = 4; water; equilibrium value) and N-acetylneuraminic acid ($[\alpha]_D^{23}$ = -115° after 7 minutes in dimethyl sulfoxide; -24° = equilibrium value), the ketosidic linkage of neuraminic acid to the galactose moiety was assigned the α-configuration (Hudson's isorotation rules) assuming that neuraminic acid belongs to the D-series (29, 30). Some questions have been raised (31) because the pyranose ring of NANA actually corresponds to an L-sugar. The structure O-α-D-N-acetylneuraminyl (2 → 3)-O-β-D-galactopyranosyl (1 → 4)-D-glucopyranose, which will be de designated NL(2 → 3), is shown in Figure 3.

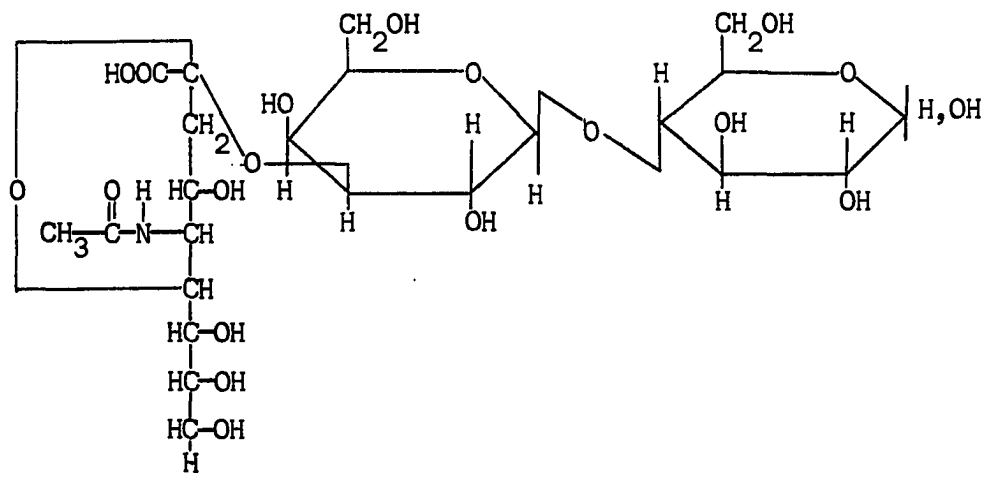


Figure 3. Structure of neuramin-lactose.

In addition to NL(2 → 3), a structural isomer of NL having the structure O-α-D-N-acetylneuraminyl-(2 → 6)-O-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose which will be designated NL(2 → 6) has been isolated as a minor component from human milk (29) and from bovine colostrum (32). A dineuraminyl lactose containing two N-acetylneuraminic acid (NANA) residues linked in an α(2 → 8) fashion has been found in human milk (33)

and in cow colostrum (34). This compound is a derivative of NL(2 → 3) having the structure NANA α(2 → 8) NANA α(2 → 3) galactose β(1 → 4) glucose.

N,O-Diacetyl-neuramin-lactose has been isolated from pig, sheep and cow colostrum, from sheep and human milk (28), and also from guinea pig mammary glands (35). Kuhn and Brossmer have shown that N,O-diacetyl-neuramin-lactose breaks down easily at its own pH to yield acetic acid, N-acetylneuraminic acid and lactose (28).

Neuramin-lactose sulfate (NLS), a sulfuric ester of neuramin-lactose, was isolated from lactating rat mammary glands by Carubelli et al. (36). Rat mammary gland is so far the only known source of NLS; analyses of milk, colostrum and mammary gland from cow and of mammary glands from guinea pig and rabbit indicated the absence of NLS (37).

The NLS content in rat mammary glands was reported to be highest in the initial part of the lactating period. A decrease in the NLS content was evident after the 12th day of lactation and no NLS was detected after 18 days of lactation. This finding parallels observations made on neuramin-lactose (NL) by Heyworth and Bacon (27).

For the preparation of NLS, Carubelli et al. (36) used rat mammary glands 1 to 6 days after delivery, which contained 0.23 mg of NLS per g of fresh tissue. The glands were extracted with hot water and the extracts were subjected to ion-exchange chromatography on a column of Dowex 1-X8 (formate). Various compounds were eluted with a gradient of formic acid from 0.1 to 4.0 M; NLS was then eluted with 0.2 M ammonium formate in 4 M formic acid. Most of the formic acid was eliminated from the effluents by repeated extraction with ether and the residual acidity

was neutralized with ammonium hydroxide. Lyophilization left a bulky residue of ammonium formate. Since repeated lyophilization did not show substantial improvements, the ammonium formate was eliminated by short term dialyses. Unfortunately some 35% of the total NLS was lost during this step. The compound was further purified with organic solvents; the dry material was extracted exhaustively with absolute methanol, and NLS was precipitated from the methanolic solution by addition of 3 volumes of anhydrous ether. After centrifugation, the residue was washed successively with ether, absolute ethanol and acetone. By repeating this purification with organic solvents, a maximum purity of 88% was obtained and the over all yield was less than 50% of the NLS present in the tissue extracts.

Analysis of constituents of NLS (36) after acid hydrolysis showed a ratio of NANA to lactose to sulfate of 1.26:1:0.96. Titration of the acid form of NLS required 2 equivalents of base per mole, corresponding to the carboxyl group of neuraminic acid and to the free acid group of a sulfate monoester respectively. The presence of a neuraminyl linkage was shown by the unchanged reactivity with the Bial reagent after reduction of NLS with borohydride, while after mild acid hydrolysis the reduced hydrolysate failed to react with the Bial reagent. The reducing power of NLS measured with hypoiodite showed one reducing group per mole of NLS. Chromatographic analysis of the hydrolysate of oxidized NLS showed the disappearance of glucose which occupies the reducing end and is thus converted into gluconic acid. Paper chromatographic studies of the products which appeared upon the progressive hydrolysis of NLS with 0.5 N sulfuric acid showed that free glucose and trace amounts of lactose appeared first

and that free galactose appeared last. This suggested that the sulfate group was attached to the galactose moiety, because a galactose-sulfate ester linkage is more stable in acid than a glycosidic linkage and thus free glucose plus galactose sulfate would be formed first followed by the hydrolysis of the latter to give free galactose.

Further studies on the structure of NLS were made (38). Enzymological studies showed that NLS is hydrolyzed by neuraminidase indicating that the neuraminyl linkage is of the α-configuration. The lactose sulfate moiety of the molecule was found to be resistant to the action of β-galactosidase, this being compatible with the presence of a substituent (sulfate) on the galactosyl moiety. During periodate oxidation of NLS, the galactosyl moiety was protected, while in the case of lactose sulfate it underwent complete oxidation. These results indicated the presence of a substituent linked to C-3 of the galactose moiety of NLS. The C-6 of the galactose moiety was shown to be occupied by the sulfate ester. This was done by isolating a galactose sulfate ester from the acid hydrolysate of NLS and comparing it with synthetic galactose-6-sulfate. Both behaved in an identical manner on periodate oxidation, and on paper chromatography and electrophoresis. These observations supported the proposed structure of NLS as O-α-D-N-acetylneuraminyl-(2 → 3)-O-β-D-galactopyranosyl 6-O-sulfate-(1 → 4)-D-glucopyranose as shown in Figure 4.

Permethylation studies are the classical and most reliable approach to the establishment of the position of the linkages in complex and substituted carbohydrates. Methylation of a complex carbohydrate is usually a difficult and painstaking task.

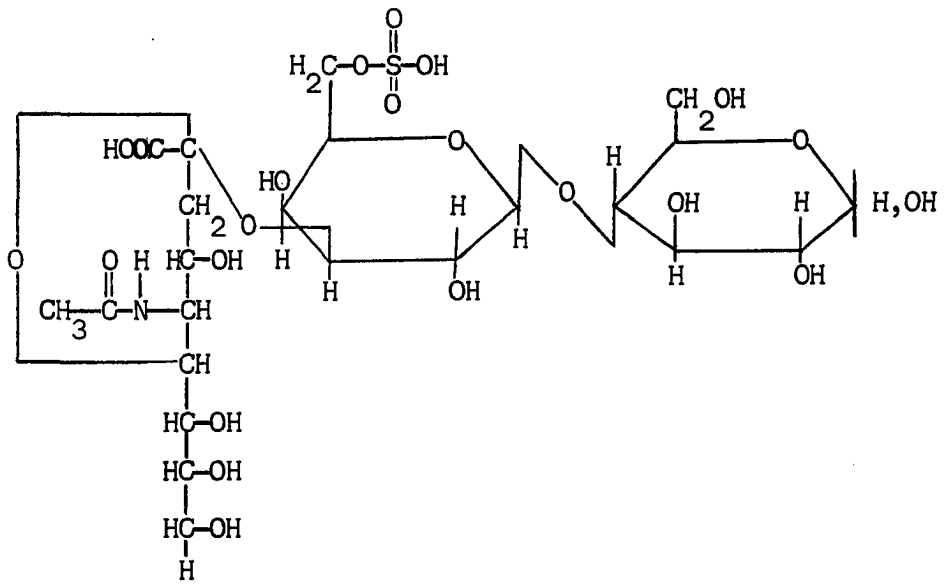


Figure 4. Structure of neuramin-lactose sulfate.

The classical Haworth (39) and Purdie (40) techniques were not successful for the total methylation of acidic oligo- and polysaccharides. Kuhn and his co-workers (41) used N,N-dimethylformamide as the solvent for permethylations with silver oxide and methyl iodide. Later these authors proposed (42) the use of N,N-dimethylformamide and dimethyl sulfoxide, separately or in admixture, as solvents for methylations with barium hydroxide and methyl iodide or dimethyl sulfate. This method, however, was not successful with uronic acid-containing materials (43). The use of dimethyl sulfoxide, with powdered sodium hydroxide and dimethyl sulfate, has been reported (44) to give high yields of almost fully methylated, neutral polysaccharides, but this technique is also less successful with uronic acid-containing materials (43).

In 1964, Hakomori (45) reported a rapid and efficient method for the permethylations of cerebroside and glycogen. In this technique, the cerebroside was fully methylated in dimethyl sulfoxide with powdered

sodium hydride and methyl iodide within one hour while glycogen was almost fully methylated by repeating the 24 hour reactions twice. Yields were very high in both experiments. With the improved Purdie type methylation method (in dimethylformamide), it was necessary to repeat the treatments at least twice to obtain fully methylated cerebroside (45).

Since Hakomori's method has been shown to be by far the most efficient method available, this method was chosen for the methylation of NLS. While this investigation was being conducted, two publications have appeared reporting the use of this method for the methylation of uronic acid-containing sugar materials (43, 46). Application of this method to such a strongly acidic sugar material as NLS has not, to my knowledge, been previously reported.

For permethylation studies, relatively large amounts of compounds with high purity are necessary. The original method of preparation of NLS has several drawbacks: a) the yields are rather low; b) the purity of the final product is under 90%; and c) the removal of formic acid with ether is laborious, expensive and hazardous. Thus a need for improved methods for the isolation and purification of NLS became very apparent.

The experimental evidence presented by Carubelli *et al.* (36, 38) in support of their proposed structure for NLS are sound. The final conclusive evidence for the structure of NLS, however, could best be substantiated by permethylation studies for the reasons described earlier in this section.

The main objectives of this investigation were:

1. To improve the methods of fractionation of acidic oligosaccharides from rat mammary gland extracts.

2. To improve the methods of purification of NLS.

3. To obtain conclusive evidence for the structure of NLS by permethylation studies.

CHAPTER II

MATERIALS AND METHODS

Materials

Mammary Glands

Inguinal and pectoral mammary glands were obtained from Sprague-Dawley albino rats which were sacrificed one to six days after delivery. The glands were pooled and stored in the frozen state for later use as the source of oligosaccharides.

Chemicals

All chemicals were reagent grade and were used as obtained except when specified otherwise.

The following chemicals were obtained from Fisher Scientific Company, Fair Lawn, New Jersey: anthrone, α-lactose, D-glucose, benzidine dihydrochloride, sodium borate, trichloroacetic acid and sulfuric acid.

p-Anisidine hydrochloride, D-galactose and anisaldehyde were obtained from Eastman Organic Chemicals, Rochester, New York.

The following chemicals were obtained from J. T. Backer Chemical Company, Phillipsburg, New Jersey: aniline oxalate, dimethyl sulfoxide and methyl iodide. The last two chemicals were subjected to further

purification prior to use in permethylation studies. Dimethyl sulfoxide was treated with anhydrous calcium chloride and redistilled *in vacuo*; methyl iodide was redistilled at atmospheric pressure.

Resorcinol was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

Synthetic crystalline N-acetylneuraminic acid, which was used as a standard sialic acid, was obtained from Sigma Chemical Company, St. Louis, Missouri.

Sodium hydride (50% oil dispersion) which was used in permethylation studies and boron tribromide which was used in demethylation studies were obtained from K & K Laboratories, Inc., Plainview, New York.

Solvents

All solvents used were reagent grade and they were used as obtained except when specified otherwise.

Absolute ethanol (U. S. Industrial Chemicals Co., New York, New York) was further purified by distilling from barium oxide.

Methylene chloride (Fisher Scientific Company, Fair Lawn, New Jersey), which was used for demethylation studies, was purified by the following method: the solvent was first washed with 5% aqueous sodium carbonate solution followed by three washings with distilled water, dried over anhydrous calcium chloride and distilled.

Isoamyl alcohol (Fisher Scientific Company, Fair Lawn, New Jersey), which was used in the determination of sialic acids by the resorcinol method, was purified by the following method: the alcohol was mixed with one-fifth volume of concentrated hydrochloric acid, allowed to stand at room temperature for several days, washed with distilled

water ten times, dried over anhydrous potassium carborate and then distilled.

Spectroquality reagent grade carbon tetrachloride and tetrachloroethylene were obtained from Matheson Coleman & Bell, Rutherford, New Jersey. These solvents were used to record the infrared absorption spectra of some methylated sugars.

Chromatographic Materials

Whatman No. 3MM chromatographic paper, from W & R Balston, Ltd., England, was used for both paper chromatography and electrophoresis. Paper used for the preparative separation of methylated isomeric monoses was previously washed (see Methods section). Silica gel G, from Research Specialties Company, Richmond, California, was used for thin layer chromatography. Sephadex G-10, G-15 and G-25, obtained from Pharmacia, Uppsala, Sweden, were used for the purification of oligosaccharides and their permethylation products by gel filtration. Dowex-50W (H^+), a strongly acidic, cation exchanger, which was used for the conversion of salt forms of acidic sugars into the free acid form and Dowex 1-X8 (Cl^-), a strongly basic, anion exchanger, which was used in column chromatographic fractionation of oligosaccharides were obtained from Baker Chemical Co., Phillipsburg, New Jersey.

Enzymes

Neuraminidase (CL. perfringens), used for hydrolysis of N-acetylneuramin-lactose sulfate, was purchased from Sigma Chemical Company, St. Louis, Missouri in a purified form (Type V). One milligram of this enzyme liberates approximately 0.16 μmole of N-acetylneuraminic acid per

minute from N-acetylneuramin-lactose at pH 5.1 at 37°.

Sulfatase (Helix pomatia) was obtained from Pierce Chemical Company, Rockford, Illinois as a lyophilized powder. This preparation contained high β-glucuronidase activity and 1,000 to 1,500 units/mg of aryl sulfatase activity. One unit causes the release of 1 μg of nitrocatechol from nitrocatechol sulfate in 1 hour at pH 5.0 at 37°.

Instruments and Equipment

Electrophoresis apparatus. A RSCO Model E-800-2 (Research Specialties Co., Berkeley, California) was used. The instrument is of the horizontal type and was operated with water cooling.

Spectrophotometers. Ultraviolet and visible spectrophotometric measurements were made using either a Beckman DU or DU-2 Spectrophotometer (Beckman Instruments Company, South Pasadena, California).

Lyophilization apparatus. Large volumes of carbohydrate solutions were lyophilized using a Freeze-Dry Apparatus, from American Instrument Co., Inc., Silver Spring, Maryland.

For small volumes, an all-glass freeze-dry apparatus K-56300, from Kontes Glass Co., Vineland, New Jersey, was utilized.

Centrifuges. Sedimentable materials were removed by centrifugation using either an International Refrigerated Centrifuge Model PR-2 or an International Centrifuge Model V, purchased from International Equipment Company, Boston, Mass.

Fraction collector. Automatic collection of fractions eluted from columns was done using a MISCO Fraction Collector, Michrochemical Specialties Co., Berkeley, California. Ten milliliter fractions were collected in test tubes and the fraction collector was used as purchased.

For large-size fractions (e.g., 100 ml each), the apparatus had to be modified. This was done by attaching tightly an aluminum foil-covered plywood board (71 cm diameter) onto the turn-table disc of the fraction collector. By placing 125-ml Erlenmeyer flasks in proper positions on the board, it was possible to collect up to 24 fractions per revolution.

Infrared spectrophotometer. The infrared absorption spectra of samples were recorded using a double-beam Recording Infrared Spectrophotometer Model 21, Perkin-Elmer Corporation, Norwalk, Connecticut.

Polarimeter. The optical rotation of sugar solutions was measured using a Carl Zeiss Polarimeter No. 369473, Carl Zeiss, Oberkochen, Wuerttemberg, Germany.

Melting point apparatus. Melting points were measured using a Melting Point Apparatus No. 4015, from The Nalge Co., Rochester, New Jersey.

Methods

Analytical Methods

Specific rotation. The desired amount of sugar was dissolved in 4.0 ml of distilled water and transferred to a polarimeter cell 8 mm diameter and 50 mm (0.5 decimeter) long. The optical rotation was measured using monochromatic light at 589 mμ (sodium D line) on the Carl Zeiss Polarimeter (see Materials). At least five readings of the sugar solution and five readings of distilled water were taken at room temperature and average values were calculated. The difference between these two average values was used as the observed rotation value for the calculation of the specific rotation, $[\alpha]_D^T$, under standard conditions, by the

following formula:

$$[\alpha]_D^T = \frac{100\alpha}{dc}$$

where α = observed rotation; d = layer thickness in decimeter (0.5); c = concentration of solute in grams per 100 ml of solution.

Resorcinol method for sialic acids (47). This method was used both for quantitative determinations and to follow the elution of neuraminic acid-containing compounds from ion-exchange columns.

Resorcinol reagent. 0.2 g crystalline resorcinol was dissolved in 10 ml of distilled water and 80 ml of concentrated hydrochloric acid and 0.25 ml of 0.1 M copper sulfate were added. The volume was then made up to 100 ml with distilled water. The reagent was prepared at least 4 hours prior to use and was stable for one week at 4°.

Isoamyl alcohol. The commercial alcohol was purified, when needed, as previously described.

Procedure. Aliquots containing approximately 0.1 μmole N-acetylneuraminic acid or an equivalent amount of a sialic acid in 1 ml aqueous solution were mixed with 1 ml resorcinol reagent in glass-stoppered (or marble covered) test tubes. The tubes were heated for 15 minutes in a boiling water bath and then cooled in ice-water to 20°. The solutions were vigorously shaken with 2.5 ml of isoamyl alcohol and chilled for 10 minutes in ice-water. The contents of the tubes were centrifuged for 5 minutes at 1,000 rpm and the isoamyl alcohol layers were transferred to 1 cm cuvettes and read at 580 mμ in a spectrophotometer against a reagent blank obtained by carrying 1 ml of distilled water through all the steps. Standards containing 0.1 μmole of NANA were analyzed simultaneously.

Micro-anthrone method for hexoses (48, 49). The basis of this method is the formation of a furfural derivative by the action of concentrated sulfuric acid on the sugar. Condensation of this furfural derivative with anthrone yields a green chromogen. This method was used for the quantitative determination of hexoses and to follow their elution from columns.

Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of concentrated sulfuric acid at least 4 hours prior to use and stored at 4^{o} for no more than 9 days.

Aliquots containing the equivalent of 10 to 20 μg of lactose were pipetted into small Pyrex tubes with ground-glass stoppers. Distilled water was added to complete 0.5 ml and after the tubes were chilled in an ice-bath for 5 minutes, 1 ml of anthrone reagent was added carefully down the test-tube wall so that it layered under the sugar solution. After mixing thoroughly the tubes were placed in a boiling water bath for 10 minutes and cooled in an ice-bath for 5 minutes. The tubes were then placed at room temperature 5 minutes and the optical density was read at 620 mμ against a blank prepared by subjecting 0.5 ml of distilled water to the same treatment. Standards containing 10 to 20 μg of lactose monohydrate were analyzed simultaneously.

Thiobarbituric acid method for free N-acetylneuraminic acid. The method developed by Warren (50) was used.

Chromatographic and ionophoretic techniques. Three main techniques were routinely used in this study.

Paper chromatography. Paper chromatography was used to characterize sugars, to follow the course of permethylation of sugars and for

the preparative isolation of methylated monoses from hydrolysates of permethylated oligosaccharides.

In analytical chromatography, Whatman No. 1 filter paper was used and the chromatogram was developed either by ascending or by descending technique. Sugar samples of approximately 0.5 μmole were spotted. For preparative chromatography, Whatman No. 3MM paper was used. The sample was applied as a band and the chromatogram was developed by ascending technique with the following solvent system: ammonium hydroxide-distilled water-methyl ethyl ketone (1:17:200) (51). After development the paper was air dried and sprayed with p-anisidine hydrochloride reagent (52). Reducing sugars appeared as pink to yellowish-brown spots upon heating the paper in an oven at 120° for 5 to 10 minutes. The spray reagent was prepared just before use by dissolving 5 g of p-anisidine hydrochloride in water-saturated n-butanol. This reagent is very sensitive, especially when the paper is viewed under ultraviolet light.

Thin layer chromatography (TLC). Silica gel G chromatoplates were used. This method is known to be rapid and sensitive and it was found to give a high resolution (greater than the conventional paper chromatography). TLC was applied frequently in studies of the rates of permethylation and hydrolysis of oligosaccharides and for the characterization of sugars in general. Since the chromatoplates can be charred after spraying with concentrated sulfuric acid, this method was of particular value for carbohydrates which were not readily detected by conventional spray reagents.

For the preparation of chromatoplates, silica gel G was slurried in water (20 g/50 ml), spread over glass plates in a uniform thickness

(250 microns), and air dried. When activation was indicated, the plates were heated in an oven at 110° for 30 minutes prior to use.

Samples containing 0.5 to 1 μg of sugar were dissolved in a suitable solvent (usually in water) and applied to the chromatoplate. The plates were often developed two or more times in the same direction by the ascending technique with one of the systems below.

Solvent systems (V/V).

Solvent 1, NH_4OH (sp.gr. 0.9)-water-methyl ethyl ketone (1:17:200) (ref. 51).

Solvent 2, benzene-ethanol (20:5) (ref. 53).

Solvent 3, NH_4OH (sp.gr. 0.9)-water-ethanol-benzene (1:15:47:200, upper phase) (ref. 54).

Solvent 4, water saturated n-butanol.

Solvent 5, n-butanol-ethanol-water (4:1:1) (ref. 55)

Solvent 6, ethyl acetate-ethanol-water (4:4:1).

Solvent 7, ethyl acetate-pyridine-water (10:5:6, upper phase) (ref. 56).

Solvent 8, ethyl acetate-acetic acid-formic acid-water (18:3:1:4) (ref. 57).

Solvent 9, n-propanol-water (85:15).

Solvent 10, isopropanol-water (4:1).

Solvents 1 to 4 were used both for separation of the permethylated oligosaccharides and for separation of isomeric methylated monoses obtained as hydrolysis products of the permethylated oligosaccharides.

Solvents 5 to 10 were used for separation of free sugars.

After the plate had been developed and air dried, it was sprayed

with one or a combination of the spray reagents listed below.

Spray reagents.

Reagent A, p-anisidine hydrochloride reagent. This reagent was prepared and used as described in the section on paper chromatography. Reducing sugars appeared as pink to yellowish-brown spots. This reagent was very sensitive for chromatoplates particularly when they are observed under ultraviolet light.

Reagent B, ninhydrin reagent. Five percent (W/V) ninhydrin in n-butanol was used to detect compounds containing free amino groups. After spraying, a pink to purple color was developed by heating in an oven at 60 to 70° for 5 to 10 minutes.

Reagent C, concentrated sulfuric acid. This was used to detect sugars (or side products) which were not detected by the spray reagents A or B. Often it was sprayed on chromatoplates which had already been treated with spray reagent A or B. The charring was done by heating in the oven at 140° for 10 minutes after spraying with sulfuric acid.

Paper electrophoresis. Whatman No. 3MM paper was used. Approximately 0.5 μmole samples of sugars were spotted on the paper and the electrophoretic analysis was run in the apparatus previously described for 4 to 5 hours at 375 volts. The buffer solutions were: for neutral sugars, 0.05 M solution of borax (pH 9.2) (58); and for acidic sugars, 0.1 M acetic acid-ammonium acetate buffer (pH 4.7). After the paper was removed and air dried, it was sprayed with benzidine reagent (59) which was prepared by dissolving 0.5 g of benzidine dihydrochloride in 10 ml glacial acetic acid and adding 10 ml of 40% (W/V) trichloroacetic acid and 80 ml of 95% ethanol. The brownish-yellow color was developed in

the oven at 110° for 5 to 10 minutes.

Fractionation of Acidic Oligosaccharides by Ion-Exchange Column Chromatography

Two methods were tested both using the strongly basic anion-exchanger Dowex 1-X8 (200 to 400 mesh).

Dowex 1-X8 (formate) column and elution with pyridinium formate. In a typical experiment, a column 8 cm in diameter and 27 cm in length was utilized to fractionate the extract prepared from 1,000 g of wet tissue. The resin, which is commercially available in chloride form, was suspended in 5 volumes of distilled water and allowed to settle for 15 minutes. The fine particles in suspension were decanted and discarded. This operation was repeated three times. The resin slurry was then transferred to the column and allowed to settle to the desired height. The resin was then converted to the formate form by passing a mixture of formic acid (sp.gr. 1.2), distilled water and ammonium hydroxide (sp.gr. 0.9) in the proportion of 4:4:1 until the removal of chloride ions was complete, as shown by testing the effluent with 5% silver nitrate solution. The column was then washed with distilled water until the effluent reached a constant pH (about 5.0).

The tissue extract (6.67 liters) was then passed through the column in a cold room (4°) at a rate of about 4 to 5 ml per minute. The column was washed with distilled water until the optical density of the effluent at 260 mμ leveled off at a low value (around 0.1). This washing usually took approximately 16 liters of water.

The column was then eluted at the rate of 2.5 ml per minute with three solutions of pyridinium formate of increasing concentration: 0.01

N, 0.15 N and 0.5 N respectively.

Fractions, about 100 ml in volume, were collected in 125-ml Erlenmeyer flasks with the modified fraction collector previously described. The elution pattern was followed by analyzing the fractions with the resorcinol reaction for sialic acid-containing compounds and with the anthrone reaction for hexoses. The fractions were then pooled and the solvents were removed by lyophilization. In a few cases the concentration was done in a rotary evaporator under diminished pressure at room temperature.

Dowex 1-X8 (acetate) column and elution with pyridinium acetate (60). The same procedure described above was followed in packing the column. The resin was converted to the acetate form by passing 2 N aqueous sodium acetate solution, until the removal of chloride ion was complete, followed by washing with distilled water until the washing was free of sodium ions (flame test).

The tissue extract was passed through the column in the cold room and the column was washed with distilled water following the same procedure as described above.

For fractionation of acidic sugars, the column was eluted with solutions of pyridinium acetate of increasing concentrations: 0.085 N, 0.50 N and 2.0 N respectively.

The course of the fractionation was followed by the resorcinol method. The resorcinol positive fractions were pooled and lyophilized.

Purification Methods

Preparation of the potassium salt of sialic acid-containing oligosaccharides. Since the pyridinium salts of NL and NLS, obtained by

lyophilization of the column effluents, were very hygroscopic, they were converted to potassium salts which were much less hygroscopic. The conversion was done by converting first to acid form with Dowex-50W (H^+) followed by neutralization with a potassium hydroxide solution. In a typical experiment, 722 mg of lyophilized NLS was dissolved in 5 ml of distilled water and the solution was passed in the cold room through a Dowex-50W (H^+) column (2.9 cm diameter and 48 cm long) which had been thoroughly washed with distilled water. The column was then washed with distilled water until the washing was neutral as checked by a pH indicator paper. The effluent was collected in an 500-ml Erlenmeyer flask in an ice-bath (total volume 300 ml). This was immediately neutralized with 1.0 N potassium hydroxide solution to pH 5.9. The clear solution obtained was then lyophilized to yield a yellow fluffy-residue. The same procedure was followed for conversion of NL to the potassium salt form.

Organic solvent method (36, 49). The dried yellow fluffy-residue (0.1 g) containing NLS in potassium salt form, NLS ($2K^+$), was extracted successively with 15 ml, 10 ml, 10 ml and 10 ml of dry absolute-methanol. After each extraction, the insoluble material was removed by centrifugation and the supernatant fraction was decanted. The combined extract was treated with 3 volumes of anhydrous ethyl ether to precipitate out NLS($2K^+$). The suspension was centrifuged and the sediment was washed twice with ethyl ether, 3 times with absolute ethanol and 3 times with anhydrous acetone. The residual acetone was removed in an evacuated desiccator over silica gel. The product was a hygroscopic, slightly yellow powder. The degree of purity was measured by the resorcinol assay and the removal of impurities with ultraviolet absorption was followed

by the decrease of the optical density at 260 mμ.

The same purification procedure was also applied to the purification of NLS as pyridinium salt contained in the lyophilized product of the NLS fraction from Dowex 1-X8 column fractionation as previously described.

NL as the potassium salt was also purified by the same procedure described above.

Gel filtration method. Sephadex is the trade name of polydextrans with a variable degree of cross-linkage. Separations by gel filtration depend primarily on differences in the molecular size between the components of a mixture. Small molecules can diffuse into the Sephadex particles and this causes a retardation of their flow rate with respect to the large molecules which are excluded (molecular sieve) and rapidly eluted from the column (61).

Sephadex G-25 (50-100 mesh), Sephadex G-15 (20-80 mesh) or Sephadex G-10 (20-80 mesh) was used. For the preparation of the columns, the dry Sephadex was allowed to swell in 0.05 M sodium chloride for half an hour and was then freed from fine-grained material by repeated sedimentations and decantations. The gel was then suspended in distilled water. For this and successive steps the distilled water utilized was previously degassed under diminished pressure. The slurry was poured into a suitable column to give a gel bed of 1.3 cm diameter and 175 cm in length. The column was packed by first filling one-fifth of the column with distilled water and then a slurry of the gel suspension in water was poured very slowly down the side of the column in order to avoid trapping air bubbles and allowed to sediment through the column of water.

This operation produced a sharp flat rising boundary of packed material. Unless this boundary is distinctly visible, the column will not be packed uniformly. After the boundary had risen approximately 5 cm, the stopcock was opened and adjusted to allow a flow-rate of 3 drops per second which was kept constant by adjusting the stopcock from time to time as the column built up. Usually after 2 to 3 hours the material was completely settled. The column was washed with distilled water for 4 to 5 hours or overnight to complete the equilibration of the column with distilled water.

The mixture to be fractionated was dissolved in 1 ml of distilled water and then applied to the column. Elution was then started with distilled water and fractions of 2 to 3 ml were collected with an automatic fraction collector at the rate of one fraction each 30 minutes. The elution of sugars was followed by the resorcinol method for sialic acid-containing compounds and by the anthrone method for hexoses. Elution of ultraviolet absorbing impurities was followed by optical density readings at 260 mμ in the purification of NL(K^+) and NLS($2K^+$). All Sephadex column chromatographies were carried out at room temperature except where stated otherwise.

Structural Studies

Hydrolysis of NLS by neuraminidase (62). A 9 mg portion of the purified NLS($2K^+$) was dissolved in 2 ml of distilled water, the pH of the solution was adjusted to 5.2 with 0.1 N hydrochloric acid and 0.25 mg of neuraminidase was then added. After adding one drop of toluene to retard bacterial growth, the tube was stoppered and placed in a water bath at 37°. No buffer was utilized in order to simplify the isolation and puri-

fication of the final products of the reaction. After incubating for 24 hours at 37°, the enzyme was denatured by heating at 95° for 5 minutes and removed by centrifugation. The hydrolysate was analyzed by paper chromatography and paper electrophoresis.

Treatment of NLS($2K^+$) with sulfatase. Two samples, each containing 3.3 mg of the purified NLS($2K^+$), were dissolved in 1 ml of distilled water in two separate test tubes and 0.4 mg of sulfatase was added to each tube. One drop of toluene was added to each solution (pH 5.0) to prevent bacterial growth and the stoppered tubes were then incubated at 37° in a water bath for 3.5 hours and for 24 hours, respectively.

After the incubation, the reaction mixtures were heated in boiling water for 5 minutes to denature the protein. The supernatant fractions were separated by centrifugation and decantation. After concentrating to a small volume, the materials were examined by electrophoresis.

Permethylation of oligosaccharides. The methylation procedure was essentially that described by Hakomori (45) wherein the methylsulfinyl carbanion (63, 64) was used to generate the oligosaccharide alkoxide prior to the addition of methyl iodide. However, due to the strongly polar (acidic) properties of the neuraminyl oligosaccharides and to the limited amount of material available, some technical problems and difficulties had to be studied and solved in order to develop a suitable modified technique.

Permethylation of NLS($2K^+$). The methysulfinyl carbanion was prepared as follows: 171 mg of sodium hydride (50%, coated with mineral oil) was weighed into a dry, 50-ml, three-necked, round-bottom flask. A

rubber serum-cap was fitted at one neck and a magnetic stirring bar was placed inside the flask. The sodium hydride was washed 3 times to remove the mineral oil by stirring with 8-ml portions of n-hexane and decanting the wash. After the third wash, the flask was fitted with a condenser at the center neck and residual n-hexane was removed by gently passing nitrogen gas *via* an 18-gauge needle inserted into the serum cap. This procedure produced a slightly greyish-white sodium hydride powder which was kept under a continuous slow stream of nitrogen. Five milliliter of dimethyl sulfoxide was added to the pwodery sodium hydride. This dimethyl sulfoxide had been distilled from calcium chloride under reduced pressure and stored over dried molecular sieves. The flask was placed in a Glasco heating mantle and stirred with a magnetic stirrer at 65^{o} until the solution had a clear green color and evolution of hydrogen gas ceased (45 minutes). The solution was then cooled to room temperature.

For generation of the oligosaccharide alkoxide, 100 mg of NLS ($2K^{+}$) dissolved in 1 ml of the dry dimethyl sulfoxide was added dropwise over a period of one minute to the sulfinyl carbanion solution. The amount of sodium hydride utilized should yield an equivalent amount of methylsulfinyl carbanion which represents a 100% excess over the amount actually needed. Upon addition of the sugar a gel often formed but gradually liquified and, after stirring at room temperature for 30 minutes, the reaction mixture appeared homogeneous and the solution changed to an orange color. The nitrogen gas was passed continuously throughout this operation. The reaction mixture was left for 8 hours at room temperature with constant stirring and passing a stream of the nitrogen. At the end of the reaction the solution had a brown color.

For the methylation reaction, the oligosaccharide alkoxide solution was cooled to 20° in an ice-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 minutes). Within a few minutes after addition of methyl iodide heat evolution ceased, the solution became clear yellow, and the viscosity was markedly reduced. The solution was stirred under nitrogen gas for 14 hours at room temperature, after which 10 ml of distilled water was added to destroy the excess methylsulfinyl carbanions. The course of the methylation reaction was followed by thin layer chromatography and completion of the reaction was confirmed by infrared spectroscopy, observing the complete disappearance of the hydroxyl band (3200-3700 cm^{-1}).

Permethylation of neuraminidase hydrolysate of NLS (NANA plus lactose-sulfate). The same procedure described for NLS was applied except for the reaction times.

Permethylation of $NL(K^{+})$. The same procedure described for NLS was applied except for the reaction times.

Permethylation lactose. The same procedure described for the permethylation of NL was used.

Isolation and purification of the permethylated oligosaccharides. Two methods were used, namely the conventional organic solvent method and gel filtration method.

Gel-filtration method. A Sephadex G-15 column 1.3 cm in diameter and 175 cm in length was used.

The reaction mixture from the permethylation reaction was transferred into a round bottom flask and evaporated in a rotary evaporator at

room temperature to remove the excess methyl iodide, the iodine formed, the excess water added and some of the dimethyl sulfoxide to give a final volume of 4 ml of a brown solution. This was placed in the column and eluted as previously described for the purification of NLS($2K^+$).

The course of the elution was followed by the anthrone reaction. This reagent gave a green color with the methylated sugars upon heating at 100° for 10 minutes, an instantaneous red color (iodine gas) at room temperature with sodium iodide and a yellow color at room temperature with dimethyl sulfoxide which changed to pink upon heating at 100° for 8 minutes. The elution of sodium iodide was also confirmed with 5% silver nitrate solution.

This gel filtration method was applied for all of the methylated sugars prepared with excellent results.

Organic solvent method for permethylated lactose and NL (65). To the permethylation reaction mixture, which was obtained from 1 g of starting material by the same procedure described for permethylation of NLS, 300 ml of distilled water was added and the solution was extracted three times with 100-ml portions of chloroform. The combined chloroform extract was washed four times with 300-ml portions of distilled water. The solution was dried over anhydrous sodium sulfate grains and the drying agent was removed by decantation and filtration. The solvent was then removed under reduced pressure on a rotary evaporator to yield a yellow syrup. The residue was dried in an evacuated desiccator containing phosphorus pentoxide and calcium chloride to give a slightly yellow "semi-crystalline" product. Several attempts to purify permethylated NLS by this method were unsuccessful.

Hydrolysis of permethylated oligosaccharides (65). A 45-mg sample of the permethylated sugar was dissolved in 3 ml of 0.5 N sulfuric acid in a 7 ml tube fitted with a condenser and a magnetic stirrer. The stirred solution was refluxed using a Glasco heating mantle for various time periods to give a heavily colored solution. The reaction mixture was then cooled to room temperature and neutralized with saturated barium hydroxide solution. The whole mixture was treated with approximately 0.1 g of activated charcoal (Nuchar C-190-N) to decolorize. After filtration through a Whatman No. 5 filter paper, the barium sulfate precipitate and charcoal on the filter paper was washed four times with 5 ml portions of hot distilled water. The colorless filtrate was evaporated in a rotary evaporator at 30° under reduced pressure.

The course of the hydrolysis was followed by thin layer chromatography as well as the paper chromatographic techniques. Preparative separation was done by the paper chromatographic method on Whatman 3MM paper.

Preparative paper chromatographic separation of the products of hydrolysis of permethylated oligosaccharides. Whatman No. 3MM paper used for these experiments was washed by descending irrigation in a big chromatographic chamber with 1 N hydrochloric acid for 24 hours, with distilled water for 18 hours and finally with the solvent mixture ammonium hydroxide-water-methyl ethyl ketone (1:17:200), and then air-dried. The material was applied as a band (approximately 1 μmole per cm) at 3 cm from the end of the paper. After development by ascending irrigation the paper was dried and three 2 mm-wide guide-strips were cut and sprayed with p-anisidine hydrochloride reagent. The sugar bands were located with

the aid of the guide-strips and the corresponding zone of the paper was cut. The pieces of paper were moistened with a drop of distilled water and were attached to a larger piece of paper which had one end in a trough of distilled water. The water was siphoned and passed through the pieces of paper thus accomplishing the elution of the sugars which were collected in test tubes. The whole system was enclosed in a chamber which was kept saturated with water vapor by placing inside a container with hot water. A dish containing chloroform was also placed inside the chamber to saturate the atmosphere with chloroform in order to retard bacterial growth. The elution was stopped when the effluent gave a negative anthrone test.

CHAPTER III

RESULTS

Isolation of Oligosaccharides

Preparation of Tissue Extracts (36, 49)

Inguinal and pectoral mammary glands were obtained from Sprague-Dawley albino rats sacrificed one to six days after delivery. The glands were pooled and stored in the frozen state until a suitable amount was accumulated. Various batches were kept frozen for different periods of time before they were extracted.

In a typical experiment (designed Experiment 5), a batch of 1,000 g of the frozen tissue which had been stored for 8 to 10 months in a deep freezer (-20°) was divided in small pieces, placed in a mixture of ethyl ether and dry-ice, and then ground in a precooled corn grinder. The powder was extracted three times successively with 3.5, 2 and 1.5 liters of distilled water at 85 to 90° for 3 minutes. The extracts were cooled in tap water to 30° and separated from the tissue by filtering through cheesecloth. Dowex 50W-X4 (H^{+}) was added to the pooled extracts (total volume of 7 liters) until a pH of 4.4 was obtained. The extract was decanted, and the resin was washed twice with a small amount of distilled water. The extract and washings were pooled and left overnight in a cold room at 4°. This caused the separation of a lipid layer on

the top, a protein precipitate in the bottom and a clear, slightly greenish-yellow solution in the middle section of the container. Most of the clear solution was removed by suction and filtered through folded Whatman No. 12 filter paper in the cold room. The remaining material (paste) was distributed in several filters in order to recover some of the remaining extract which drained rather slowly through the filters. Occasionally the components of the extract did not separate satisfactorily and the suspension had to be subjected to centrifugation for 5 minutes at 350 x g in a refrigerated centrifuge because otherwise the filtration of the extract became too slow. The pooled clear solution was neutralized to pH 7 with concentrated ammonium hydroxide (approximately 1.7 ml) and allowed to stand overnight in the cold room during which time a cloudy, white precipitate appeared in the bottom of the container. The supernatant was decanted and filtered through the Whatman No. 12 filter paper as described above.

Ion-Exchange Column Fractionation

The pooled extract containing 4.80 mmoles of total sialic acids in 6.67 liters was passed through a Dowex 1-X8 (formate) column of 8 cm in diameter and 27 cm in length. The column was eluted with pyridinium formate by collecting fractions of approximately 100-ml each as described previously. The fractions were assayed by both resorcinol and anthrone methods. The elution pattern is shown in Figure 5.

Fractions 9-167 (Peak 1), eluted with 0.01 N pyridinium formate, gave both anthrone and resorcinol positive tests. The sugar present in this peak was further identified as neuramin-lactose (NL) by its electrophoretic mobility and thin layer chromatographic behavior, which were

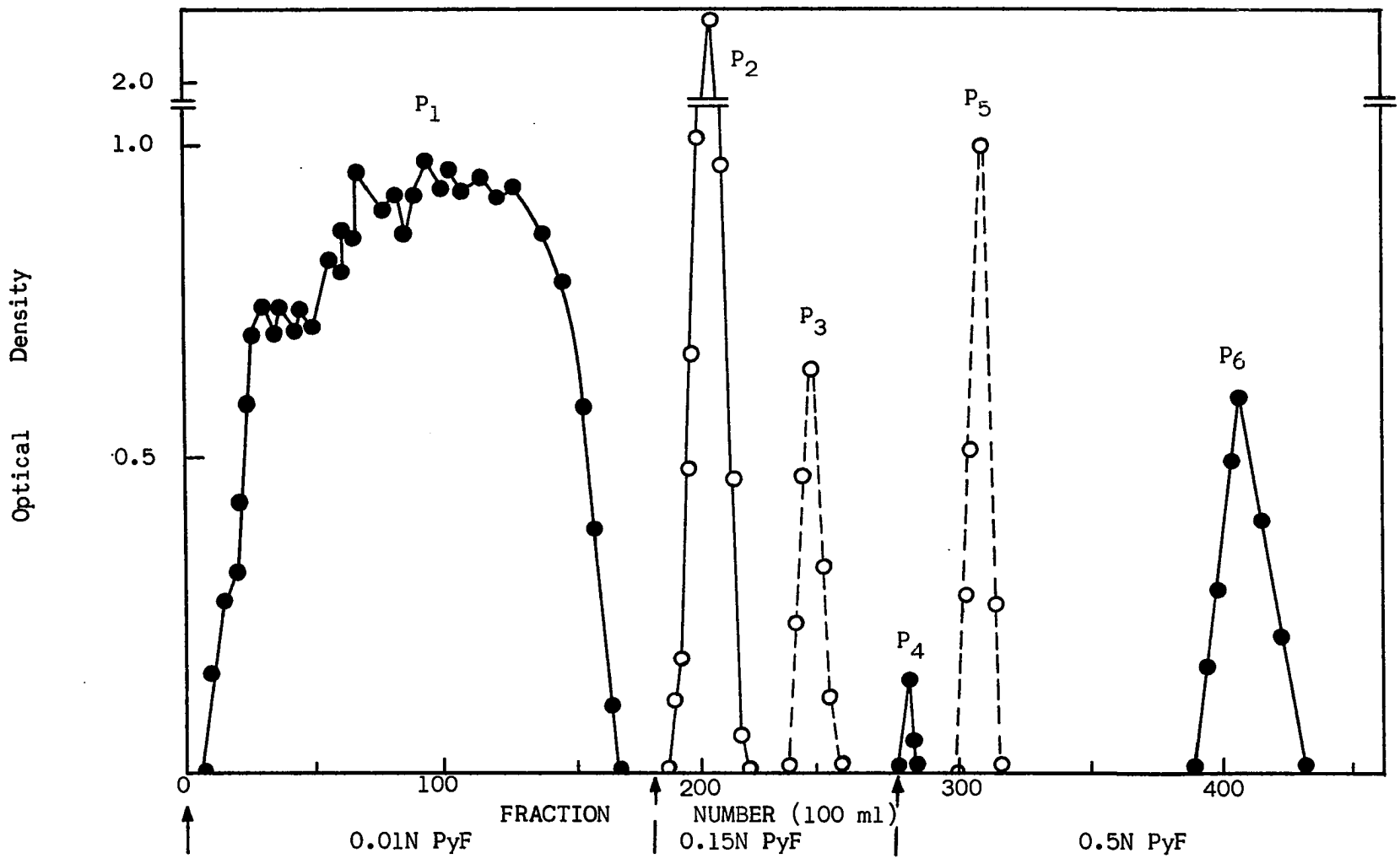


Figure 5. Dowex 1-X8 (formate) column chromatography of acidic oligosaccharides from the extract of 1,000 g rat mammary glands (tissue stored at -20° for 8-12 months), Exp. 5. —●—●—, OD_{580} mμ for the resorcinol reaction (neuraminic acid-containing compounds) and anthrone positive. —○—○—, OD_{580} mμ for the resorcinol reaction but anthrone test negative. --○--○--, OD_{620} mμ for the anthrone reaction (hexoses). PyF, Pyridinium formate.

identical to those of standard NL. The pooled fraction (15,140 ml) containing 3,693 μmole of NL, was concentrated in the rotary evaporator to 50 ml and then lyophilized yielding a yellow fluffy-residue.

Fractions 186-213 (Peak 2), eluted with 0.15 N pyridinium formate, gave anthrone negative but resorcinol positive tests. This compound gives a positive thiobarbituric acid assay and it was further identified as free N-acetylneuraminic acid (NANA) by its electrophoretic mobility, which was identical to that of standard NANA, and by the failure to yield a positive thiobarbituric acid test after treatment with sodium borohydride. Fractions 235-254 (Peak 3) gave anthrone positive but resorcinol negative tests. The sugar material in this peak, which will be designated as P_3, was also eluted with 0.15 N pyridinium formate solution. The fractions corresponding to these 2 peaks were pooled separately: the total volume of Peak 2 was 2,940 ml (containing 698 μmole of NANA) while the volume of Peak 3 was 2,200 ml. The pooled solutions were concentrated in the rotary evaporator under diminished pressure at 30° to a final volume of 50 ml, and then lyophilized twice to yield a light-brown sticky-gum.

Fractions 281-284 (Peak 4) gave both anthrone and resorcinol positive tests. The sugar material in this peak, which will be designated as P_4, and fractions 302-314 (Peak 5), which gave anthrone positive but resorcinol negative tests (the sugar in the peak will be designated as P_5), were eluted by 0.5 N pyridinium formate solution. The fractions corresponding to these 2 peaks were pooled separately giving volumes of 300 ml and 1,360 ml respectively. The pooled solutions were concentrated to small volumes and lyophilized twice to remove most of the pyridinium

formate, yielding a brown sticky-gum. Fractions 389-432 (Peak 6), eluted also with 0.5 N pyridinium formate, gave both anthrone and resorcinol positive tests. The sugar present in this peak was further identified as neuramin-lactose sulfate (NLS) by its electrophoretic mobility, which was identical to that of a standard NLS. The pooled fractions (4,820 ml) containing 371.14 μmoles of NLS were lyophilized, leaving a light-yellow syrup. By repeating the lyophilization a light-yellow sticky-gum was obtained.

The recovery of neuraminic acid and its derivatives from the column was 99.3% (see Table 1).

The typical experiment described above was the method finally adopted. This method was developed taking into account the findings of the experiments described below.

Experiment 1. The objective of this experiment was to test the Dowex 1-X8 (acetate) column in the fractionation of neuraminic acid-containing compounds from rat mammary glands.

Mammary glands (100 g) which had been stored in the deep freezer for 12-18 months were extracted following the procedure previously described but on a one-tenth scale. The extract (568 ml) containing 226 μmoles of total sialic acids was passed through a Dowex 1-X8 (acetate) column (3.3 x 16.6 cm) in the cold room and the column was eluted with pyridinium acetate solutions, collecting 10-ml fractions. The elution of sialic acid-containing compounds was followed by the resorcinol method and the elution pattern is shown in Figure 6. Three peaks of resorcinol-positive material were eluted, the first two peaks are slightly asymmetric, and the second and third peaks which appeared rather close together,

TABLE 1

AMOUNT OF SIALIC ACIDS IN THE TISSUE EXTRACTS

Exp. No.	Period of Storage (Month)	Amount of Tissue (g)	Total Sialic Acid in Extract (μmoles)	μmoles per g Wet Tissue			Recovery[a] (%)
				NL	NANA	NLS	
1	12-18	100	226	1.46	0.286	0.285	89.6
2	12-18	1,000	5,300	4.30	0.715	0.282	100
3	0-½	100	-----	0.75[b]	0.000	0.292	---
4	0-2	1,000	-----	----	-----	0.250	---
5	8-12	1,000	4,802	3.69	0.698	0.371	99.3

[a]Recovery after column fractionation.

[b]This includes only the main of the two NL-containing peaks eluted from this column.

NL, Neuramin-lactose. NANA, N-acetylneuraminic acid. NLS, Neuramin-lactose sulfate.

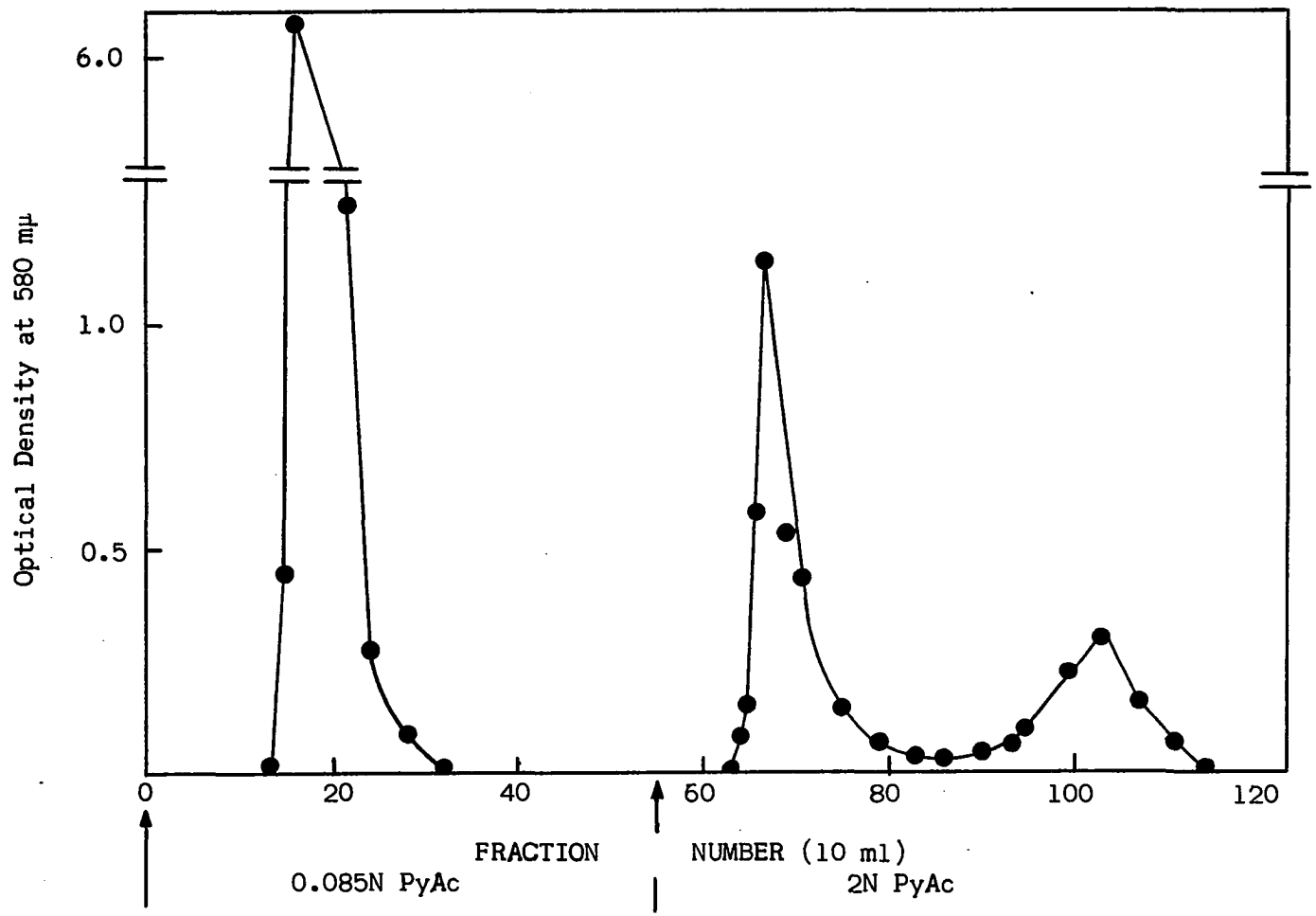


Figure 6. Dowex 1-X8 (acetate) column chromatography of acidic sugars from the extract of 100 g rat mammary glands (stored at -20° for 12-18 months), Experiment 1. $OD_{580\ m\mu}$ for the resorcinol reaction (neuraminic acids).

PyAc, pyridinium acetate.

did not separate completely because of the tailing of the second peak that never reached the base line.

The electrophoretic mobility of the resorcinol positive material in each peak corresponded to NL in the first peak, free NANA in the second and NLS in the third peak.

Fractions 15-33 (first peak), eluted with 0.085 N pyridinium acetate, were pooled (186 ml) and the resorcinol assay indicated a total of 146.0 μmoles of NL. After lyophilization, a light-yellow, fluffy residue was obtained.

Fractions 63-83 (second peak), eluted with 2.0 N pyridinium acetate, were pooled giving a total volume of 192 ml and the resorcinol assay indicated 28.6 μmoles of free NANA. After dilution with an equal volume of distilled water to allow freezing, the solution was lyophilized twice to obtain a yellow, fluffy residue.

Fractions 93-119 (third peak), also eluted with 2.0 N pyridinium acetate, were pooled giving a total volume of 290 ml, and containing 25.5 μmoles of NLS as determined by the resorcinol assay. The solution, after dilution with an equal volume of distilled water, was lyophilized twice to yield a yellow, fluffy residue.

The recovery of total sialic acids after the column chromatography was 89.6% (see Table 1).

Experiment 2. Although a reasonably good fractionation was obtained in Experiment 1, a better separation between the second and third peaks was desirable. Thus, a modification of the eluting system was tested in this experiment.

Mammary glands (1,000 g) which had been stored in the deep freez-

er for 12-18 months were extracted following the same procedure previously described. The extract (5 liters) containing 5,300 μmoles of total sialic acids was passed through a Dowex 1-X8 (acetate) column (8 x 12.5 cm) as described in Methods and eluted successively with pyridinium acetate solutions 0.085 N, 0.5 N and 2.0 N. The elution rate was 2.5 ml per minute and 100-ml fractions were collected with the modified fraction collector (see Methods). The elution of sialic acid-containing compounds was followed by the resorcinol assay. The elution pattern is shown in Figure 7. The resorcinol positive material in these peaks was identified as NL in the first peak, free NANA in the second and NLS in the third peak by their electrophoretic mobilities, which were identical to those of the corresponding standards.

The material in each peak was treated essentially the same as in Experiment 1 and the quantitative data are shown in Table 1.

Experiment 3. One of the methods tried for the purification of the NLS obtained in Experiments 1 and 2 was the use of a Dowex 1-X8 (formate) column and pyridinium formate eluting solvents. This column chromatography resulted in the separation of a sugar-contaminant from the NLS present in Peak 3 and the system was therefore directly applied for the fractionation of rat mammary gland extracts.

A batch of 100 g of fresh rat mammary glands, which had been accumulated and stored in the deep freezer during the previous two weeks was extracted following the procedure already described. The 600 ml tissue extract obtained, which contained 267 μmoles of total sialic acids, was passed in the cold room through a Dowex 1-X8 (formate) column (8 x 14 cm), which was prepared as described in Methods. After washing, the col-

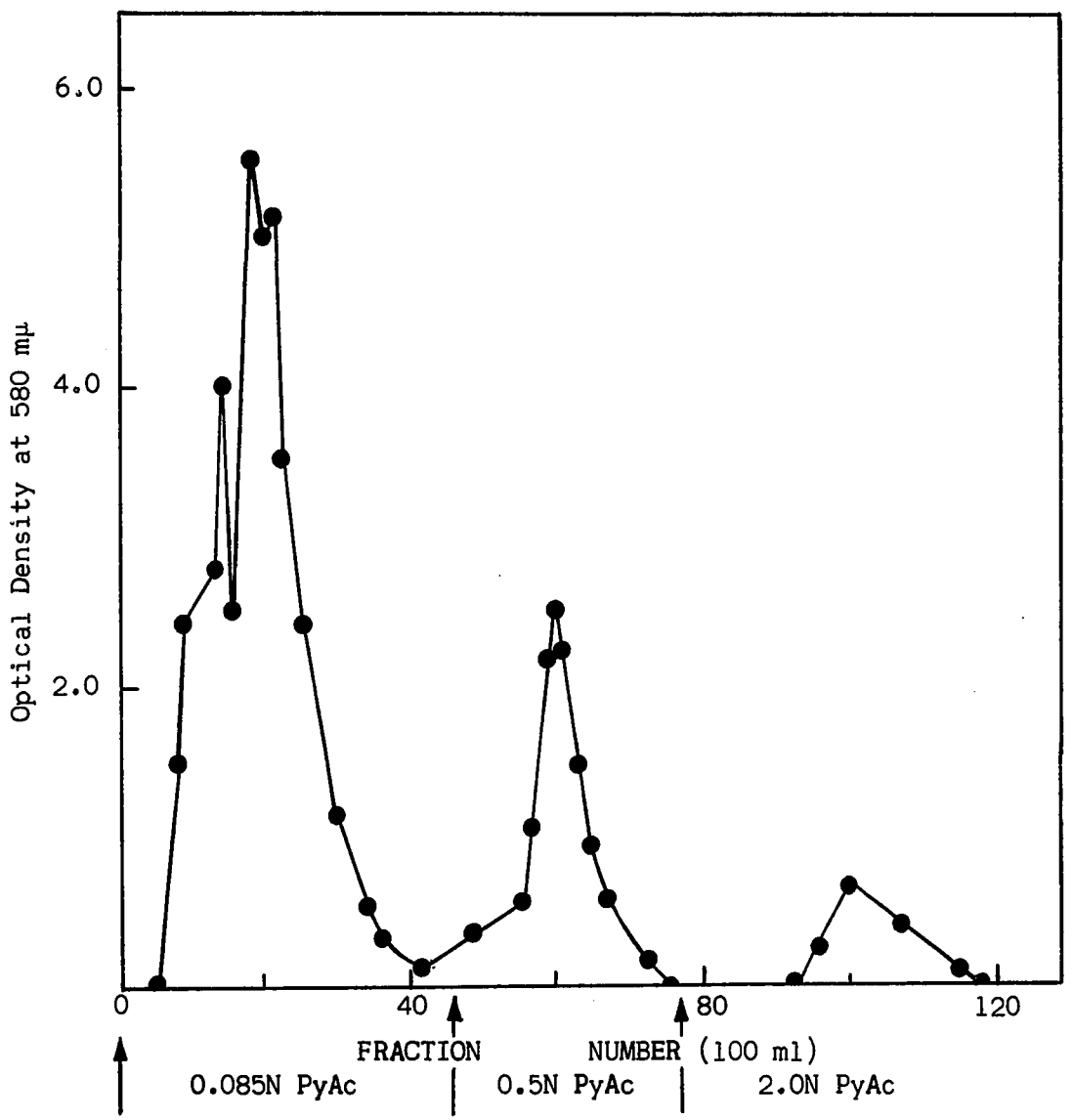


Figure 7. Dowex 1-X8 (acetate) column chromatography of acidic sugars from the extract of 1,000 g rat mammary glands (stored at -20° for 12-18 months), Experiment 2.

$OD_{580\ m\mu}$ for the resorcinol reaction (neuraminic acids).

PyAc, pyridinium acetate.

umn was eluted with solutions of pyridinium formate of increasing concentration: 0.01 N, 0.15 N, 0.20 N and 0.30 N, respectively. The rate of elution was 0.5 ml per minute, and 8-ml fractions were collected. The fractions were analyzed by both the resorcinol and anthrone assay and the elution pattern is shown in Figure 8.

The first peak (fractions 11-45) and a minor component preceding the first peak (fractions 3-10) were eluted with 0.01 N pyridinium formate solution. These fractions gave both resorcinol and anthrone positive tests. Fractions 11-45 were pooled (237 ml) and the sialic acid containing sugar (67.2 μmoles) was identified as NL by its electrophoretic mobility. Fractions (3-10) eluted prior to the main peak were pooled giving a total volume of 57 ml. The sugar(s) in this pool had an electrophoretic mobility identical to that of the main peak (NL). Although the nature of this minor component was not studied further, it is most likely the NL (2 → 6) isomer, which is known to be present in human milk (29) and also in cow colostrum (32).

The second peak (fractions 83-88) and the third peak (fractions 95-106), which were eluted with 0.15 N pyridinium formate solution, gave anthrone positive but resorcinol negative tests. The elution of these sugars was followed by the anthrone assay. Prior to the anthrone determination, it was necessary to evaporate an aliquot from each tube in order to remove all the pyridinium formate. Unless the pyridinium formate was eliminated, the bubbles formed in the presence of the anthrone reagent made it impossible to measure the optical density. The fractions in the two peaks were pooled separately and evaporated in the rotary evaporator under diminished pressure at room temperature to yield light-

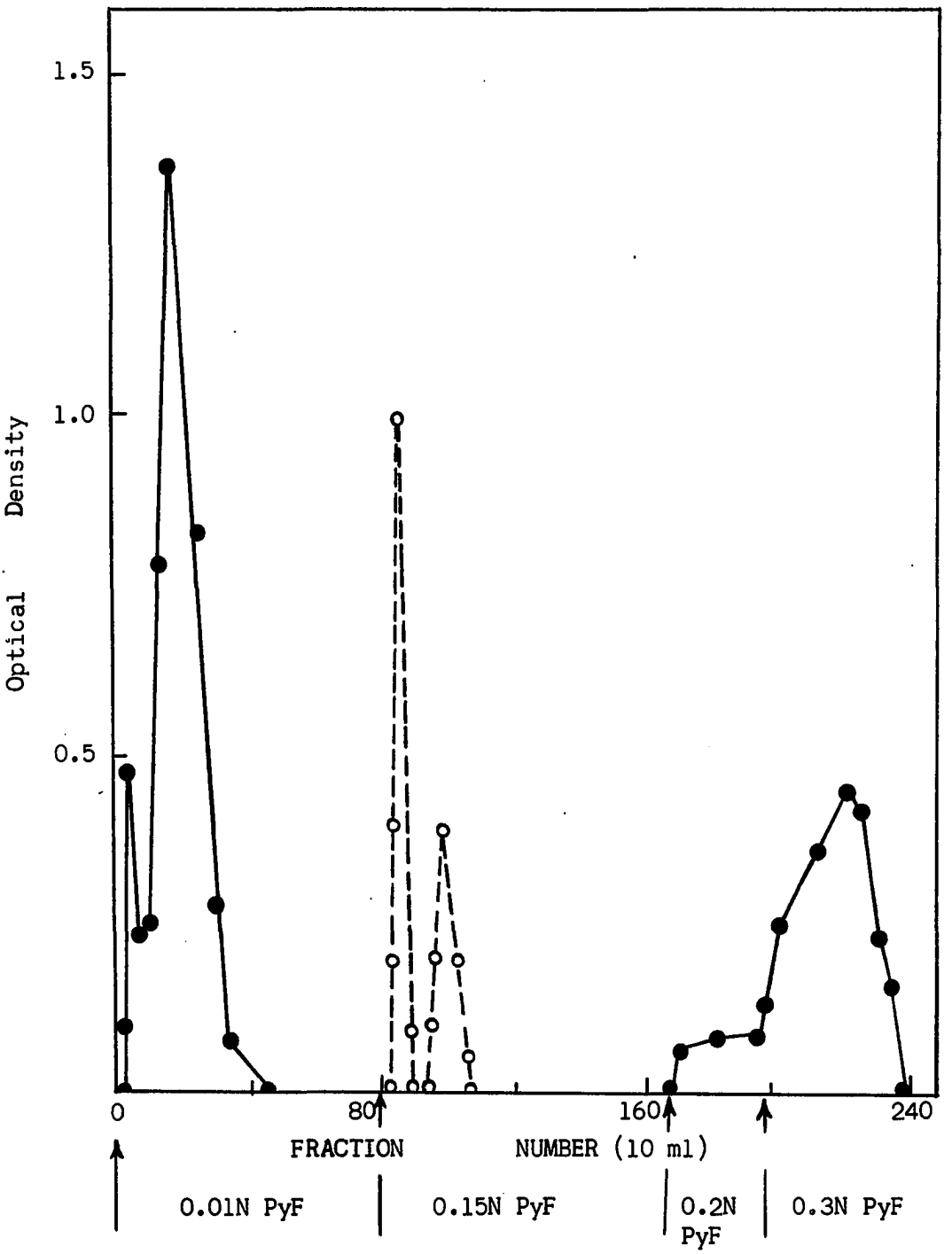


Figure 8. Dowex 1-X8 (formate) column chromatography of acidic sugars from the extract of 100 g rat mammary glands (stored at -20° for 2 weeks), Experiment 3.

—●—●—, $OD_{580\ m\mu}$ for the resorcinol reaction (neuraminic acids) and anthrone test positive. --○--○--, $OD_{620\ m\mu}$ for the anthrone reaction (hexoses). PyF, pyridinium formate.

brown residues.

The fourth peak started to appear from tube 173 eluted with 0.2 N pyridinium formate. Since the resorcinol values leveled off at low readings the concentration of pyridinium formate was increased to 0.3 N. This resulted in the elution of a sharp peak. Fractions 198-236 comprised the main portion of the fourth peak and gave both resorcinol and anthrone positive tests. The pooled fractions gave a total volume of 348 ml and the sugar material was identified as NLS by its electrophoretic mobility. The solution, containing 292 μmoles of NLS, was lyophilized three times to obtain a light-brown, sticky gum.

Experiment 4. This experiment was carried out using 1,000 g of tissue which had been collected and stored in the deep freezer within the previous 2 months. The extract obtained was passed through a Dowex 1-X8 (formate) column (8 x 14 cm) as previously described. The column was eluted successively with 0.01 N, 0.15 N, 0.3 N and 0.5 N pyridinium formate solutions at a rate of 2.5 ml per minute and 100-ml fractions were collected. The elution was followed by both the anthrone and resorcinol methods.

Since the main purpose of this experiments was the preparation of NLS, only the fractions of this peak were processed as previously described. The results for NLS are shown in Table 1.

Purification of Isolated Sugars

Organic Solvent Purification Method

Purification of NL. The NL(py^+), NL in pyridinium salt form, obtained from the Dowex 1-X8 (acetate) column (Experiment 1) was 49.0%

pure. After conversion to the potassium salt form, $NL(K^+)$, via a cation-exchanger column as described in the Method Section, the purity improved to 75.0% with 100% recovery. This material was then subjected twice to the organic solvent purification method. This yielded a white powdery $NL(K^+)$, 96.1% pure. The recovery, based on the NL content in the lyophilized product, was 64.1%. The results are summarized in Table 2. The improvement of the purity (from 75.0% to 96.1%) during organic solvent purification was closely correlated to the reduction of the ratio between the $O.D._{260\ m\mu}$ and the $O.D._{580\ m\mu}$ (resorcinol assay) which fell from 0.218 to 0.080. Thus a major impurity contained in the 75.0% pure $NL(K^+)$ was ultraviolet absorbing material, presumably nucleotides. Several attempts at further purification by repeating the organic solvent purification procedure did not improve the purity and did not reduce the optical density ratio.

Purification of NLS. The organic solvent method was tested for the purification of NLS in both the potassium salt form, $NLS(2K^+)$, and pyridinium salt form, $NLS(2py^+)$.

As $NLS(2py^+)$. A portion (3.88 mg) of lyophilized material from the NLS peak of the Dowex 1-X8 (acetate) column (Experiment 1), which had a 35.9% purity, was subjected twice to the organic solvent purification method. Unlike NL, the solubility of NLS in dry methanol was not very high. Therefore, it was necessary to increase the number of methanolic extractions. The crude material was extracted with methanol five times and the NLS was then precipitated out by addition of 3 volumes of ethyl ether to the combined extract.

After the first purification, the purity was 51.6% with 71.4%

TABLE 2

PURIFICATION OF NEURAMIN-LACTOSE BY ORGANIC SOLVENT

Isolation Method Used	Purification Step	$\frac{O.D._{260\ m\mu}}{O.D._{580\ m\mu}}$	Purity (%)	Recovery[a] (%)
Dowex 1-X8 (Acetate) Column System (Exp. 1)	Lyophilized	-----	49.0	100
	Conversion to K^+ Salt Form	0.218	75.0	100
	1st Organic Solvent Purification	-----	88.0	71.8
	2nd Organic Solvent Purification	0.080	96.1	64.1

[a]The percent recoveries were based on the NL content in the lyophilized material.

recovery based on the NLS content of the lyophilized material. The second purification improved the purity to 60.4% but the recovery based on the NLS content in the lyophilized material was only 41.8%. The results are summarized in Table 3.

As NLS($2K^+$). Since the NLS in pyridinium salt form, NLS($2py^+$), was very hygroscopic, while the potassium salt form was not, the lyophilized material was converted to the potassium salt form via a Dowex-50W(H^+) column as described previously. This process had a further advantage as evidenced by the improvement of the purity of the material from 60.4% to 71.3%. Apparently some positively charged impurities are removed by this process. Further treatment of the 71.3% pure NLS($2K^+$) by the organic solvent purification method did not improve the purity. Attempts to purify the material further by various methods (which will be described later) were unsuccessful.

The 71.3% pure NLS($2K^+$) material was then subjected to Dowex 1-X8 (formate) column chromatography. This separated a sugar contaminant from NLS($2K^+$) thus increasing its purity to 83.0%. This experiment was done in the following manner: a solution of the 71.3% pure sugar material was applied to a Dowex 1-X8 (formate) column (0.5 x 16 cm) and eluted successively with increasing concentrations of pyridinium formate. The contaminant, an anthrone positive but resorcinol negative material (later identified as lactose sulfate) was eluted with 0.15 N pyridinium formate while NLS was eluted with 0.3 N pyridinium formate. After lyophilization of the NLS($2py^+$), containing fraction, the sugar was converted to the potassium salt. This yielded the 83.0% pure NLS($2K^+$) (Table 3).

The lyophilized material containing 48.0% pure NLS($2py^+$) obtained

TABLE 3

PURIFICATION OF NEURAMIN-LACTOSE SULFATE BY ORGANIC SOLVENT

Isolation Method	Salt Form of NLS	Purification Step	$\frac{OD_{260\ m\mu}}{OD_{580\ m\mu}}$	Purity (%)	Recovery[a] (%)
Dowex 1-X8 (Acetate) PyAc Elution	NLS($2py^+$)	Lyophilized	-----	35.9	100
		1st Organic Solvent Purification	-----	51.6	71.4
		2nd Organic Solvent Purification	-----	60.4	41.8
	NLS($2K^+$)	Conversion to K^+ Form	-----	71.3	---
		Dowex 1-X8 (Formate) Column with PyF Elution, then Conversion to K^+ Form	0.582	83.0	---
Dowex 1-X8 (Formate) Column with PyF Elution (Exp. 3)	NLS($2py^+$)	Lyophilized	-----	48.0	100
	NLS($2K^+$)	Organic Purification Twice	0.265	87.5	50

[a]The percent recoveries were based on NLS content of lyophilized residue.

PyF, pyridinium formate. PyAc, pyridinium acetate. py^+, pyridinium. NLS, neuramin-lactose sulfate.

from the Dowex 1-X8 (formate) column (Experiment 3) was converted to NLS ($2K^+$). Upon subjecting this material to the organic solvent purification twice, 87.5% pure NLS($2K^+$) with about 50% recovery was obtained (Table 3). Repetition of the organic solvent purification did not improve the purity any further and the 87.5% purity was the maximum purity obtained by the organic solvent purification method. Electrophoretic analysis of the 87.5% pure NLS($2K^+$) showed that it still contained an U.V. absorbing impurity (Figure 9).

Other Methods of Purification Tested

At this point, three methods commonly used for the purification of carbohydrates, for ion-exchange separation of sulfated-sugars and for removal of nucleotides respectively, were tested in the purification of NLS: a) Adsorption chromatography on a column of charcoal-celite followed by elution with a gradient of aqueous ethanol (66, 67); b) Ion-exchange chromatography on Amberlite IRA-400 (acetate) using a gradient of sulfuric acid for elution (68); c) Treatment with a zinc sulfate-barium hydroxide mixture (69).

None of these 3 procedures afforded any significant increase in purity or any decrease of the ratio of $O.D._{260\ m\mu}$ to the $O.D._{580\ m\mu}$ (resorcinol).

Purification by Gel Filtration

Sephadex G-25. An aqueous solution of 56.1% pure NLS($2py^+$) containing 101 μmoles of NLS in 1.64 ml was applied to a Sephadex G-25 column (1.8 x 100 cm) prepared as described previously. The column was eluted with distilled water at a rate of 0.33 ml per minute and 5-ml

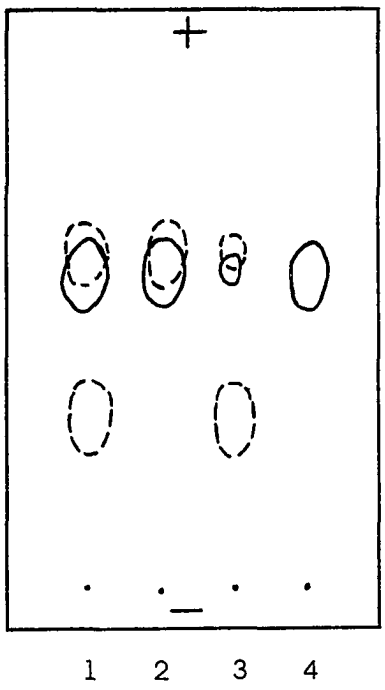


Figure 9. Electrophoretic analyses of neuramin-lactose sulfate preparations. The electrophoresis was run in 0.1 M ammonium acetate buffer (pH 4.5) at 375 volts for 5 hours.

Spots outlined with dotted line: UV abosrbing spots.
Spots outlined with solid line: benzidine-spray positive spots.

Key to the Spots

1 = Crude lyophilized NLS material from ion-exchange column fractionation of the mammary gland extracts.

2 = Organic solvent-purified NLS.

3 = Methanol-insoluble material.

4 = Sephadex G-15 column-purified NLS($2K^+$).

fractions were collected. The elution of NLS was followed by the resorcinol test and the optical density at 260 mμ was used to detect the ultraviolet absorbing impurities. The elution pattern is shown in Figure 10. The sugar was eluted as a single sharp peak while the optical density readings at 260 mμ showed two overlapping peaks: the main peak had a sharp maximum at tube No. 20, coinciding with the maximum of the sugar peak; the second peak was eluted behind the main peak but without complete separation between them.

Fractions 12 to 28 were pooled and the ratio of $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) of the pooled solution was 4.25, as compared with 4.95 before passing through the column. The small improvement of the optical density ratio corresponded to the removal of some of the ultraviolet absorbing material of the second peak. The pooled eluate was lyophilized to produce a white, fluffy residue and the purity was 60.0%. The elution of the sugar peak and most of the impurities in the same fractions indicated that the Sephadex G-25 column was not suitable for further purification.

Sephadex G-10. A sample containing 173 μmoles of $NLS(2K^+)$, which had been subjected to one cycle of purification by the organic solvent method, was dissolved in 2 ml of distilled water and applied to a Sephadex G-10 column (1.4 x 55 cm). The column was eluted at a rate of 0.5 ml per minute and 10-ml fractions were collected. The elution pattern is shown in Figure 11. Although there is a slight displacement of the maxima, the sugar peak and the $O.D._{260\ m\mu}$ peak were eluted in the same fractions and the ratio of $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) of the pooled fractions was identical (1.52) with that obtained before pass-

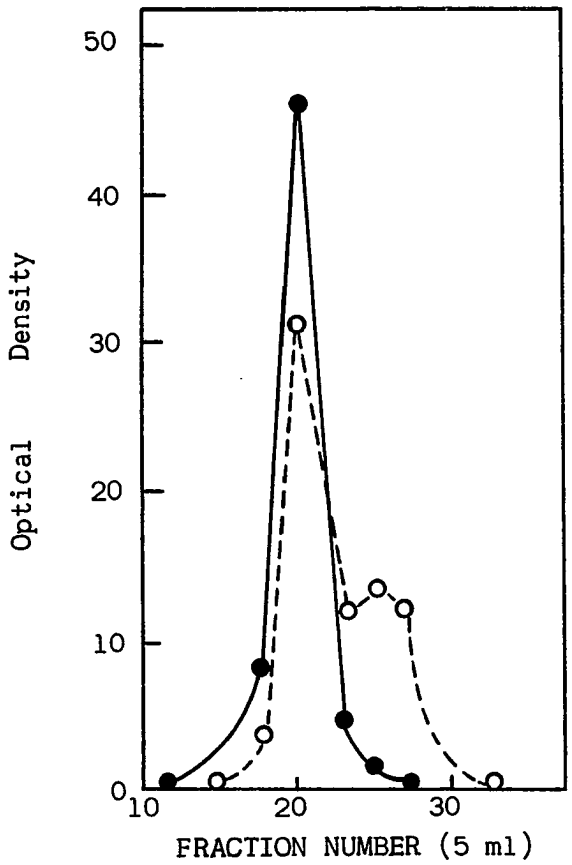


Figure 10. Chromatography of 101 μmole of neuramin-lactose sulfate ($2py^+$) on Sephadex G-25 column (1.8 x 100 cm). Flow rate, 0.33 ml per minute.

—●—●—, $OD_{580\ m\mu}$ (resorcinol reaction). --O--O--, $OD_{260\ m\mu}$.

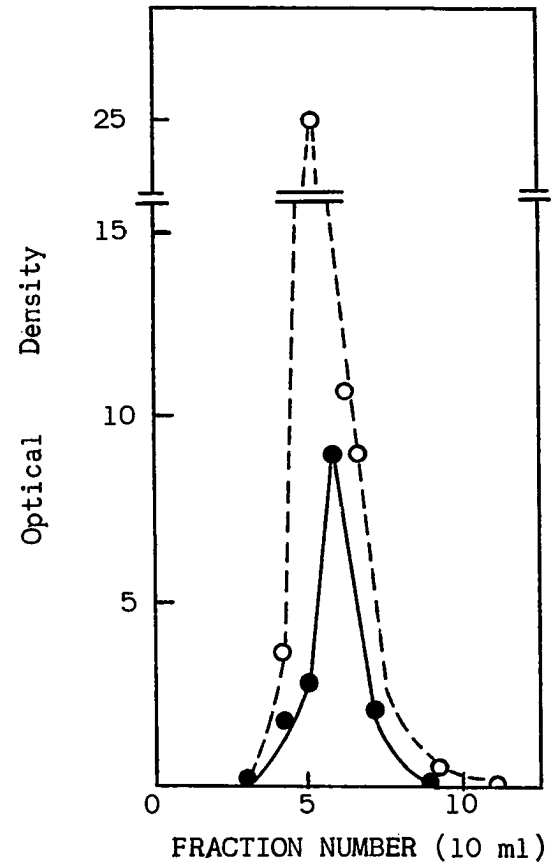


Figure 11. Chromatography of 173 μmole of neuramin-lactose sulfate ($2K^+$) on Sephadex G-10 column (1.4 x 55 cm). Flow rate, 0.5 ml per minute.

—●—●—, $OD_{580\ m\mu}$ (resorcinol reaction). --O--O--, $OD_{260\ m\mu}$.

ing through the column.

Sephadex G-15. Both $NL(K^+)$ and $NLS(2K^+)$ were purified by this method.

$NL(K^+)$. A 200 mg sample of $NL(K^+)$ dissolved in 1 ml of distilled water was applied to a Sephadex G-15 column (1.3 x 175 cm). The column was eluted at a rate of 0.25 ml per minute and 5-ml fractions were collected. The elution pattern (see Figure 12) shows that the sugar peak had its maximum at tube No. 26, while the $O.D._{260\ m\mu}$ peak had its maximum at tube No. 21. The two peaks, however, were not completely separated from each other. The pool of tubes 25 to 30 was analyzed and the ratio of the $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) was only 0.026 as compared with a ratio of 1.67 in the original material.

$NLS(2K^+)$. The $NLS(2K^+)$ sample used in this experiment was the fluffy residue obtained by lyophilization of the pooled effluents of the Sephadex G-10 column (Figure 11). This material contained 155 μmole of $NLS(2K^+)$ as measured by the resorcinol method, and the ratio $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) was 1.52.

The whole sample was dissolved in 2 ml of distilled water and applied to a Sephadex G-15 column (1.3 x 107 cm) in the cold room. The column was eluted at a rate of 4.5 ml per hour, collecting 4.5-ml fractions. The elution pattern is shown in Figure 13. The ultraviolet absorbing material was resolved into two peaks, the first of which overlapped about half of the sugar peak. The second $O.D._{260\ m\mu}$ peak contained the material which was removed by the organic solvent purification procedure and was the slow migrating U.V. absorbing spot in the electrophoretic analysis shown in Figure 9. The $O.D._{580\ m\mu}$ maximum was located

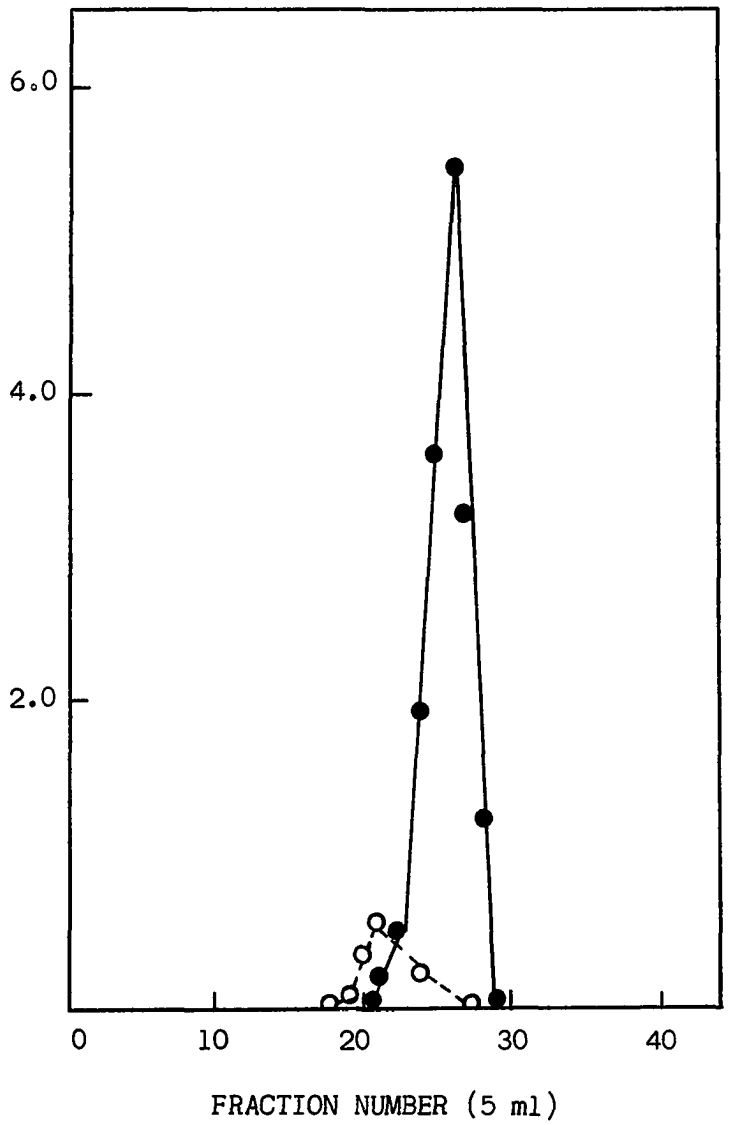


Figure 12. Chromatography of neuramin-lactose (K^+) on Sephadex G-15 column (1.3 x 175 cm). Flow rate, 0.25 ml per minute.

—●—●—, $OD_{580\ m\mu}$ (resorcinol reaction). —o—o—, $OD_{260\ m\mu}$.

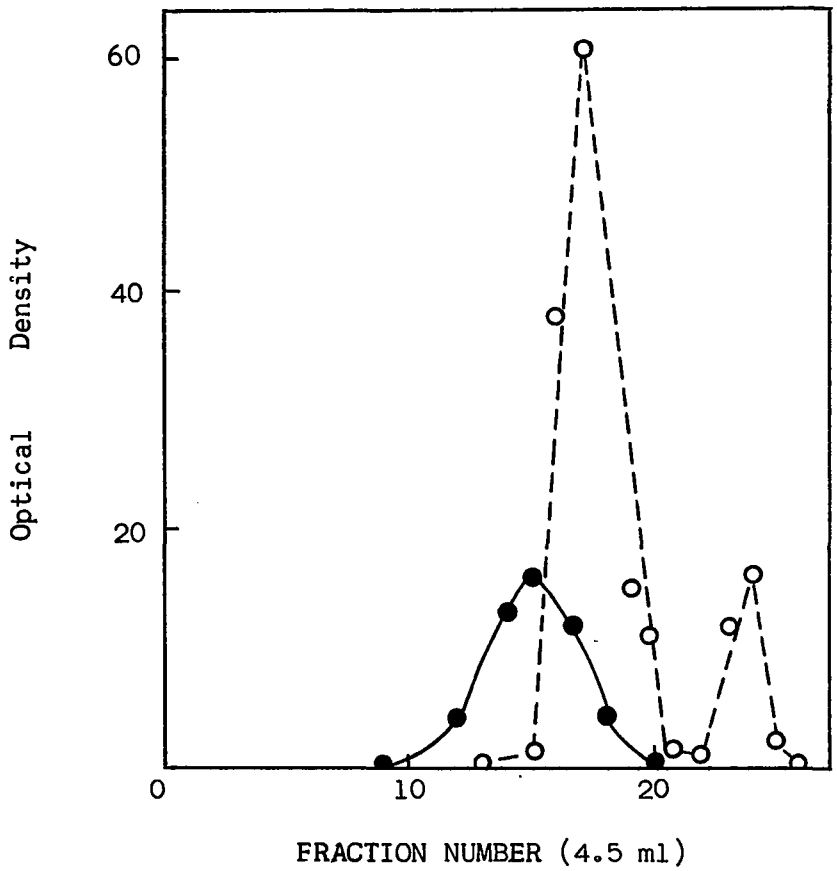


Figure 13. Chromatography of neuramin-lactose sulfate($2K^+$) on Sephadex G-15 column (1x3 x 107 cm).

Flow rate, 4.5 ml per minute.

—●—●—, $OD_{580\ m\mu}$ (resorcinol reaction).
-o--o-, $OD_{260\ m\mu}$.

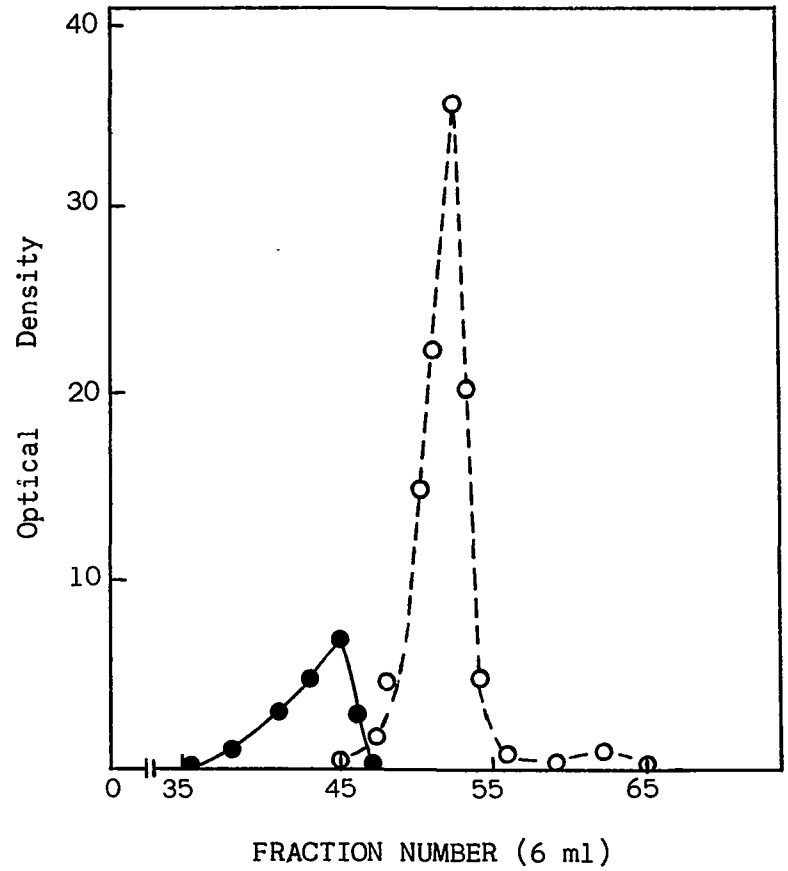


Figure 14. Chromatography of neuramin-lactose sulfate($2K^+$) obtained from fractions 16-21 (Fig. 13) on column of Sephadex G-15 (1.6 x 150 cm).

Flow rate, 0.15 ml per minute.

—●—●—, $OD_{580\ m\mu}$ (resorcinol reaction. -o--o-, $OD_{260\ m\mu}$.

at the tube No. 15, while the O.D.$_{260\ m\mu}$ of this tube was very low.

Fractions 10 to 15 were pooled and the ratio O.D.$_{260\ m\mu}$ to O.D. 580 mμ (resorcinol) was 0.002. The lyophilized white, powdery product was 100% pure NLS($2K^+$) as determined by the resorcinol method.

Fractions 16 to 21 were pooled separately and the optical density ratio of this pool was 3.01. The lyophilized, slightly yellow residue was 41.8% pure. This material was subjected to a second Sephadex G-15 column chromatography using a column 1.6 x 150 cm. This column was eluted at room temperature at the rate of 0.15 ml per minute collecting 6-ml fractions. The elution pattern is shown in Figure 14. This time the resorcinol positive peak is almost completely separated from the O.D.$_{260\ m\mu}$ peak. Thus, by increasing the length of the column and using a smaller sample size it was possible to free the sugar from the 260 mμ absorbing material. The optical density ratio of the pool of tubes 36 to 45 was virtually zero and a 100% pure NLS($2K^+$) was recovered by lyophilization.

Efficiency of the Improved Isolation and Purification Methods

On the basis of the findings described in the previous experiments an improved method for the isolation and purification of NLS was developed. A careful study of the recovery of the various steps of the new method was carried out in Experiment 5. A tissue extract was prepared, as previously described, utilizing 1,000 g of mammary glands which had been stored for 8 to 10 months in the deep freezer. The extract was fractionated using the Dowex 1-X8 (formate) column and the elution pattern is shown in Figure 5.

Lyophilization of the pool of NLS-containing fractions produced a light yellow sticky gum containing 371.14 μmoles of NLS($2py^+$).

In order to obtain the potassium salt of NLS the NLS($2py^+$) residue was dissolved in 20 ml of distilled water and passed through a Dowex-50W(H^+) column (2.9 x 48 cm). The column was eluted thoroughly with water until the effluent showed neutral pH. The effluent was immediately neutralized to pH 5.9 with 1 N potassium hydroxide solution. The final solution containing 357.51 μmoles of NLS($2K^+$) in a volume of 407 ml was lyophilized. The product obtained at this stage was a white crystalline residue weighing 2.763 g. The purity of NLS($2K^+$) was only 10.2% and the main impurity was potassium formate. The ratio $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) was 0.365.

The residue was dissolved in a minimum amount of water (8 ml) and applied to a Sephadex G-15 column (2 x 175 cm). The column was then eluted at a rate of 0.67 ml per minute and 10-ml fractions were collected. The elution was followed by the resorcinol assay and by the $O.D._{260\ m\mu}$. The elution pattern (Figure 15) shows that the resorcinol positive peak ($O.D._{580\ m\mu}$) partially overlaps one of the two peaks of $O.D._{260\ m\mu}$. All sugar-containing fractions (tubes 16 to 31) were pooled and analysis of the pool gave an $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) ratio of 0.283 and a 100% recovery of NLS($2K^+$). Lyophilization yielded 428 mg of white powdery residue. The purity of NLS($2K^+$) was 65.2%.

The lyophilized material was dissolved in 2 ml of distilled water and rechromatographed on the same column. The elution rate was 0.7 ml per minute and 7-ml fractions were collected. The elution pattern (Figure 16) shows that the sugar peak was virtually free of ultraviolet

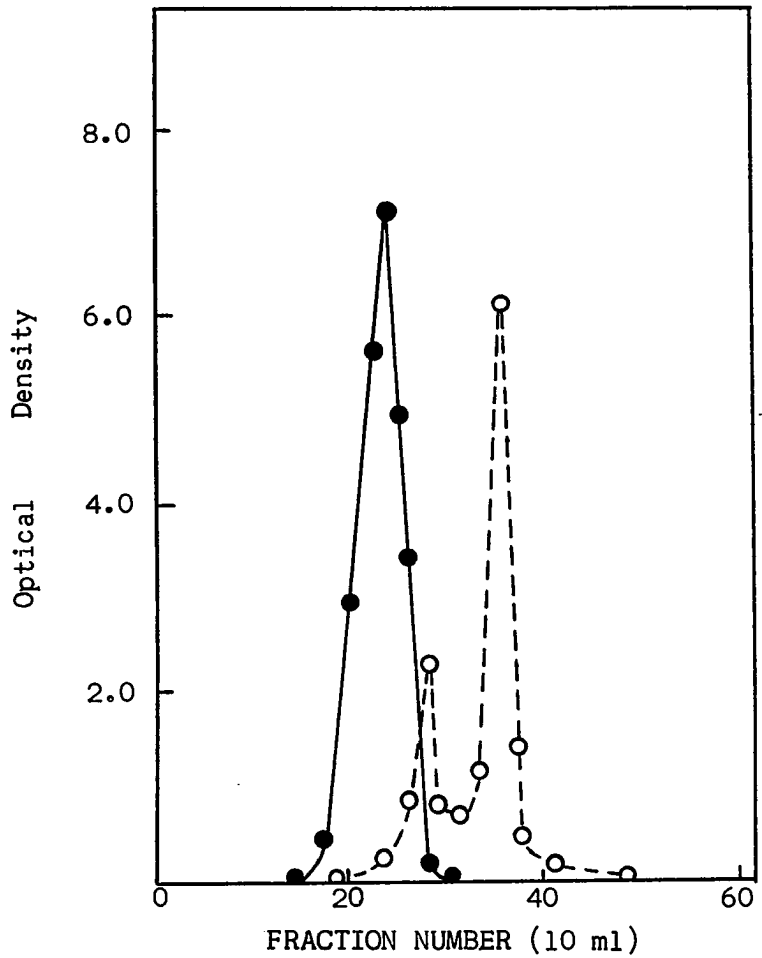


Figure 15. Chromatography of 357.5 μmole of neuramin-lactose sulfate($2K^+$) obtained from Dowex 1-X8 (formate) column (Exp. 5) on column of Sephadex G-15 (2 x 175 cm).

Flow rate, 0.67 ml per minute. —●—●—, OD_{580} mμ (resorcinol reaction. —○—○—, OD_{260} mμ.

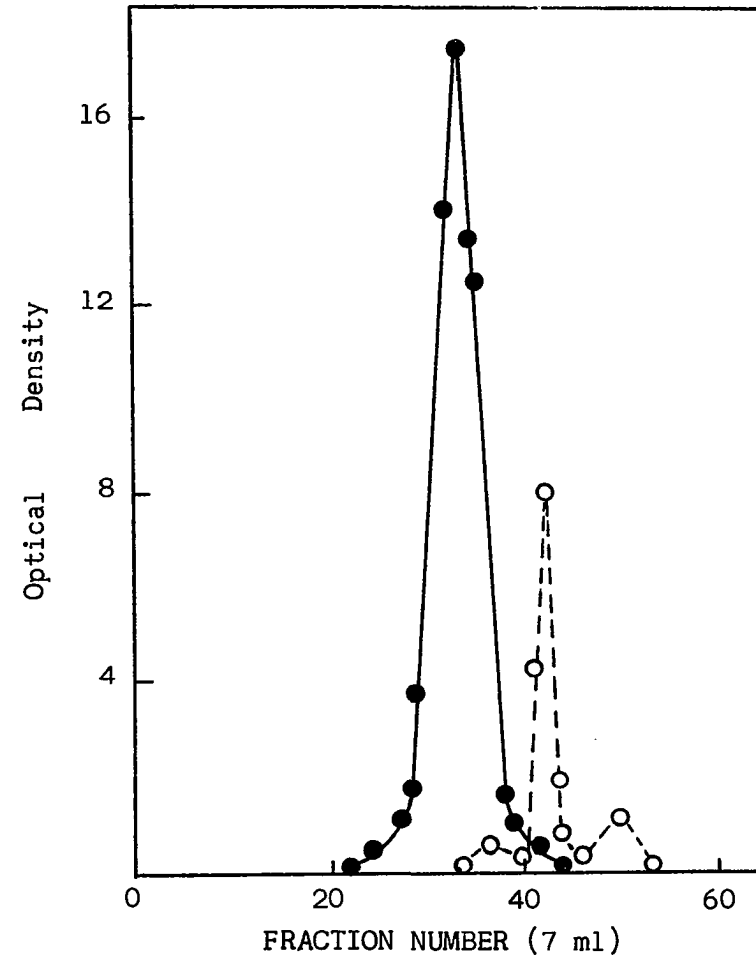


Figure 16. Chromatography of 357.5 μmoles of neuramin-lactose sulfate($2K^+$) obtained from previous Sephadex purification (Fig.15) on column of Sephadex G-15 (2 x 175 cm).

Flow rate, 0.7 ml per minute. —●—●—, OD_{580} mμ (resorcinol reaction). —○—○—OD_{260} mμ.

absorbing impurities. The small impurity peak within the sugar peak was negligible as further evidenced by the very low optical density ratio of the pooled fractions. Tubes 24 to 40 were pooled to give a total volume of 115.8 ml. The pool contained 340.3 μmoles of NLS($2K^+$) and had an $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) ratio of 0.013. This solution was then lyophilized to yield 277.10 mg of a white powdery residue, and the final purity of NLS($2K^+$) was 97.3%. The quantitative data and recoveries of each step are summarized in Table 4.

The over all recovery during the purification of NLS after its isolation by ion-exchange column chromatography was 91.7%, and the final purity was 97.3%. These results were far superior to those obtained by the conventional organic solvent method. The latter method gave a 41.8% recovery of 60.4% pure NLS($2py^+$) from the material isolated by Dowex 1-X8 (acetate) column system and approximately 50% recovery of 87.5% pure NLS($2K^+$) from the material fractionated by Dowex 1-X8 (formate) column system.

Structural Studies

Analysis of Neuramin-lactose Sulfate

Molar ratio of NANA to lactose. The content of NANA and lactose in NLS was determined by the resorcinol and anthrone methods, respectively. The average value for the molar ratio, obtained from three preparations of NLS was 1.03, as shown in Table 5.

Determination of sulfate. The amount of sulfate ester in the 97.3% pure NLS preparation (Experiment 5) was determined spectrophotometrically by the method of Jones *et al.* (70) after mineralizing a 1.196

TABLE 4

QUANTITATIVE DATA AND STEPWISE RECOVERIES IN EXPERIMENT 5

Step	Salt Form	Total Sialic Acids (μmole)	Dry Weight (gram)	$\frac{OD_{260\ m\mu}}{OD_{580\ m\mu}}$*	Purity (%)	Recovery (%)
Tissue Extraction	----	4,802.0	------	1.35	----	100
Column Fractionation On Dowex 1-X8 (Formate)	NL(py^+)	3,692.6	------	-----	----	
	NANA(py^+)	698.0	------	-----	----	99.3[a]
	NLS($2py^+$)	371.1	------	-----	----	
Conversion to K^+ Salt	NLS($2K^+$)	357.5	2.7630	0.365	10.2	96.3[b]
1st Sephadex	NLS($2K^+$)	353.6	0.4280	0.283	65.2	95.2[b]
2nd Sephadex	NLS($2K^+$)	340.3	0.2710	0.013	97.3	91.7[b]

[a]Over all recovery of sialic acids (NL, NANA, NLS) after column fractionation.

[b]NLS recoveries are based on the NLS obtained by column fractionation (371.1 μmoles). Recovery of NLS during Sephadex purification was 95.3%.

*Resorcinol test.

μmole sample of the NLS with nitric acid (71). The result (Table 5) shows 1.06 moles of sulfate group per mole of NLS.

Determination of nitrogen. Nitrogen content in this NLS preparation was determined by Galbraith Laboratories Inc., Knoxville, Tennessee. The value, expressed in number of atoms of nitrogen per mole of NLS, was 0.92, as shown in Table 5.

Specific rotation. Optical rotations were measured as previously described using a concentration of 1.54 g NLS per 100 ml of aqueous solution. The specific rotation remained fairly constant at room temperature, as shown in Table 5.

TABLE 5

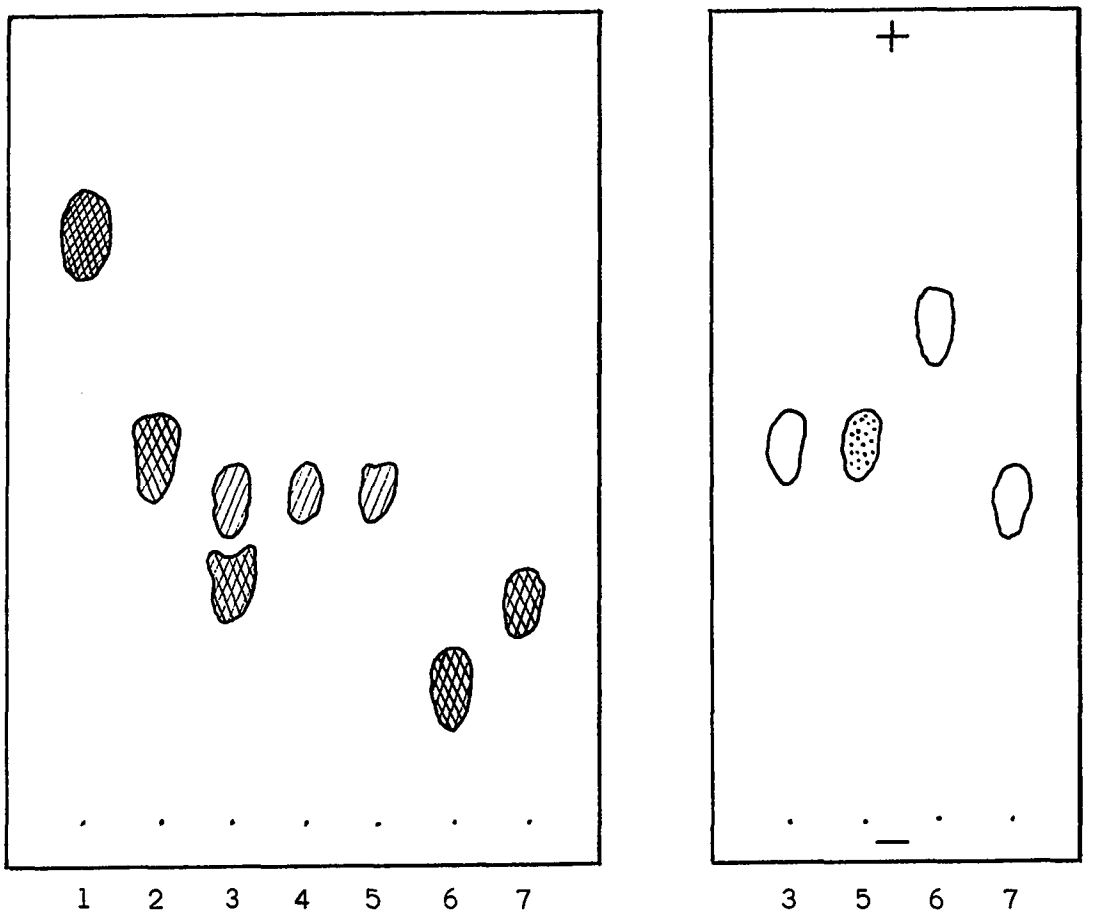
ANALYSIS OF NEURAMIN-LACTOSE SULFATE

$\frac{N\ (atom)}{NLS(mole)}$	$\frac{SO_4(mole)}{NLS(mole)}$	$\frac{NANA(mole)}{Lactose(mole)}$	$[\alpha]_D^{24}$, H_2O, C = 1.54
0.92[a]	1.06	1.03[b]	+22.79 → +23.24 (5 min) (30 min) → +23.83 → +23.83 (1 hr) (24 hr)

[a]Analyzed by Galbraith Laboratories, Inc., Knoxville, Tenn.

[b]Average value of three preparations of NLS.

Neuraminidase hydrolysis of NLS. A sample (8.96 mg) of purified NLS was incubated for 24 hours at 37° with bacterial neuraminidase at pH 5.2. After the removal of the enzyme, the hydrolysate was analyzed by paper chromatography and paper electrophoresis. The results are shown in Figure 17.



A. Paper Chromatography B. Electrophoresis

Figure 17. Paper chromatography and paper electrophoresis of neuraminidase-hydrolysate of neuramin-lactose sulfate.

A. Paper chromatography: solvent 10, Isopropanol-H_2O (4:1); descending technique; aniline-diphenylamine spray; , blue; , pink.

B. Paper electrophoresis: 0.1 M ammonium acetate-acetic acid buffer (pH 4.5); 375 volt; 5 hr; benzidine spray; , brown; , bluish grey.

Key to the spots

1 = Glucose.
2 = Lactose.
3 = Neuraminidase hydrolysate of NLS.
4 = Crystalline NANA (from rat mammary gland extract).
5 = Crystalline NANA (commercial synthetic).
6 = NLS.
7 = NL.

The paper chromatogram of the enzyme hydrolysate produced two spots, one corresponded with free NANA and the other (lactose sulfate) had a migration close to that of NL. No residual (unhydrolyzed) NLS was observed. The electrophoretic analysis of the enzymic hydrolysate showed a single spot which migrated as free NANA (free NANA and lactose sulfate are not separated by this system). No residual NLS was observed, thus confirming the complete enzyme hydrolysis of NLS.

Permethylation

Lactose. The permethylation of lactose was carried out in order to find experimental conditions suitable for the permethylation of carbohydrates of low molecular weight with this new method.

The methylsulfinyl carbanion (62.5 meq) was generated by reacting 1.5 g of powdered sodium hydride with 50 ml of dry dimethyl sulfoxide at 70° for 30 minutes as described under Methods. For the preparation of 5 meq of lactose alkoxide, 1.8 g of lactose in 5 ml of dry dimethyl sulfoxide was added to the carbanion solution and allowed to react at room temperature for 20 minutes. The alkoxide was methylated by addition of an excess of redistilled methyl iodide (10 ml), maintaining the reaction mixture at room temperature. The time course of the methylation reaction was studied by taking out 0.5-ml aliquots at 0, 20, 75, 135 and 180 minutes after the addition of methyl iodide was completed. The reaction was stopped by adding 0.5 ml of water to each aliquot. The methylated sugar was isolated by chloroform extraction and analyzed by thin layer chromatography with solvents 1 and 4. Every sample showed two spots migrating close to each other, while the spot of unreacted lactose was always absent. Thus, lactose was completely permethylated when the

addition of the methyl iodide was completed. Typical thin layer chromatograms of permethylated lactose are shown in Figure 18. The two spots observed in the chromatograms of permethylated lactose (PML) correspond to the α- and β-methyl glycosides, respectively.

The isolated PML was dried overnight at room temperature in an evacuated desiccator over phosphorus pentoxide. The yield of dry slightly yellow powdery product was 83.2%.

A solution of the powdery product in spectroquality carbon tetrachloride was placed in a sodium chloride cell and the infrared spectrum was recorded. The complete disappearance of the OH band (3200-3700 cm^{-1} region) confirmed the complete permethylation of lactose.

Neuramin-lactose(K^+). A solution containing 1.49 mmoles of NL(K^+) in 2 ml of dry dimethyl sulfoxide (17.88 meq of hydroxyl and carboxyl groups in this NL sample) was added dropwise to a solution containing 31.2 meq of methylsulfinyl carbanion in 50 ml of dry dimethyl sulfoxide and the mixture was allowed to react at room temperature for 40 minutes. An excess of dry methyl iodide (12 ml) was added dropwise to the alkoxide solution, maintaining the reaction mixture at room temperature. The mixture was kept at room temperature and aliquots were removed at: 2, 15, 25, 50, 90 minutes and 26.5 hours, respectively. Examination of the sugar products present in these aliquots by thin layer chromatography indicated that the permethylation reaction was complete within 25 minutes, i.e., the aliquots taken at 25, 50, 90 minutes and 26.5 hours had only two spots with R_f-values identical to those shown in Figure 18. The sample taken after 2 minutes had 3 spots: one dark spot at the origin and two light spots which coincided with those of the 25

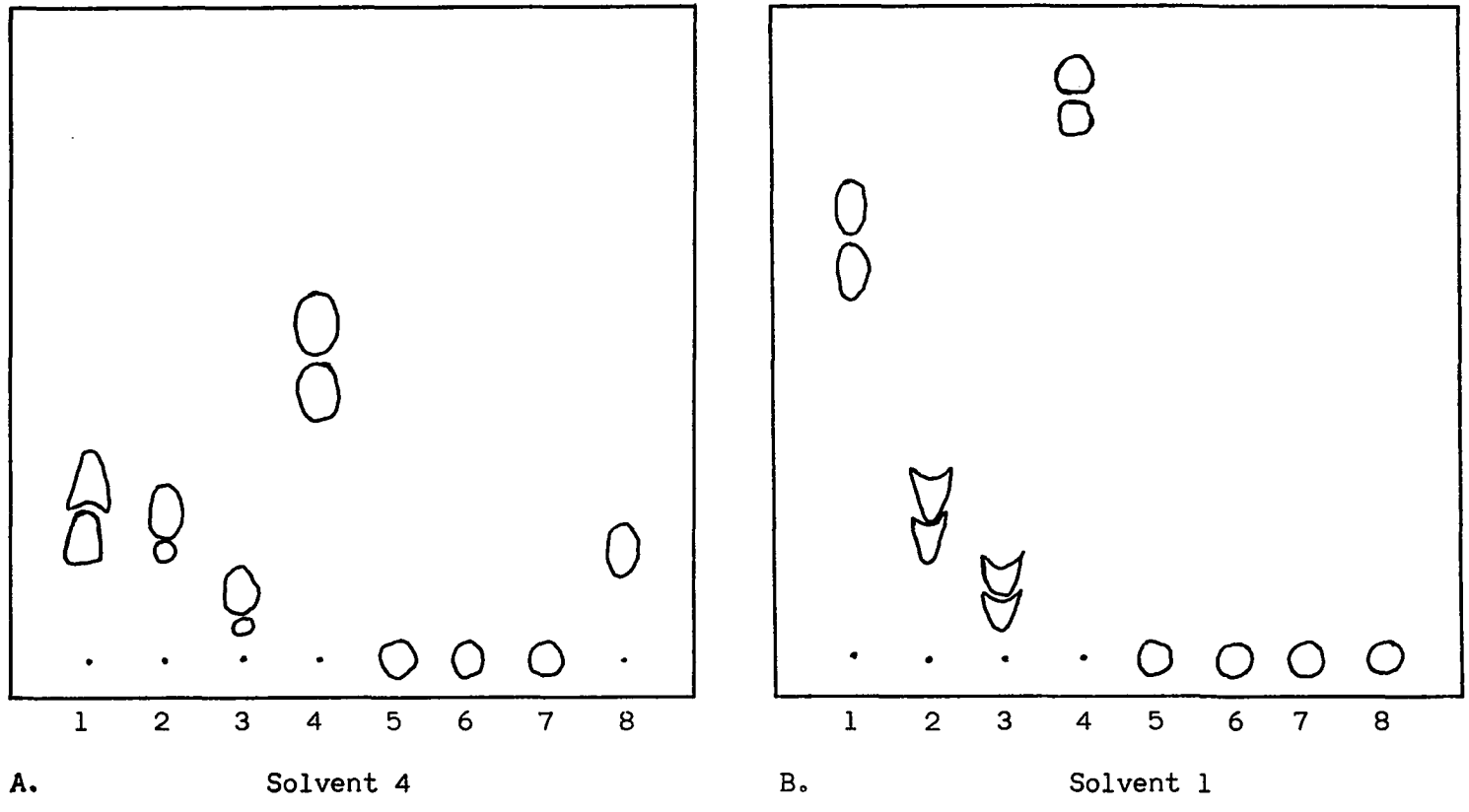


Figure 18. Thin layer chromatography of permethylated sugars. The plates were developed unidimensionally twice: Plate A with Solvent 4, water-saturated n-butanol, and Plate B with Solvent 1, ammonium hydroxide-water-methyl ethyl ketone (1:17:200). The plates were charred after spraying with concentrated sulfuric acid.

Key to the spots

1 = Permethylated NL.
2 = Permethylated neuraminidase-hydrolysate of NLS.
3 = Permethylated NLS.
4 = Permethylated lactose.
5 = NL(K^+).
6 = NLS($2K^+$).
7 = Neuraminidase-hydrolysate of NLS.
8 = Lactose.

minute sample. The 15 minute specimen also had 3 spots, but the spot at the origin was light while the spots with the same R_f as those of the 25 minute sample were dark. As shown in Figure 18, permethylated neuramin-lactose (PMNL) also had two spots which migrated close together and which are also believed to correspond to α- and β- methyl glycosides of PMNL, respectively.

The PMNL was isolated from the reaction mixture by the chloroform extraction method, as described under Methods. The yellow syrup obtained was dried overnight in an evacuated desiccator over phosphorus pentoxide. The final product was a yellow sticky gum and the yield was 92.5% (Table 6).

This yellow sticky gum containing permethylated NL was dissolved in 1 ml of distilled water and applied to a Sephadex G-15 column (1.3 x 170 cm). The column was eluted with distilled water at a rate of 0.5 ml per minute and 9-ml fractions were collected. The elution was followed by the anthrone method, and the fractions containing the PMNL were pooled. The pool was evaporated to a small volume in the rotary evaporator under diminished pressure at room temperature and then lyophilized to yield a white powdery product.

The sticky gum isolated by chloroform extraction and the powdery product obtained by Sephadex column chromatography were dissolved in spectroquality carbon tetrachloride and the infrared spectra were recorded. The disappearance of the OH band (3200-3700 cm^{-1} region) in both spectra indicated that the permethylation of NL was complete (Figure 19). Figure 20 was placed in the same page to make comparison.

Neuramin-lactose sulfate($2K^+$). Three separate permethylation

TABLE 6

YIELDS OBTAINED IN THE PERMETHYLATION OF ACIDIC OLIGOSACCHARIDES AND FRACTIONATION OF THEIR RESPECTIVE ACID HYDROLYSATES BY PREPARATIVE PAPER CHROMATOGRAPHY

Reaction	Starting Material	Product	Molar Ratio	Yield (%)
Permethylation	NL	PMNL	----	92.5
	NLS	PMNLS	----	92.8
Acid Hydrolysis	PMNL	2,3,6-TMGlu	1.00	84.7 (78.3)[a]
		2,4,6-TMGal	1.00	84.6 (78.2)[a]
	PMNLS	2,3,6-TMGlu	1.00	80.2 (74.4)[a]
		2,4-DMGal	1.07	85.5 (79.3)[a]

[a]Over all % yields of methylated monoses based on the amounts of initial free acid oligosaccharides (NL, NLS).

PMNL, permethylated NL; PMNLS, permethylated NLS; 2,3,6-TMGlu, 2,3,6-Tri-O-methyl-D-glucose; 2,4,6-TMGal, 2,4,6-Tri-O-methyl-D-galactose; 2,4-DMGal, 2,4-Di-O-methyl-D-galactose.

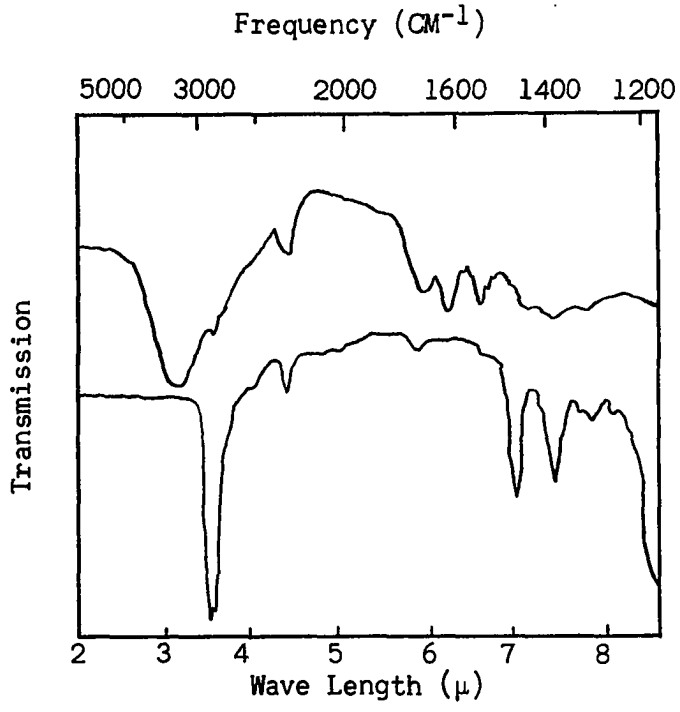


Figure 19. Infrared spectra of neuramin-lactose in KBr pellet (upper curve) and permethylated neuramin-lactose in CCl_4 (lower curve).

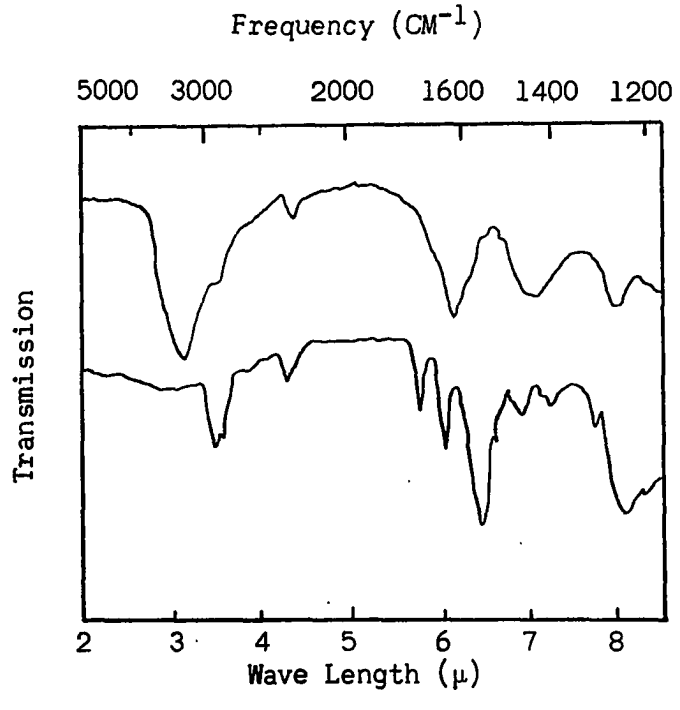


Figure 20. Infrared spectra of neuramin-lactose sulfate in KBr pellet (upper curve) and permethylated neuramin-lactose sulfate in CCl_4 (lower curve).

experiments were carried out, and the final results were identical in each.

In the first experiment, which was carried out as a pilot experiment, a 1 to 9 ratio of functional groups in NLS to sodium hydride was used. For generation of the methylsulfinyl carbanion, 1.98 meq of powdered sodium hydride in 3 ml of dimethyl sulfoxide was heated at 70° for 35 minutes. To generate NLS alkoxide, 14 mg of NLS($2K^+$) dissolved in 1 ml of dimethyl sulfoxide was added to the carbanion solution and allowed to react for 8 hours at room temperature. The methylation reaction was carried out using 2 ml of methyl iodide. After addition of the reagent, the mixture was kept for 15 hours at room temperature. The reaction was stopped by addition of 0.5 ml of water. After isolation, the product was analyzed by thin layer chromatography.

In the second experiment, a lower ratio of functional groups in NLS($2K^+$) to sodium hydride (1:2) was used. The methylsulfinyl carbanion was generated by heating 85.6 mg of powdered sodium hydride suspension in 5 ml of dimethyl sulfoxide for 55 minutes at 70°. A solution of 100.55 mg of 100% pure NLS($2K^+$) in 2 ml of dimethyl sulfoxide was added to the carbanion solution and allowed to react at room temperature for 8 hours. For methylation, 2 ml of methyl iodide was added to the alkoxide solution and the mixture was kept at room temperature for 15 hours. The product was isolated and examined by thin layer chromatography and by infrared spectroscopy.

In the third experiment, a 53.1 mg portion of the permethylated neuramin-lactose sulfate (PMNLS) obtained from the second experiment was permethylated for the second time. The methylsulfinyl carbanion was gen-

erated by heating 45 mg of powdered sodium hydride in 4 ml of dimethyl sulfoxide at 70° for one hour. The PMNLS, dissolved in 0.6 ml of dimethyl sulfoxide, was added to the carbanion solution and kept at room temperature for 21 hours. The methylation reaction was carried out by addition of 1.5 ml of methyl iodide and the mixture was kept at room temperature for 5 hours. After isolation, the product was examined on thin layer plates and the infrared spectrum was recorded.

Several attempts to isolate PMNLS by the organic solvent extraction method utilized for permethylated lactose and permethylated neuramin-lactose were not successful because PMNLS is more soluble in water than in chloroform. Therefore, the PMNLS remains in the aqueous layer in a chloroform-water partition system. In order to isolate the PMNLS a modified extraction procedure was developed. The reaction mixture was first evaporated in the rotary evaporator at room temperature under diminished pressure until just a few ml of volume was left (mostly dimethyl sulfoxide). This was then extracted three times with 10-ml portions of chloroform, which removed the dimethyl sulfoxide, PMNLS and some of the sodium iodide. The bulk of the sodium iodide, however, was not extracted and remained as a white crystalline residue. The chloroform was removed under diminished pressure and the brownish oil obtained was extracted three times with 10-ml portions of ethyl ether, which removed most of the dimethyl sulfoxide and left the PMNLS as a gum residue. By repeating this process once more, most of the dimethyl sulfoxide and sodium iodide could be removed from the reaction mixture. This method was utilized only for the rapid preparation of small samples for thin layer chromatography. For the isolation of pure preparations of PMNLS, the Sephadex G-15 column

chromatographic method was used, as described under Methods.

The reaction mixture obtained in the second permethylation experiment, for example, was concentrated to 3 ml by evaporation under diminished pressure, applied to the Sephadex G-15 column, and eluted with distilled water. Upon lyophilization of the sugar fractions, a white powdery product was obtained with 92.8% over-all yield (Table 6).

Analyses of the products obtained from the three permethylation experiments by thin layer chromatography showed the same spots observed in Figure 18 and no spot of free unreacted NLS was detected. The chromatoplates of PMNLS also showed two closely-migrating spots which correspond to the α- and β-methyl glycosides, respectively. To confirm the complete methylation, infrared spectra were recorded of saturated solutions of PMNLS in spectroquality methylene chloride and carbon tetrachloride solutions, using a sodium chloride cell. The infrared spectrum in carbon tetrachloride (Figure 20) showed no appreciable absorption in the OH band region (3200-3700 cm^{-1}), indicating that the methylation was complete.

Attempts to record the infrared spectrum of PMNLS in other solvents, e.g., chloroform, tetrachloroethylene and carbon disulfide, were hindered by the extremely low solubility of PMNLS in these solvents. Attempts to prepare potassium bromide discs were abandoned because the discs were always cloudy, perhaps due to the extreme hygroscopicity of PMNLS. This property is probably also responsible for the failure of the "mull" technique using a mineral oil (Nujol).

Neuraminidase-hydrolysate of neuramin-lactose sulfate. The hydrolysate (NANA plus lactose-sulfate) obtained from 8.90 mg of NLS($2K^+$)

was permethylated using essentially the same procedure described for the permethylation of NLS($2K^+$).

The methylsulfinyl carbanion was generated by heating 0.177 meq sodium hydride in 2 ml dimethyl sulfoxide at 70^o for 30 minutes. The dried enzyme hydrolysate (0.011 meq) dissolved in 1 ml of dimethyl sulfoxide was added to the cooled carbanion solution and stirred at room temperature for 18 hours. The solution was then treated with 2 ml of methyl iodide and stirred for another 18 hours at room temperature.

After stopping the reaction by addition of 0.5 ml of water, the solution was evaporated in vacuo in the rotary evaporator to a final volume of 3 ml. The oil residue was then extracted with 10-ml portions of ethyl ether to remove most of the dimethyl sulfoxide and leave a yellow gum residue. The methylated sugar was then purified by Sephadex G-15 column chromatography and lyophilization of the sugar-containing fractions gave a white powdery product. The product was examined on thin layer chromatographic plates (Figure 18) and showed two closely migrating spots, while unreacted NANA and lactose sulfate (which remain at the origin with these solvent systems) were not detected.

Acid Hydrolysis of Permethylated Sugars

Permethylated lactose (PML). A solution containing 1.6 g of PML in 15 ml of 0.5 N sulfuric acid was refluxed and 7.5-ml aliquots were taken out after 4 and 6 hours of hydrolysis, respectively. The hydrolysates were decolorized with activated charcoal which was then removed by filtration. The clear solutions were neutralized with saturated barium hydroxide solution and filtered to remove the precipitate of barium sulfate. The sugar materials were then examined by the thin layer and paper

chromatographic methods.

Typical thin layer chromatograms in three different solvent systems are shown in Figure 21, and they were obtained with the 6 hour hydrolysate described above. Two spots were observed: the spot with high R_f-value is that of 2,3,4,6-Tetra-O-methyl-D-galactose and the spot with a lower R_f-value corresponds to 2,3,6-Tri-O-methyl-D-glucose. The 4 hour hydrolysate gave the same results. These two spots were detected by p-anisidine hydrochloride spray reagent, which reacts only with reducing sugars. By charring the chromatogram with concentrated sulfuric acid, an unidentified additional faint spot appeared slightly ahead of the tetramethyl galactose. Paper chromatograms of both hydrolysates, which were developed in solvent 1 and sprayed with p-anisidine hydrochloride also showed the same two sugar spots.

Permethylated neuramin-lactose (PMNL). A sample of PMNL (0.372 g) was hydrolyzed by refluxing in 10 ml of 0.5 N aqueous sulfuric acid solution. Aliquots were taken after 4 hours and 6 hours, respectively. The hydrolysates were processed and chromatographed as previously described. The thin layer chromatogram of both samples treated with p-anisidine spray showed two spots which had R_f-values identical with those of authentic 2,3,6-Tri-O-methyl-D-glucose and 2,4,6-Tri-O-methyl-D-galactose, respectively, as shown in Figure 21. An unidentified additional faint spot slightly ahead of the 2,3,6-Tri-O-methyl-D-glucose spot appeared after spraying with concentrated sulfuric acid and charring. Paper chromatograms developed in solvent 1 by both ascending and descending techniques showed two spots by p-anisidine spray reagent. These spots had R_f-values identical to authentic 2,3,6-Tri-O-methyl-D-glucose and 2,4,6-

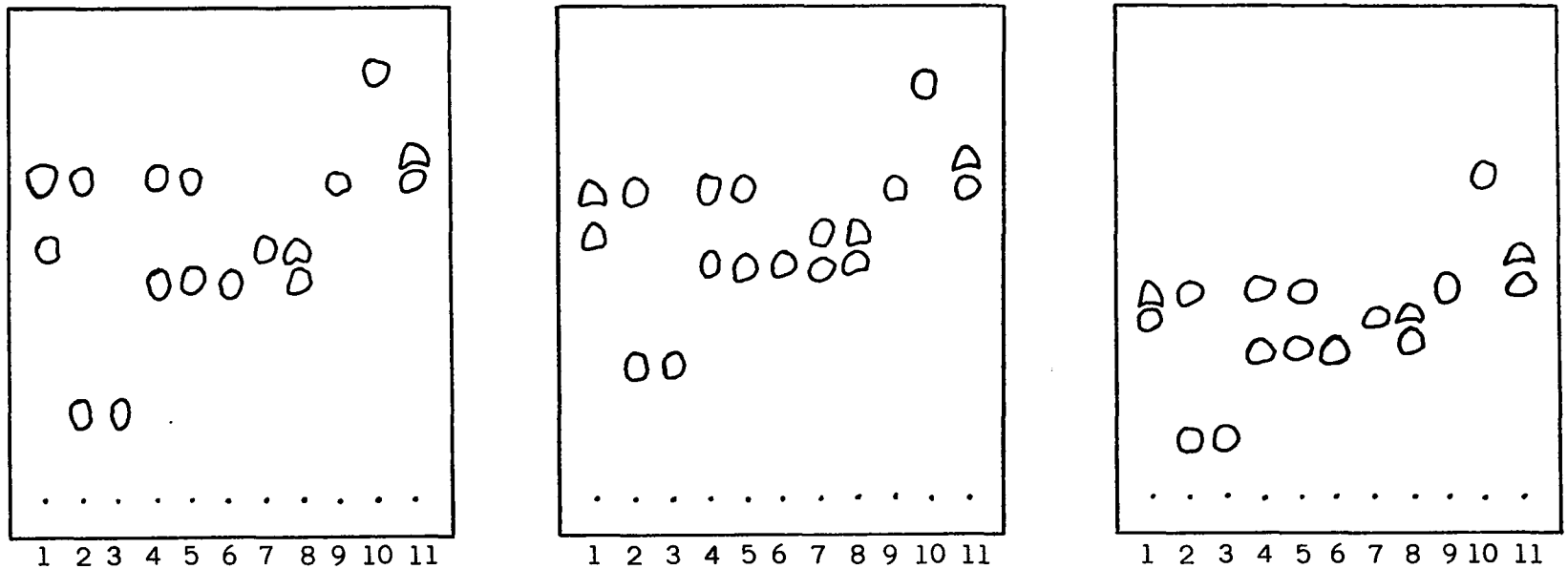


Figure 21. Thin layer chromatography of acid hydrolysates of permethylated sugars. The chromatoplates were developed unidimensionally by ascending technique and sprayed with p-anisidine hydrochloride reagent.

Plate A was developed 3 times in Solvent 1, NH_4OH-H_2O-methyl ethyl ketone (1:17:200).
Plate B was developed 4 times in Solvent 2, benzene-ethanol (20:5).
Plate C was developed 5 times in Solvent 3, benzene-ethanol-water-NH_4OH (200:47:15:1).

Key to the spots

1 = Acid hydrolysate of PMNL.
2 = Acid hydrolysate of PMNLS.
3 = Authentic 2,4-Di-O-methyl-D-galactose.
4 = Acid hydrolysate of permethylated neuraminidase-hydrolysate of NLS.
5 = Acid hydrolysate of permethylated P_5 material in Exp. 5 (see Fig. 5).
6 = Authentic 2,3,4-Tri-O-methyl-D-galactose.
7 = Authentic 2,4,6-Tri-O-methyl-D-galactose.
8 = Mixture of 6 and 7.
9 = Authentic 2,3,6-Tri-O-methyl-D-glucose.
10 = Authentic 2,3,4,6-Tetra-O-methyl-D-glucose.
11 = Acid hydrolysate of permethylated lactose.

Tri-O-methyl-D-galactose, respectively. PMNL hydrolysates obtained by refluxing in 1.0 N sulfuric acid for 4 hours gave the same results.

Permethylated neuramin-lactose sulfate (PMNLS). A sample of 45.05 mg of purified PMNLS was hydrolyzed by refluxing in 3.0 ml of 0.5 N aqueous sulfuric acid for 7 hours. The hydrolysate was neutralized with saturated aqueous barium hydroxide, filtered, and concentrated to a small volume in the rotary evaporator at 30°. The sugar solution was then examined by thin layer chromatography with four different solvent systems. The results obtained with three different solvent systems are shown in Figure 21. The chromatogram developed with water-saturated butanol (not shown in Figure 21) also have good separation of the methylated isomeric monoses. As shown in Figure 21, the chromatograms of the hydrolysate had two spots; the fast moving spot had an R_f-value identical to authentic 2,3,6-Tri-O-methyl-D-glucose, while the slow spot had an R_f-value identical to authentic 2,4-Di-O-methyl-D-galactose. Paper chromatographic analysis of the hydrolysate in solvent 1 also produced the same two spots.

Ionophoretic analysis of the hydrolysate in 0.05 M borate buffer (pH 9.2) showed one spot which migrated as authentic 2,4-Di-O-methyl-D-galactose, $M_{glucose}$ = 0.19 (uncorrected), and another spot of 2,3,6-Tri-O-methyl-D-glucose that remained at the origin as expected.

Thus, refluxing of PMNLS in 0.5 N sulfuric acid for 7 hours caused the complete hydrolytic cleavage of all neuraminyl, glycosidic and sulfate ester linkages.

Analysis of another hydrolysate in which PMNLS was refluxed in 0.5 N sulfuric acid for only 5 hours showed, in addition to the two spots observed in Figure 21, an additional spot at the origin. The additional

spot most likely contained 2,4-Di-O-methyl-D-galactose-6-sulfate, since the sulfate ester linkage is relatively resistant to acid hydrolysis.

Permethylated neuraminidase-hydrolysate of NLS. The permethylated product of the neuraminidase-hydrolysate of 8.90 mg NLS($2K^+$) was dissolved in 0.8 ml of 0.5 N sulfuric acid and refluxed for 7.5 hours. The hydrolysate was cooled, neutralized with saturated aqueous barium hydroxide and filtered as described under Methods. Thin layer chromatograms of the hydrolysate in three different solvent systems (Figure 21) showed two spots: the fast moving spot has an R_f-value identical to that of authentic 2,3,6-Tri-O-methyl-D-glucose and the slow moving spot has an R_f-value identical to that of authentic 2,3,4-Tri-O-methyl-D-galactose.

Fractionation of the Acid Hydrolysates by Preparative Paper Chromatography

Hydrolysate of permethylated neuramin-lactose (PMNL). A sample (0.1949 g) of the light-yellow residue obtained from the hydrolysis of the PMNL was dissolved in 1 ml of methanol and applied, as a narrow band, on a sheet of prewashed Whatman No. 3MM paper (46.5 cm wide and 60 cm long). The chromatogram was developed by ascending irrigation in solvent 1, and the two sugars were located and identified with the aid of four 5 mm wide guide-strips. The zones corresponding to each sugar were cut out and the sugars were eluted separately with distilled water (total 300 ml each). The slightly yellow eluates were decolorized with activated charcoal (Nuchar, C-190-N) and the charcoal was removed by filtration through Whatman No. 5 filter paper. After washing the charcoal on the filter paper twice with 2-ml portions of hot distilled water, the

washings were combined with the respective filtrates. The identity of the sugars in each solution was confirmed as chromatographically pure 2,3,6-Tri-O-methyl-D-glucose and 2,4,6-Tri-O-methyl-D-galactose, respectively, using thin layer chromatographic technique. Lyophilization of the solutions yielded an equimolar ratio of white powdery products: 45.75 mg of 2,3,6-Tri-O-methyl-D-glucose (84.7% yield) and 45.70 mg of 2,4,6-Tri-O-methyl-D-galactose (84.6% yield). The results are summarized in Table 6.

Hydrolysate of permethylated neuramin-lactose sulfate (PMNLS). The acid hydrolysate from 45.05 mg of purified PMNLS was dissolved in 0.4 ml of distilled water and applied to prewashed Whatman 3MM paper (15.6 cm wide and 50 ml long) as a narrow band. The paper was developed in solvent 1 and the two sugar spots were located with the aid of three 2-mm wide guide-strips. The zones corresponding to each sugar were cut out and the sugars were eluted with distilled water as described previously. The slightly yellow eluates (approximately 70 ml each) were decolorized with activated charcoal and the charcoal was removed by filtration and washed. The identity of the sugar contained in each solution was confirmed as chromatographically pure 2,3,6-Tri-O-methyl-D-glucose and 2,4-Di-O-methyl-D-galactose, respectively, by thin layer chromatography. Upon lyophilization of these solutions, 8.96 mg of 2,3,6-Tri-O-methyl-D-glucose (80.2% yield), and 8.98 mg of 2,4-Di-O-methyl-D-galactose (85.5% yield) were obtained, representing a molar ratio of 1.00 to 1.07 of the slightly yellow products. The results are summarized in Table 6.

Further Characterization of the Isolated Methylated Monosaccharides

Specific rotation. Specific rotation of the methylated monoses

from both PMNLS and PMNL were measured.

2,4-Di-O-methyl-D-galactose (from PMNLS). The 8.98 mg of the sugar obtained by preparative paper chromatography was dissolved in 4 ml of distilled water (0.2242 g per 100 ml) and the solution was equilibrated at room temperature (24°). Five readings of optical rotation were taken after equilibration for one hour and 24 hours, respectively, and the specific rotations were calculated as described under Methods. The results, in close agreement with those found in the liberature, are shown in Table 7.

2,3,6-Tri-O-methyl-D-glucose (from PMNLS). The 8.96 mg of the methylated sugar obtained by preparative paper chromatography was dissolved in 4 ml of water (0.2240 g per 100 ml) and the solution was equilibrated at room temperature (24°). Five readings of optical rotation were taken after equilibration for 5 minutes, 1, 6 and 24 hours, respectively. The specific rotations are listed in Table 7 and they agree with the values given in the literature for 2,3,6-Tri-O-methyl-D-glucose.

2,3,6-Tri-O-methyl-D-glucose (from PMNL). The 32.14 mg of the sugar obtained by preparative paper chromatography was dissolved in 4 ml of distilled water (0.805 g per 100 ml). After the solution was equilibrated at room temperature (24°), five readings of optical rotation were taken at 10 minutes, 1, 4, 6 and 24 hours, respectively. The specific rotations are shown in Table 7. They agree with the values given in the literature for 2,3,6-Tri-O-methyl-D-glucose and with the results obtained with the same sugar isolated from PMNLS.

2,4,6-Tri-O-methyl-D-galactose (from PMNL). The 27.80 mg of the sugar obtained by preparative paper chromatography was dissolved in 4 ml

TABLE 7

SPECIFIC ROTATION OF METHYLATED SUGARS FROM PERMETHYLATED OLIGOSACCHARIDES

Methylated Sugars	Found $[\alpha]_D^{24}$ in H_2O	Literature $[\alpha]_D$ in H_2O	Ref.
2,4,6-Tri-O-methyl-[a] D-galactose	+91.0(c,0.694) (24 hr)	+100 → +91 +124 → +90.4	(72) (73)
2,3,6-Tri-O-methyl-[a]	+63.4 → +66.2 → +69.3 → +68.4(c,0.805) (10 min) (1 hr) (6 hr) (24 hr)	+90 → +87 (5 min) (10 min) → +74 → +68.9 (1.5 hr) (14 hr)	 (65)
2,3,6-Tri-O-methyl-[b] D-glucose	+66.1 → +67.9 → +69.7 → +69.7(c,0.2240) (5 min) (1 hr) (6 hr) (24 hr)	+70 (equil.)	(74,75)
2,4-Di-O-methyl-[b]	+38.7 → +84.4(c,0.2242) (1 hr) (24 hr)	+22 → +85.6 +85.7	(76) (77)

[a]From the hydrolysate of permethylated neuramin-lactose.

[b]From the hydrolysate of permethylated neuramin-lactose sulfate.

of distilled water (0.694 g per 100 ml) and the solution was equilibrated for 24 hours at room temperature (24°) before taking optical rotation readings. Five readings were taken and the specific rotation was calculated. The result is shown in Table 7, and agrees with the value found in the liberature for 2,4,6-Tri-O-methyl-D-galactose.

Crystallization of methylated monosaccharides. Methylated monoses from both PMNLS and PMNL were crystallized.

2,4,-Di-O-methyl-D-galactose from PMNLS. Approximately 2 mg of the methylated sugar was dissolved in 2 ml of ethyl acetate. The solvent was evaporated by heating on a heating-mantle to a final volume of 0.3 ml. The concentrated solution was cooled slowly to room temperature and then placed in an ice-bath (76, 78). A small amount of white needles crystallized on the wall of the test tube. After decanting the mother liquor, the crystalline material was washed in the ice-bath with two drops of a cold mixture of ethyl acetate and petroleum ether (b.p. 38.4 to 52.3°). The crystals were then placed in a precooled desiccator containing phosphorus pentoxide and dried in vacuo to give about 0.1 mg of the white needle crystalline material.

The melting point of these crystals was measured on a microscope hotstage. The melting-point range (uncorrected) is listed in Table 8 and agrees closely with the literature values for 2,4-Di-O-methyl-D-galactose.

2,3,6-Tri-O-methyl-D-glucose from PMNLS. Approximately 2 mg of the methylated sugar was dissolved in 0.1 ml of hot ethyl acetate and a small amount of white needles crystallized out upon cooling gradually to room temperature. The tube was then placed in the refrigerator for 2

TABLE 8

MELTING POINT OF CRYSTALLINE METHYLATED SUGARS ISOLATED FROM PERMETHYLATED OLIGOSACCHARIDES

Methylated Sugars	Found		Literature	
	Melting Point[a] (°C)	Mixed Melting Point[a] (°C)	Melting Point (°C)	Ref.
2,4,6-Tri-O-methyl-[b] D-galactose	88-91	88-91	88-91 95-96	(72) (73)
2,3,6-Tri-O-methyl-[b] D-glucose	114-116	113-116		
			121-123 113-115	(74,75) (66)
2,3,6-Tri-O-methyl-[c] D-glucose	113-115	113-115		
2,4-Di-O-methyl-[c] D-galactose	101-103	-------	103 100-103	(78) (77)

[a]Melting points were not corrected.

[b]From the acid hydrolysate of permethylated neuramin-lactose.

[c]From the acid hydrolysate of permethylated neuramin-lactose sulfate.

hours to complete the crystallization. The crystals were sedimented in the refrigerated centrifuge and the mother liquor was removed by decantation. The material was then dried over phosphorus pentoxide *in vacuo*, yielding about 0.2 mg of the white needle crystalline material.

Melting points (uncorrected) are listed in Table 8. The melting-point range and mixed melting-point range agree with each other and with one of the literature values for 2,3,6-Tri-O-methyl-D-glucose.

2,3,6-Tri-O-methyl-D-glucose from PMNL. Approximately 10 mg of the methylated sugar was dissolved in 3 ml of boiling ethyl acetate and a white needle crystalline material was obtained following the same procedure described above.

The simple and mixed melting-point ranges (Table 8) agree with each other, with the values for the trimethyl glucose from PMNLS, and with one of the literature values for 2,3,6-Tri-O-methyl-D-glucose.

2,4,6-Tri-O-methyl-D-galactose from PMNL. 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 solution was cooled slowly to room temperature and then the test tube was placed in an ice-bath while the wall was scratched with a glass-rod to initiate the crystallization. White crystals started to appear within 2 hours. The solution was then placed in a refrigerator overnight to allow the crystals to grow slowly. White elongated, hexagonal-plate crystals were formed on the wall of the test tube. The mother liquor was separated by decantation, and the crystalline material was washed with a cold mixture of ethyl acetate and petroleum ether, and dried over phosphorus pentoxide *in vacuo*, yielding approximately 0.5 mg of white crystalline material.

Melting points (uncorrected) are listed in Table 8. The melting-point range agrees exactly with the mixed melting-point range and closely with the values found in the literature for 2,4,6-Tri-O-methyl-D-galactose.

Anilide derivatives of the methylated monosaccharides. Anilide derivatives of the methylated monoses from both PMNLS and PMNL were made.

2,4-Di-O-methyl-N-phenyl-D-galactosylamine. Approximately 0.8 mg of 2,4-Di-O-methyl-D-galactose obtained from the acid hydrolysis products of permethylated NLS was placed in a ground-joint test tube (1.3 cm inside diameter and 5 cm in length) fitted with a reflux condenser connected to a calcium chloride drying-tube. The sugar was dissolved in 0.3 ml of dry ethanol (freshly redistilled from barium oxide). After refluxing with 0.01 ml of freshly redistilled aniline for 30 minutes, using a Glas-Col heating mantle, the condenser was removed and the heating was continued until the volume was reduced to half (55, 79). The solution was cooled to room temperature slowly (crystals started to appear at this point). The tube was then placed in a desiccator containing phosphorus pentoxide and left overnight in the refrigerator to complete the crystallization. The white needle-shaped crystalline material was separated by centrifugation and decantation, and the crystals were washed once with cold dry ethanol. The crystalline material was dried in vacuo, yielding approximately 1 mg of white needle-shaped crystals.

Melting points (uncorrected) are listed in Table 9. The melting-point range agrees exactly with the mixed melting-point range and closely with the literature values for 2,4-Di-O-methyl-N-phenyl-D-galactosylamine.

TABLE 9

MELTING POINT OF ANILIDE DERIVATIVES OF METHYLATED SUGARS
FROM PERMETHYLATED OLIGOSACCHARIDES

Anilides	Found		Literature	
	Melting Point[a] (°C)	Mixed Melting Point[a] (°C)	Melting Point (°C)	Ref.
2,4,6-Tri-O-methyl-[b] N-phenyl-D-galactosylamine	174-175	-------	175 179	(80) (81)
2,3,6-Tri-O-methyl-[b] N-phenyl-D-glucosylamine	Liquid[d]	-------	-------	
2,3,6-Tri-O-methyl-[c] N-phenyl-D-glucosylamine	Liquid[d]	-------	-------	
2,4-Di-O-methyl-[c] N-phenyl-D-galactosylamine	217-218	217-218	216 214-217	(77) (82,83)

[a]Melting points were not corrected.

[b]From permethylated neuramin-lactose.

[c]From permethylated neuramin-lactose sulfate.

[d]Not confirmed.

2,4,6-Tri-O-methyl-N-phenyl-D-galactosylamine. Approximately 1.5 mg of 2,4,6-Tri-O-methyl-D-galactose isolated from the acid hydrolysate of permethylated NL was converted to the anilide derivative by refluxing with 0.01 ml of freshly redistilled aniline in 0.5 ml of dry ethanol as described above. Sharp needle-crystals were obtained but the initial crystallization did not start until after 1 hour in the refrigerator. The crystalline material was separated by centrifugation and decantation, washed once with cold dry-ethanol, and then recrystallized as follows: the dried crystalline material was dissolved first in hot ethyl acetate and then petroleum ether (b.p. 38.4 to 59.3°) was added dropwise until the solution became slightly opalescent while hot. The solution was heated until it became clear again and then it was cooled slowly to room temperature before being placed in a desiccator in the regrigerator. The white needle crystalline material obtained was separated and dried in vacuo, yielding approximately 1.3 mg.

Melting points (uncorrected) are shown in Table 9. They agree closely with the literature values for 2,4,6-Tri-O-methyl-N-phenyl-D-galactosylamine.

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 PMNL and PMNLS, were subjected to the same operations described above, but no crystalline derivative was obtained. No literature report was found on the physical properties of 2,3,6-Tri-O-methyl-N-phenyl-D-glucosylamine, and from these experiments it would appear that this compound is a liquid.

Demethylation of methylated monosaccharides with boron tri-

bromide (84). Five-tenth milligram of thoroughly dried methylated monosaccharide was dissolved in 0.1 ml of dry dichloromethane (see Materials) in a long test tube (15 ml size) fitted with a calcium chloride drying tube. The solution was chilled in an ice-bath and then 0.1 ml of precooled boron tribromide was added dropwise. Extreme precautions should be taken during the addition of the boron tribromide because of its explosive reactivity in moist air. The reaction mixture was then allowed to warm up gradually and it was kept for 18 hours at room temperature under anhydrous conditions to give a slightly yellowish-orange solution.

The solvent and boron tribromide were then removed under diminished pressure at room temperature. The glassy residue obtained was evaporated three times in vacuo with 1-ml portions of dry methanol in order to eliminate the residual reagent. The final product was dissolved in a small volume of water and examined by thin layer chromatography on silica-gel plates. The results obtained with three different solvent systems are shown in Figure 22. Demethylation of 2,4,6-Tri-O-methyl-D-galactose produced a main spot with an R_f-value identical to that of galactose and a very faint unidentified spot with an R_f-value close to that of 2,3,4,6-Tetra-O-methyl-D-glucose. Demethylation of 2,4-Di-O-methyl-D-galactose produced a single spot with an R_f-value identical to that of galactose. Demethylation of 2,3,6-Tri-O-methyl-D-glucose produced a main spot with an R_f-value identical to that of glucose and a very faint unidentified spot with an R_f-value close to that of 2,3,4,6-Tetra-O-methyl-D-glucose.

These results indicate that the products isolated from the hydrolysates of the permethylated oligosaccharides were, indeed, methylated

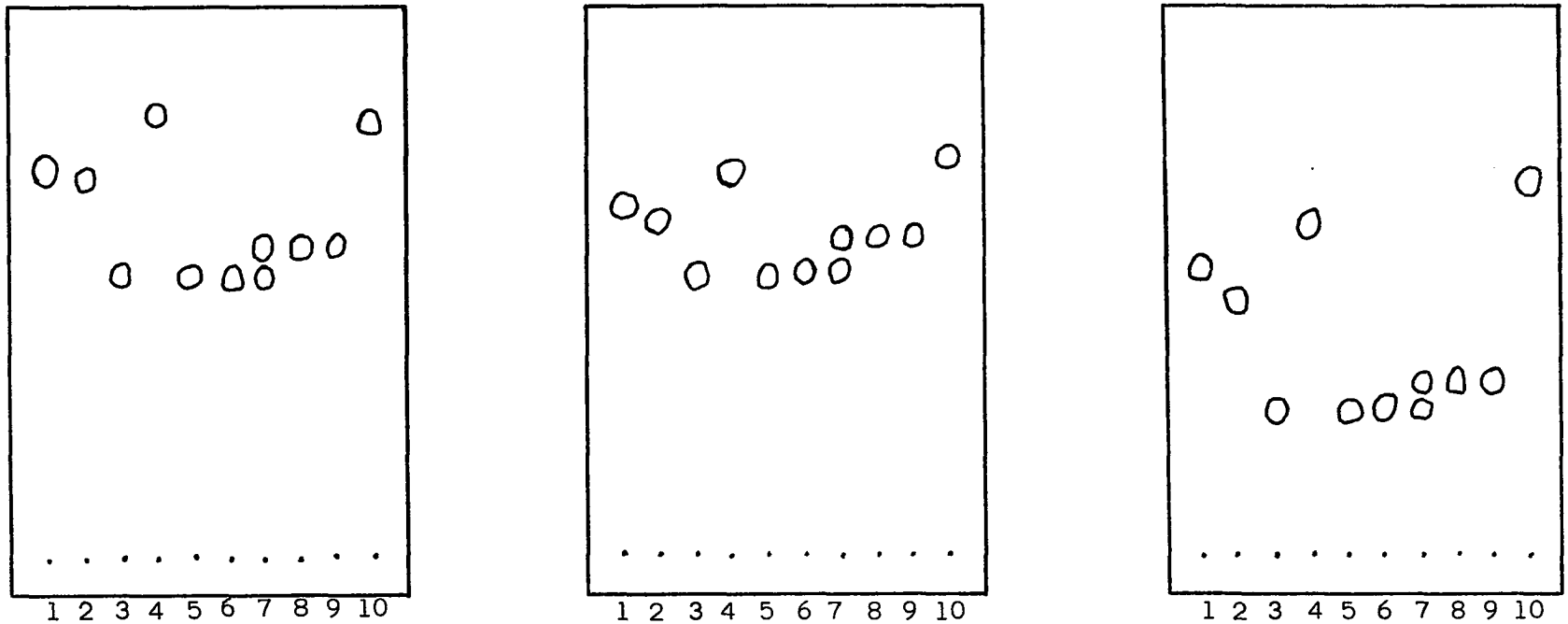


Figure 22. Thin layer chromatography of methylated monoses before and after demethylation with boron tribromide. All plates were developed unidimensionally twice and sprayed with p-anisidine reagent. Solvent 6, ethyl acetate-ethanol-water (4:4:1). Solvent 9, n-propanol-water (85:15). Solvent 4, water saturated n-butanol.

Key to the spots

1 = 2,4,6-Tri-O-methyl-D-galactose.
2 = 2,4-Di-O-methyl-D-galactose.
3 = Galactose.
4 = 2,3,6-Tri-O-methyl-D-glucose.
5 = Demethylation product of 2,4,6-Trimegal.
6 = Demethylation product of 2,4-Dimegal.
7 = Mixture of galactose and glucose.
8 = Demethylation product of 2,3,6-Tri-O-methyl-D-glucose.
9 = Glucose.
10 = 2,3,4,6-Tetra-O-methyl-D-glucose.

sugars and the original monoses did not undergo chemical alterations throughout the permethylation and acid hydrolysis procedures.

Other Acidic Oligosaccharides Isolated During the Fractionation by the Dowex 1-X8 (Formate) Column (Experiment 5)

P_5 Material

The brownish oily residue, obtained by lyophilization of Peak 5 (fractions 302-314) in Experiment 5 (Figure 5), was dissolved in 1.5 ml of water and purified on a Sephadex G-15 column (2.0 x 175 cm) following the procedure described for the purification of NLS. Lyophilization of the sugar-containing fractions yielded a slightly yellow powdery residue weighing 274 mg.

Preliminary studies. The P_5 material gave a positive reaction with the anthrone reagent and a negative reaction with the resorcinol reagent. Electrophoretic analysis of the material in acetate buffer (pH 4.5) at 375 volts produced a benzidine positive brown spot with a mobility identical to that of the neuraminidase hydrolysate of NLS (see Figure 17 for the relative mobility). Paper and thin layer chromatography of this compound with several solvent systems (solvents 5, 6, 9, 10) showed a single sugar spot with R_f-value identical to that of lactose-6-sulfate, which was obtained by neuraminidase hydrolysis of NLS. A typical paper chromatogram is shown in Figure 23.

Progressive acid hydrolysis. A 46 mg sample of P_5 was dissolved in 1 ml of 0.5 N sulfuric acid solution in a long test tube fitted with a reflux condenser. The tube was placed in a boiling-water bath, and 0.2-ml aliquots were taken out at 0.5, 1, 2 and 3 hours, respectively.

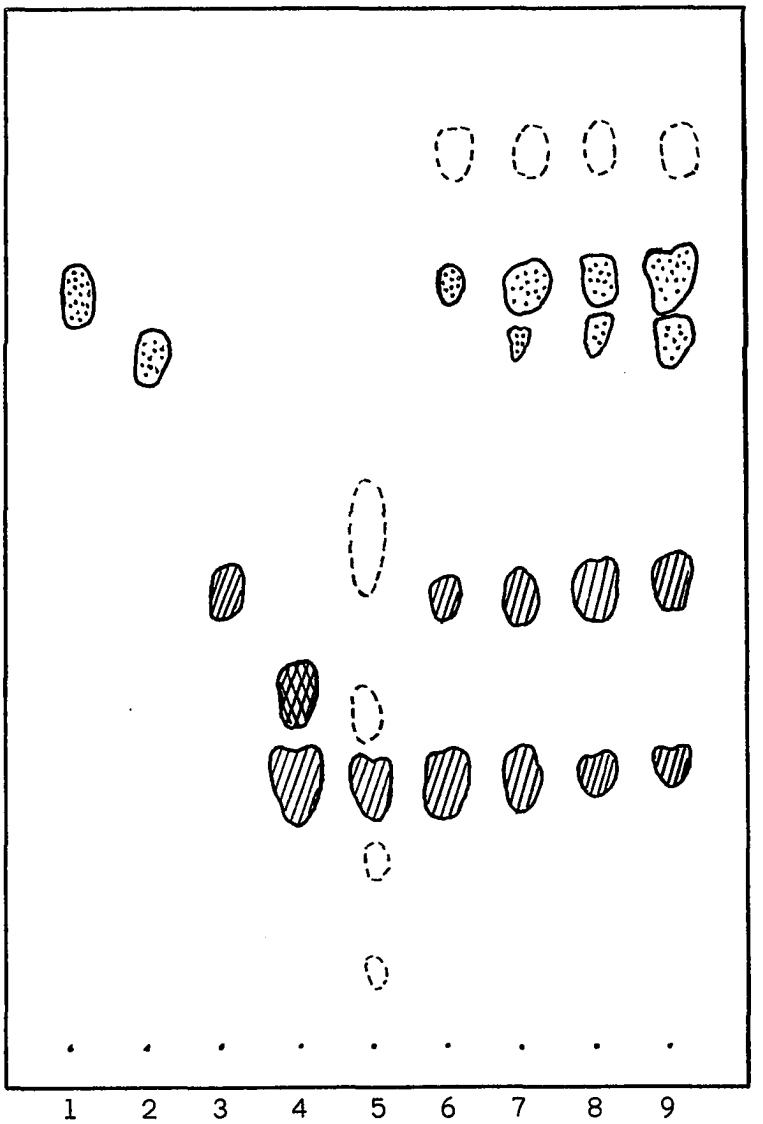


Figure 23. Paper chromatography of the products of progressive hydrolysis of P_5 (lactose sulfate) from Exp. 5 (see Fig. 5).

Solvent 10, isopropanol-H_2O (4:1); descending technique, aniline-diphenylamine spray.

, UV spot; , pink; , blue; , green.

Key to the spots

1 = Glucose.
2 = Galactose.
3 = Lactose.
4 = Neuraminidase-hydrolysate of NLS.
5 = P_5, 0 min. hydrolysis.
6 = P_5, 30 min. hydrolysis.
7 = P_5, 1 hr. hydrolysis.
8 = P_5, 2 hr. hydrolysis.
9 = P_5, 3 hr. hydrolysis.

These aliquots were neutralized with saturated barium hydroxide solution and centrifuged. The hydrolysates were concentrated to a small volume and subjected to paper and thin layer chromatography with several solvent systems. The paper chromatogram, developed in solvent 10 and sprayed with aniline-diphenylamine spray reagent, is shown in Figure 23. Lactose and glucose started to appear after 30 minutes of hydrolysis while galactose appeared after 1 hour of hydrolysis. Thin layer chromatograms in three different solvent systems gave the same results. These results indicate that the P_5 material is composed of lactose sulfate and some ultraviolet absorbing impurities. Since the sulfate ester linkage is known to be more resistant to acid hydrolysis than the glycosidic linkage, the slight delay in the appearance of free galactose suggested attachment of the sulfate ester to the galactosyl moiety of the molecule. While this investigation was in progress, Barra et al. (85) reported the isolation of a compound similar to P_5 from rat mammary glands and proposed the tentative structure "6-O-sulphonyl-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose" based on infrared spectra, acid hydrolysis and periodate oxidation studies.

Permethylation studies. A 10.57 mg sample of the powdery P_5 material was permethylated following the same procedure described for the permethylation of the neuraminidase hydrolysate of NLS and the permethylated product was isolated by the Sephadex G-15 column procedure previously described. The yellow powdery residue of permethylated P_5 was then hydrolyzed in 1 ml of 0.5 N sulfuric acid solution at 100° for 7 hours. After neutralization with saturated barium hydroxide solution and centrifugation, the hydrolysate was concentrated to a small volume and analyzed

by thin layer chromatography with three different solvent systems. The hydrolysate produced two spots (Figure 21), which were identical with those observed in the hydrolysate of the permethylated neuraminidase-hydrolysate of NLS. The R_f-values are identical to those of authentic 2,3,4-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose, respectively.

The results of the progressive hydrolysis and permethylation studies indicate that the galactose moiety is linked to the C-4 position of glucose and that the sulfate ester is linked to the C-6 position of galactose.

The P_5 material was thus identified as lactose sulfate, and its structure was shown to be O-β-D-galactopyranosyl 6-O-sulfate-(1 → 4)-D-glucopyranose, which agrees with the structure postulated by Barra *et al.* (85).

P_3 and P_4 Materials

Very little is known about these materials, the findings of some preliminary studies are described below.

P_3 material. When subjected to electrophoresis in acetate buffer (pH 4.5) at 375 volts, it migrated between free NANA and NLS. The material was very unstable. After a few hours at room temperature a neutral sugar was cleaved off. The component released behaved as ribose on paper and thin layer chromatography with several solvent systems, and gave a positive reaction with the Bial's (orcinol) reagent for pentoses. No ninhydrin positive spot was observed on thin layer chromatoplates.

P_4 material. The P_4 material (Figure 5) was present in such a small amount that, when 100 g of rat mammary gland was used for the column

fractionation (Figure 8), it could not be detected. When subjected to thin layer chromatography with solvents 7 and 9, compound P_4 gave a single ninhydrin positive spot.

CHAPTER IV

DISCUSSION

Isolation of Oligosaccharides

Ion-Exchange Column Fractionation

Several methods have been reported for the isolation of neuramin-lactose (NL) from various sources (36, 65, 67, 86) by the use of ion-exchange chromatography.

For isolation of neuramin-lactose sulfate (NLS), the original method of Carubelli *et al.* (36) was the only one reported up to now. These authors utilized ion-exchange chromatography on a column of Dowex 1-X8 (formate) and elution with strongly acidic formate solutions.

In the work reported here, neuraminyl-oligosaccharides and other acidic sugars present in the tissue extracts were fractionated under milder conditions. Two fractionation systems were tested, both using Dowex 1-X8 resin.

The first system, Dowex 1-X8 (acetate) column and pyridinium acetate elution, is the same system used by Schneir *et al.* (60) for cow colostrum. Fractionation of mammary gland extracts with this system gave three well separated major peaks of resorcinol positive material, plus a minor peak preceding and incompletely separated from the first peak (Figure 7). The resorcinol positive material in the first peak (main por-

tion) was identified as NL by electrophoresis, paper and thin layer chromatography. This NL was homogeneous in all systems tested, indicating the presence of only one isomer of NL whose structure was determined as NL(2 → 3). The structure of the sugar in the minor peak (Figure 7) was not studied in detail. Since it is both resorcinol and anthrone positive, it might be an isomer of NL, most likely NL(2 → 6), which is known to occur together with the NL(2 → 3) form (29, 32).

Purification of NL(K^+) by the organic solvent method gave a final product 96.1% pure. During this purification, the ratio $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) decreased from 0.218 to 0.080 (Table 2), indicating that the contaminant consisted mostly of methanol-insoluble, ultraviolet-absorbing material. The purity obtained here is comparable with that obtained by other investigators (26, 36).

The material in the third peak was found to be NLS contaminated with a small amount of an acidic sugar, later identified as lactose sulfate, in addition to the ultraviolet-absorbing impurities. The maximum purity of the NLS obtained by organic solvent purification method from material fractionated by this system was 71.3%. This value is much lower than that obtained with the formate-column fractionation system.

The Dowex 1-X8 (formate) column and pyridinium formate elution is the system developed during this investigation and adopted for the preparation of NLS. This system has higher resolution than the acetate system, and has distinct advantages over the formate system previously utilized by Carubelli *et al.* (36). The pyridinium formate solution used for elution had pH 4.4, and furthermore, due to its high volatility and relatively low concentration, most of the pyridinium formate can be re-

moved easily by lyophilization of the column effluents. Thus, this system offers not only milder conditions for the fractionation but also has special advantages of simplicity in the subsequent removal of the eluting agent. The elution pattern (Figures 5, 8) shows separation of the acidic oligosaccharides into well defined peaks which upon analysis revealed the presence of a single homogeneous sugar in each peak. The organic solvent purification of the NLS isolated by this system gave a higher final purity (87.5%) than the maximum purity (71.3%) obtained from the material fractionated by the acetate-column system (Table 3). The recovery of neuraminic acid-containing compounds from the column was almost quantitative (99.3%).

When the mammary glands used had been stored in the deep freezer for more than 8 months prior to extraction, peaks of free NANA were observed (Figures 5, 6 and 7), while no free NANA was detected when using fresher tissue (less than 2 weeks of storage) which had been quickly chilled upon removal (Figure 8). These results suggest that fresh mammary glands contain only bound NANA. The appreciable amount of free NANA observed in samples stored for prolonged periods of time probably originates by enzymatic hydrolysis (neuraminidase) (62) of one or both of the neuraminyl oligosaccharides (NL and/or NLS) due to lack of precautions and improper handling of the tissue during its removal and prolonged storage. Data in Table 1 show that the amount of NLS per g of the wet tissue was similar in both aged and fresh tissue (average 0.296 μmole per g tissue), while at the same time it was observed that the amount of free NANA (per g of wet tissue) was equal to or greater than that of NLS. These results rule out NLS as the primary source of the

free NANA. The amount of NL per g of wet tissue, on the other hand, fluctuated markedly and it was always very high as compared with that of NLS and free NANA (Table 1). From these considerations, NL appears as the most probable source for the observed free NANA. Experiments with rat mammary gland neuraminidase, however, show that NLS is hydrolyzed about three times faster than NL (62).

On the average, the tissue contained 2.12 mg of neuramin-lactose (NL) and 0.23 mg of neuramin-lactose sulfate (NLS) per g of wet tissue.

Purification

The two methods previously utilized for the purification of NL are the charcoal column method (26, 65, 67) and the organic solvent purification method (26, 36).

The organic solvent method was used for the purification of NL in this study and produced a 96.1% pure NL with 64.1% recovery (Table 2). Although the recovery of NL by this purification is rather low, the method is quick, simple and suitable for handling large samples.

Whenever nearly quantitative recoveries and higher purities are required, the Sephadex G-15 column purification method is the best choice. This method, however, can handle limited amounts of material with columns of common size, and it is slower and requires more equipment.

For the purification of NLS, the organic solvent method produced a maximum purity of 87.5% with only about 50% recovery (Table 3).

The ratio $O.D._{260\ m\mu}$ to $O.D._{580\ m\mu}$ (resorcinol) was 0.265, indicating that the 87.5% pure NLS material still contained a considerable amount of ultraviolet absorbing impurities (Table 3). The electrophoretic

analysis of NLS at various stages during the purification procedure (Figure 9) shows that the crude lyophilized material from the ion-exchange column has two U.V. absorbing spots, i.e., one with a slow mobility and the other with a mobility close to that of NLS. The slow U.V. absorbing impurity was removed by the organic solvent purification process, but the fast moving U.V. absorbing spot was still present in the purified material and overlapped the NLS spot (Figure 9). Even after repeating several times the organic solvent purification process, the relative amounts of NLS and U.V. absorbing material did not seem to change appreciably and the purity of NLS (around 87.5%) did not increase. From these observations it may be concluded that the organic solvent purification system could not separate NLS from the residual ultraviolet-absorbing material, and that 87.5% is the maximum purity which could be obtained by this method. Electrophoretic analysis of the methanol-insoluble material after exhaustive methanol-extraction, shows some NLS in addition to the two U.V. absorbing spots (Figure 9). This incomplete extraction by methanol was the major cause of the low recovery of NLS in the organic solvent purification method.

As evidenced in these studies, the organic solvent purification method is not efficient for the purification of NLS, although it is a satisfactory method for purification of NL.

Among the gel filtration methods tried, the Sephadex G-15 column turned out to be the most efficient one. By using the proper column size and elution rate for a given amount of NLS, it was possible to obtain almost quantitative recoveries of close to 100% pure material.

This method offers mild conditions for the purification of the

oligosaccharides, and since the elution is carried out with distilled water, the purified sugar can be easily recovered by lyophilization.

Data in Table 4 shows that the recovery of NLS from the Sephadex G-15 column was 95.3% and the purity of the NLS obtained was 97.3%. These results represent a great improvement over the results obtained by the conventional organic solvent method where the maximum purity was 87.5% with approximately 50% recovery (Table 3). The ratio of the O.D. 260 mμ to O.D.$_{580}$ muμ (resorcinol) was 0.013. This low value indicates that the Sephadex G-15 column chromatographic process effectively removed the troublesome 260 mμ absorbing impurity which could not be removed completely by the organic solvent system. The U.V. absorbing impurity apparently has a similar ratio of negative charge to mass (Figure 9) and a similar solubility in methanol to that of NLS, but its molecular weight probably is slightly smaller than that of NLS as evidenced by its somewhat longer retention time in the Sephadex G-15 column chromatography (Figure 16).

The results of the over all performance of the isolation and purification methods developed, i.e., the fractionation system using the Dowex 1-X8 (formate) column and pyridinium formate elution coupled with the Sephadex G-15 column purification system, are shown in Table 4. The data clearly shows that with these methods nearly 100% pure NLS can be isolated almost quantitatively from the rat mammary gland extracts, conveniently and quickly. Analysis of NLS (Table 5) showed the expected equivalent amounts of NANA, lactose and sulfate ester.

Permethylation Studies

Because of the stability of methyl ethers of sugars toward acid

and alkali, the methylation of oligo- and polysaccharides has proved invaluable for the elucidation of the position of the linkages between the different sugar residues. Characterization of the individual methylated sugars in the hydrolysate of a permethylated oligosaccharide reveals the presence of any unmethylated hydroxyl groups. The carbon atoms carrying these free hydroxyl groups were blocked in the original oligosaccharide and hence they point out the positions at which a glycosyl group or another acid-labile substituent was attached.

Permethylation studies alone usually are not sufficient to establish the molecular structure of a complex carbohydrate. In the case of NLS the composition, some properties and a considerable amount of knowledge with regard to the structure of this compound were already available. On the basis of chemical and enzymic studies the structure O-α-D-N-acetylneuraminyl-(2 → 3)-O-β-D-galactopyranosyl-6-O-sulfate-(1 → 4)-D-glucopyranose had been postulated by Carubelli et al. (36, 38). It was however recognized that final proof for this structure would require permethylation studies (38).

The basic idea underlying the use of this method for the determination of the structure of NLS is as follows: assuming that the proposed structure of NLS is correct (see Figure 4), hydrolysis of permethylated NLS (PMNLS) in acid medium should cause the cleavage of the glycosidic and ketosidic linkage as well as the sulfate ester linkage, and the final hydrolysate should contain 2,3,6-Tri-O-methyl-D-glucose and 2,4-Di-O-methyl-D-galactose. The methylated sialyl group is completely degraded by the action of the hot mineral acid.

The characterization of 2,4-Di-O-methyl-D-galactose in the hy-

drolysate of PMNLS points out that the hydroxyl groups at C-3 and C-6 of the galactosyl moiety of NLS were substituted but it does not provide any clues as to the nature of the substituents. Since glucose was established to have its reducing end free in NLS (36, 38), the positions 3 and 6 of galactose are blocked by the neuraminyl group and the sulfate ester, which leads to two possible structures: the sulfate group could be linked to the C-6 of galactose and the neuraminyl group to the C-3 of galactose, or vice versa. The results of the studies with permethylated NLS would not allow by themselves a distinction between these two alternatives. However, this problem can be solved by removing first one of the two groups involved, and then carrying out the permethylation studies. Due to the extreme lability of the neuraminyl linkage, selective removal of the sulfate ester group by acid hydrolysis is impossible. On the other hand, since good preparations of glycosulfatase are not commercially available, the enzymic removal of the sulfate group of NLS could not be accomplished. The removal of the neuraminyl group by the action of neuraminidase on NLS could be easily done and it was adopted for this study. After hydrolysis with bacterial neuraminidase, the hydrolysate of NLS was permethylated and subjected to acid hydrolysis. The acid hydrolysate now should contain 2,3,4-Tri-O-methyl-D-galactose if the neuraminyl group was attached to C-3, and 2,4,6-Tri-O-methyl-D-galactose if the neuraminyl linkage involved C-6. Characterization of the 2,3,4-Tri-O-methyl-D-galactose proved that the C-6 position is occupied by the sulfate ester group, while the C-3 of the galactose moiety of NLS is occupied by the neuraminyl linkage which, due to its susceptibility to neuraminidase, is known to be in the α-anomeric configuration.

Characterization of 2,3,6-Tri-O-methyl-D-glucose in the hydrolysate of PMNLS indicates that the hydroxyl group at C-4 of the glucose moiety of NLS is substituted. The conclusions reached above from the studies on the galactosyl moiety of PMNLS leave galactose as the only possible substituent. The presence of a lactose type of linkage (β 1 $\rightarrow$ 4) between galactose and glucose is supported by the finding that the reducing power of NLS resides on the glucose, which disappears after hypoiodite treatment (36), and by the actual isolation of small amounts of lactose from the products of partial hydrolysis of NLS (38).

The experimental findings described here prove beyond any doubt that the structure of NLS corresponds to O-α-D-N-acetylneuraminyl-(2 $\rightarrow$ 3)-O-β-D-galactopyranosyl 6-O-sulfate-(1 $\rightarrow$ 4)-D-glucopyranose (Figure 4) as previously proposed (36).

Acid hydrolysis of PMNL produced an equimolar ratio of 2,4,6-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose in high yields (Table 6). Since the neuraminyl linkage of NL is split by neuraminidase and the products of hydrolysis were characterized as NANA and lactose, the permethylation studies described here indicate that the structure of the NL isolated from rat mammary glands is O-α-D-N-acetylneuraminyl-(2 $\rightarrow$ 3)-O-β-D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose (Figure 3), which is identical to the NL (2 $\rightarrow$ 3) from cow colostrum studied by Kuhn *et al.* (65).

As for the P_5 material obtained by the Dowex 1-X8 (formate) column fractionation of the rat mammary gland extracts (see Figure 5), the studies of progressive acid hydrolysis in 0.5 N sulfuric acid at 100° (Figure 23) plus other studies indicated that the material is lactose-

sulfate. Permethylation of the P_5 material followed by acid hydrolysis produced 2,3,4-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose, thus establishing the structure of lactose sulfate as O-β-D-galactopyranosyl 6-O-sulfate-(1 $\longrightarrow$ 4)-D-glucopyranose. This is in agreement with the structure tentatively proposed by Barra *et al.* (85).

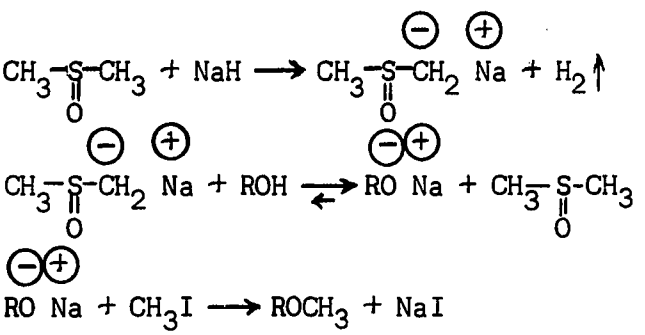
A brief discussion of the new method of permethylation used in these studies seems appropriate at this point. Classical methylation methods (39, 40) have relied upon the initial conversion of the hydroxyl groups to alkoxides by reacting the sugar with a base in aqueous solution. The methylating reagent then reacts with the alkoxides to yield methyl ethers. Any free base in the reaction mixture competes with the alkoxide for the alkylating reagent. In the initial reaction, an equilibrium is established at a point dependent on the strength and concentration of the base, B^-.

$$ROH + B^- \rightleftharpoons RO^- + BH$$

Complete conversion to the alkoxide requires a base stronger than OH^-. However, the strongest base that can exist in aqueous solution is the OH^- ion since stronger bases will react with water to form this ion. Thus, in the classical methylation procedures the extent of ether formation is influenced by the point of equilibrium in the base-catalyzed reaction which in turn is limited by the base strength of OH^-. In the method applied here, these limitations are overcome by the use of the strongly basic methylsulfinyl carbanion (64) in non-aqueous medium to generate the oligosaccharide alkoxide. It is apparent that equilibrium in this reaction lies almost completely in the direction of alkoxide formation. The

competing reaction between the excess methylsulfinyl anion and methyl iodide does not interfere as long as methyl iodide is added in excess of the total base.

The reaction sequence of the permethylation system used here is as follows:



where ROH represents the oligosaccharide. For generation of the methylsulfinyl carbanion, the reaction normally takes about 30 minutes at 70° under the conditions used in this study. For formation of the sugar alkoxide the reactions were carried out at room temperature, and the reaction time varies with the type of sugars. For lactose (a neutral sugar), the complete conversion of hydroxyl groups to alkoxides took 20 minutes. For NL (a weakly-acidic oligosaccharide), a period of 40 minutes was required to complete the alkoxide formation. For the sulfate ester-containing, strpngly-acidic oligosaccharides (NLS, lactose sulfate from neuraminidase hydrolysate of NLS and P_5 material), the alkoxide formation reactions were carried out for 8 hours.

The time required for the reaction of the alkoxides with methyl iodide, which were carried out at room temperature, varies with the type of sugar alkoxide involved. In the case of lactose, the reaction was complete as soon as the addition of methyl iodide was finished, while the

NL alkoxide was completely methylated in approximately 20 minutes. NLS and lactose-sulfate alkoxides were allowed to react with methyl iodide for 15 hours. This may be longer than actually needed because the minimum reaction time required for the completion of the alkoxide formation and the methylation reactions of these two compounds have not been determined. Prolonged reaction times were used for the strongly acidic oligosaccharides because it has been shown that the permethylation of sulfate ester-containing polysaccharide is extremely difficult. These prolonged reaction times appeared to be safe since studies on the permethylation of lactose and NL under various conditions had shown no evidence for cleavage of glycosidic or ketosidic linkages, or any other side reactions.

Although the isolation of permethylated lactose and NL could be accomplished satisfactorily with the chloroform extraction method, this method was not satisfactory for the isolation of the PMNLS. By utilizing the Sephadex G-15 column method, however, PMNLS could be isolated from the reaction mixture almost quantitatively and in a highly-pure form.

Thin layer chromatography indicated that the permethylation was complete (Figure 18) and this was confirmed by the infrared spectral analyses (Figures 19 and 20). The yields of permethylated products were approximately 90% of the theoretical values in all cases (Table 6).

Acid hydrolysis of the glycosidic and ketosidic linkages of permethylated oligosaccharides in 0.5 N sulfuric acid solution at 100^{o} was complete in 4 hours while the complete cleavage of the sulfate ester linkage took 7 hours under these experimental conditions. Paper and thin layer chromatographic analyses of the acid hydrolysates in several solvent systems (Figure 21) indicated that the hydrolysates contained:

2,3,4,6-Tetra-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose from permethylated lactose; 2,4,6-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose from the PMNL; 2,4-Di-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose from the PMNLS; 2,3,4-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose from the permethylated product of the neuraminidase-hydrolysate of NLS; and, 2,3,4-Tri-O-methyl-D-galactose and 2,3,6-Tri-O-methyl-D-glucose from the permethylated product of P_5 (lactose sulfate). When the hydrolysis of the PMNLS was carried out for 5 hours under the same conditions, one additional spot was observed at the origin, which presumably was that of 2,4-Di-O-methyl-D-galactose-6-O-sulfate because the sulfate ester linkage is expected to be the last one to be cleaved.

Fractionation of the hydrolysate of PMNLS by preparative paper chromatography produced a 1.00 to 1.07 molar ratio of 2,3,6-Tri-O-methyl-D-glucose and 2,4-Di-O-methyl-D-galactose, and yields of 80.2 and 85.5%, respectively, based on the starting amount of PMNLS (Table 6). The high yields of methylated sugars assure that the molar ratio found for the methylated sugars accurately reflects their actual proportion in the fully methylated NLS. The recovery of the equimolar ratio of the methylated sugars further indicates that the permethylation of NLS was complete. Undermethylation, if occurs, would be more likely to occur on the galactose moiety than on the glucose moiety in view of electronic and steric effects of the neighboring groups. A negatively charged sulfate group would exert a strong electronic repulsion to the approaching methylsulfinyl carbanion in the alkoxide formation. Since the sulfate is on the galactose moiety, the electron-repulsive effect would be stronger on the

reactions involving the galactose moiety than on those involving the glucose moiety. Furthermore, since the galactose moiety is situated between bulky sugar groups, the methylation reaction of the galactose moiety would be effected by the steric hindrance of the neighboring groups. The recovery of an equimolar ratio of the methyl sugars, however, reassures that undermethylation did not occur.

In addition to paper and thin layer chromatographic studies, the methylated sugars obtained by acid hydrolysis of both PMNL and PMNLS were further characterized by their specific optical rotations, melting points of the crystallized methylated sugars and of their anilide derivatives (Tables 7, 8 and 9). The values obtained are consistently in agreement with those found in the literature. Finally, the demethylation studies on the isolated methylated sugars (Figure 22) proved that the products isolated from the hydrolysates of PMNL and PMNLS were, indeed, methylated monoses and that the original monoses did not undergo chemical alteration throughout the permethylation and the acid hydrolysis procedures.

CHAPTER V

SUMMARY

An improved method for the isolation of acidic oligosaccharides from rat mammary gland extracts has been developed. This method involves fractionation by ion-exchange chromatography using a Dowex 1-X8 (formate) column which was eluted with a gradient of pyridinium formate.

The isolated sugars were purified by gel filtration on Sephadex G-15 columns, eluting with distilled water. By using these improved isolation and purification methods, almost quantitative isolation of nearly 100% pure neuramin-lactose and neuramin-lactose sulfate was obtained conveniently, quickly and under mild conditions.

The chemical structures of three acidic oligosaccharides, isolated and purified as described above, were established by permethylation studies in which permethylation of the acidic sugars was achieved with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide solution. On the basis of the results of these studies, the following structures were assigned:

1) Neuramin-lactose sulfate; O-α-D-N-acetylneuraminyl-(2 → 3)-O-β-D-galactopyranosyl 6-O-sulfate-(1 → 4)-D-glucopyranose.

2) Neuramin-lactose; O-α-D-N-acetylneuraminyl-(2 → 3)-O-β-D-galactopyranosyl (1 → 4)-D-glucopyranose.

3) Lactose sulfate; O-β-D-galactopyranosyl 6-O-sulfate-(1 → 4)-D-gluccpyranose.

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