The Structure and Chemistry of Colominic Acid*

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Received August 9, 1963

Evidence is presented to show that colominic acid is a homopolymer of *N*-acetylneuraminic acid having 2–8 ketosidic linkages between the polymer units. A portion of the carboxyl groups is not titratable until after treatment with alkali. These carboxyl groups are probably linked to the hydroxyl of the neighboring monomer unit by an ester group to the 7 or 9 positions. Neuraminidase from *Clostridium perfringens* catalyzes the complete hydrolysis of colominic acid which has been pretreated with alkali. Since this neuraminidase is probably an α-ketosidase, the linkage between the polymer units is probably α.

Colominic acid was isolated originally from *Escherichia coli* K235 by Barry and Goebel (1957). It was shown to be a macromolecular substance which gave a direct Ehrlich reaction indicating the presence of sialic acid. Subsequently, Barry (1958) hydrolyzed colominic acid and obtained only *N*-acetylneuraminic acid in 26% yield.

Cassidy *et al.* (1962) found that neuraminidase preparations from *Clostridium perfringens* or *Vibrio cholerae* cleaved colominic acid. *N*-acetylneuraminic acid was the only product of the reaction. Aminoff *et al.* (1963) found that neuraminidase cleaved 65% of colominic acid in 24 hours. These results are evidence that the steric configuration of the C-2 ketosidic linkage may be α.

This paper presents evidence that colominic acid is a low-molecular-weight homopolymer of *N*-acetylneuraminic acid. The monomers are probably linked by an α-2,8 ketosidic linkage. Some of the C-1 carboxyl groups are esterified and are easily saponified with 0.1 N NaOH at 25°. A high yield of *N*-acetylneuraminic acid is obtained in 1 hour when colominic acid which has been treated with alkali is incubated with neuraminidase from *Clostridium perfringens*. Only 20% of *N*-acetylneuraminic acid is liberated from unsaponified colominic acid in 1 hour.

Materials and Methods

Colominic acid was prepared by growing *Escherichia coli* K235 L+O in the medium described by Barry (1958). The culture was grown in 100-liter batches in fermenters[1] with rapid agitation and aeration at 37° and at a *p*H maintained at 7.0. After 18 hours the culture was filtered and the filtrate was concentrated to about 5 liters under reduced pressure at 40°. The remaining steps were identical with those used by Barry (1958).

Sialic acid was determined by the modified Ehrlich procedure of Werner and Odin (1952) and also by the procedure of Warren (1959). Colominic acid was determined by the direct Ehrlich procedure. Color development was slow and increased continually for more than 2 hours. Color development with sialic acid was complete within 30 minutes. A heating time of 30 minutes as used in the following experiments gave values for colominic acid which were 10–15% low. Heating times of 1 hour or more gave values which exceeded 100% when sialic acid was used as a standard for comparison. Colominic acid gave no color with the Warren reagent. Reducing values were measured by the method of Schales and Schales (1945) and were compared to *N*-acetylneuraminic acid standards. Acetyl values[2] were determined by the method of Ludowieg and Dorfman (1960). *N*-Glycolyl groups were determined by the method of Klenk and Uhlenbruck (1957) employing the Eegrewie reagent. Phosphorus was measured by the method of Fiske and Subbarow (1925). The Lowry *et al.* (1951) and biuret methods were used for the detection of proteins and peptides. The ninhydrin procedure of Moore and Stein (1954) was used for the detection of amino acids. The orcinol procedure of Mejbaum (1939) was used to determine pentoses and uronic acids. The anthrone method of Trevelyan and Harrison (1952) was used to determine carbohydrate. Periodate oxidations were carried out at 4° by the procedure of Dyer (1956) using a 2–5 molar excess of periodate per mole of NAN.[3] The periodic acid consumption was followed by the bicarbonate-arsenite titration method. Seventy mg to 500 mg of colominic acid was used for each determination. The periodate oxidations were carried out in water solutions of colominic acid (*p*H 2.6) except for the oxidation at *p*H 4.6 which was conducted in 0.2 M sodium acetate buffer (*p*H 4.6). A reagent blank, which contained all reagents except colominic acid was run. Formic acid was determined by titration of 0.5-ml aliquots with 0.01 N NaOH. Prior to the base titration periodic acid was decomposed with 1.0 ml of ethylene glycol. A zero-time blank was subtracted to account for the acidity of colominic acid. This method measures the increase in total acidity without identifying the acid released. The ferric chloride–hydroxylamine reagent of Weissmann and Meyer (1954) was used to determine the presence of esters or lactones.

The exoenzyme neuraminidase was prepared by the method of Popenoe and Drew (1957) from the culture filtrate of *Clostridium perfringens*. The enzyme activity was determined using neuraminlactose as the substrate in 0.1 M acetate buffer (*p*H 4.9) in a total volume of 0.2 ml. The incubation was stopped by the addition of 0.1 ml of the periodate reagent of Warren (1959). One-tenth ml of the enzyme preparation

* This communication is part of a dissertation submitted by Edward J. McGuire to the graduate faculties of the College of Medicine of the University of Illinois in partial fulfillment of the requirement for the Ph.D. degree. This work was supported by a grant (USPHS E 3380) from the United States Public Health Service. A preliminary account of this work was presented before the meeting of the Federation of American Biologists, April, 1962, Atlantic City, New Jersey.

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[1] The authors would like to express their appreciation to Abbott Laboratories, North Chicago, Illinois, for the concentrated culture filtrates of *Escherichia coli* K235 L+O.

[2] Acetyl values were confirmed by the Clark Microanalytical Laboratory, Urbana, Illinois. Alkoxyls and C, H, N analyses were performed by the Spang Microanalytical Laboratory, Ann Arbor, Michigan.

[3] Abbreviation used: NAN, *N*-acetylneuraminic acid.

liberated 0.036 μmole *N*-acetylneuraminic acid per minute from 0.16 μmole neuraminlactose at *p*H 4.9 in 0.1 M acetate buffer (final volume 0.2 ml). One ml of the enzyme solution contained 3.25 mg protein.

The methyl ester methyl glycoside of *N*-acetylneuraminic acid was prepared according to the procedure of Blix *et al.* (1956) with the exception that colominic acid was used as the starting material in place of *N*-acetylneuraminic acid. Three g of colominic acid (70% NAN by the Ehrlich method) was dissolved in 200 ml water; 30 g Dowex-50 × 8 (H^+ form) was added and the reaction mixture was heated with stirring at 100° for 10 minutes, cooled, and dried by lyophilization. Absolute methanol (400 ml) was added and the reaction mixture was heated under reflux for 20 hours. The Dowex-50 was removed by filtration and the filtrate was evaporated to dryness *in vacuo* at 40°. The resulting yellow-white solid was dissolved in water, placed on a Dowex-1 × 4 (acetate form) column (43 × 2 cm) and the column was washed with 2000 ml water. The water wash was saved and dried by lyophilization. The resulting white solid was crystallized from methanol-ether 1:1.5. After recrystallization, 435 mg of an analytically pure compound was obtained.

Anal. Calcd. for $C_{13}H_{23}NO_9$ (337): C, 46.29; H, 6.82; N, 4.15. Found: C, 46.13; H, 6.92; N, 4.13. Decomposition point: 97–98°; $[\alpha]_D^{24.5} = -39.2$ (5% in methanol). Methyl ester methyl glycoside of NAN gave a negative reaction to the Warren reagent, did not reduce ferricyanide, and gave a molar color yield with the hydroxylamine–ferric chloride reagent for esters.

Results

Preparation and Analysis of Colominic Acid.—From 50 liters of culture medium, 5.25 g of a product which contained 4.05 g of sialic acid as determined by the Ehrlich procedure was obtained. The product gave no color by Warren's procedure. There was a 7% loss in weight when the product was dried at 100° for 3 hours. There was 4.4% ash in the compound. Products prepared by passing a solution of colominic acid of this purity through Dowex-50 × 8 (H^+ form), drying by lyophilization, and drying for 10 hours at 65.5° under vacuum contained 89% *N*-acetylneuraminic acid by the Ehrlich procedure. The product contained no substances which absorb ultraviolet light in the 260–280 mμ range. No color was produced with the Lowry Folin-phenol reagent at a concentration of 6 mg/ml or by the biuret method. No phosphorus was detected in a 610 μg sample. The infrared spectrum was identical with that found by Barry (1958). The purified colominic acid contained no material which reacted with the orcinol reagent of Mejbaum (1939). Colominic acid contained about 1% anthrone-positive material based on a glucose standard.

Anal. Calcd. for $C_{11}H_{19}NO_9$ (*N*-acetylneuraminic acid): C, 42.74; H, 6.19; N, 4.53. Calcd. for $C_{11}H_{17}NO_8$: C, 45.39; H, 5.88; N, 4.81. Calcd. for $C_{11}H_{15}NO_7$: C, 48.35; H, 5.53; N, 5.12. Found: C, 44.61; mole/μmole H, 6.14; N, 5.01. Acetyl 0.84 μmole/μmole NAN. Glycolyl, negative; alkoxyl, negative.

The empirical formula for the monomeric unit of colominic acid is represented by $C_{11}H_{17}NO_8$ or $C_{11}H_{15}NO_7$ depending on whether one or two molecules of water are eliminated in joining two units.

Molecular Weight Determination.—The average molecular weights of colominic acid samples by ultracentrifuge technique of Archibald as described by Schachman (1957) were found to range from 3000 to 4000. This procedure also indicated the preparations were polydisperse. This molecular weight range was confirmed by the C^{14}-labeled cyanide procedure of Robert *et al.* (1962) (1 mole NAN reacted with 1 mole cyanide).[4] If the 7, 8, and 9 positions of the terminal NAN unit of the colominic acid homopolymer are free (see Table III) and the remaining NAN units are unreactive toward periodate, an uptake of 0.2 mole of periodate per NAN unit would also indicate a molecular weight of about 3000. The loss of colominic acid on prolonged dialysis also indicates a low molecular weight.

Hydrolysis of Colominic Acid to NAN.—Method A. Acid Catalysis.—One g of colominic acid, containing 630 mg of *N*-acetylneuraminic acid as determined by the Ehrlich method, was hydrolyzed at 100° with 10 g Dowex-50 × 8 (H^+ form) for 2 hours, filtered, and dried by lyophilization. The material was suspended in 10 ml glacial acetic acid and dissolved by rapidly heating and adding 1 ml of water. After standing at 0° for 48 hours, the crude product was removed by filtration and dried. The yield of NAN was 504 mg. Products prepared by this method contained a purplish-blue impurity which was difficult to remove by washing the crystalline material with dry ether or by charcoal treatment of the material in solution. To obtain a pure compound, the hydrolysate was purified by chromatography on a Dowex-1 × 4 (acetate form) column (24 × 2 cm). Elution was carried out using 500 ml of 1 M pyridyl acetate (*p*H 4.5) which fed into a reservoir of water (2000 ml) connected to the column. Twenty-ml fractions were collected and assayed for NAN by the Warren (1959) procedure. One major peak was obtained between tubes 34 and 47. The contents of tubes 34–47 were combined, concentrated to 1 ml *in vacuo* at 35°, and then dried by lyophilization. Upon crystallization from glacial acetic acid and water, a 50% over-all yield of *N*-acetylneuraminic acid of high purity was obtained.

The crystalline material had identical R_F values with authentic NAN on paper chromatography in three solvent systems: (1) methylethylketone–acetone–water–formic acid (3:1:1:0.1); (2) sec. butanol–acetic acid–water (4:1:5); (3) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). The infrared spectrum of isolated NAN was identical to the spectrum of authentic NAN.[5] The isolated NAN, when treated with *N*-acetylneuraminic acid aldolase isolated from *Clostridium perfringens* by the method of Comb and Roseman (1960), yielded the same amount of pyruvate as an authentic sample of *N*-acetylneuraminic acid.[6] $[\alpha]_D^{24.5} = -32°$ (5% in H_2O).

Method B. Enzymatic.—A colominic acid preparation (170 mg) was dissolved in water and 0.09 N sodium hydroxide was added to the solution until the *p*H remained constant at 11.0. The *p*H was then reduced to 5.0 with 0.1 N HCl. The volume was brought to 100 ml with 70 ml of 0.1 M acetate buffer (*p*H 4.9). An Ehrlich assay indicated that 120 mg sialic acid was present. Five ml of *Clostridium perfringens* neuraminidase was added and the solution was incubated at 37°. Two drops of merthiolate were added to retard bacterial growth. After incubation for 5 hours a Warren assay indicated 131 mg of free sialic acid was present. The enzymatic hydrolysate (104 ml) was adjusted to *p*H 8–9 with 1 M NH_4OH and placed on a Dowex-1 × 4 (formate form) column (35 × 3 cm). The column was washed with 1 liter of water and then eluted with a linear gradient of 0.9 M formic acid (500 ml) into water

[4] Performed by Dr. L. Robert.

[5] A sample of authentic NAN was secured from Dr. R. J. Winzler of this department and from the Nutritional Biochemicals Corporation.

[6] Kindly performed by Mr. R. Gantt.

TABLE I

PERIODATE OXIDATION OF COLOMINIC ACID AND METHYL ESTER METHYL GLYCOSIDE OF NAN

Time	μmoles Periodate Used per μmole NAN[a] pH 2.6	(OH)pH 2.6[b]	μmole Formic Acid per μmole Periodate pH 2.6
Colominic Acid			
5 min	0.212	0.210	0.40
30 min	0.229	0.222	0.47
1 hr	0.21	0.224	—
2 hr	0.229	0.229	0.49
24 hr	0.34	0.238	—
Methyl Ester Methyl Glycoside of NAN			
1 min	1.83	1.82	0.449
10 min	1.79	1.82	0.489
30 min	1.79	1.82	0.52
1 hr	1.79	1.82	0.57
2 hr	1.76	1.82	—

[a] Calculations based on monomer weight of 291. [b] Sample of colominic acid was adjusted to pH 11.0, let stand 1 hour, and adjusted to pH 2.6.

(500 ml). Twenty-ml fractions were collected and each fraction was assayed by the Ehrlich procedure. Peak I (NAN) contained 108 mg. Peak II (unknown) contained 1.7 mg (by Ehrlich). Peak I was dried by lyophilization and the resulting white material was crystallized by dissolving in 2 ml H_2O and diluting with 10 ml glacial acetic acid. A total of 90 mg of crystalline NAN was isolated. Decomposition point, 186–187°. $[\alpha]_D^{24.5} = -32°$. It had identical R_F values to authentic NAN in three paper chromatographic systems.

Neutralization Equivalent.—Results obtained from titration experiments were dependent on the time required for manipulation. The neutralization equivalent obtained in one case in an automatic recording titrimeter where the time of titration was very short was 1357. When the titration was carried out more slowly and followed potentiometrically to an end point of pH 8, a value of 500 was obtained. Above pH 9, anomalous behavior was encountered with the release of more titratable groups. If the solution was then back-titrated with HCl a neutral equivalent of 273 was obtained. When the solution was titrated again with NaOH the neutral equivalent remained at 273. These results have been interpreted as representing the saponification of a labile ester or lactone which was subsequently titrated. Quantitative ester determinations on the same preparation before alkali treatment gave a value of 0.4 μmole ester/μmole *N*-acetylneuraminic acid using ethyl acetate as a standard. These data indicated that a portion of the carboxyl groups was involved in ester or lactone formation.

Periodate Oxidations.—Table I shows the results obtained from the oxidation of colominic acid and the methyl ester methyl glycoside of *N*-acetylneuraminic acid with periodate. The uptake of 0.2 μmole of periodate per μmole of NAN in colominic acid corresponded to the oxidation of one NAN per 10 units. The formation of 0.5 μmole of formic acid per μmole of periodate used is in accordance with this conclusion. Since there was a possibility that an ester or a lactone linkage might interfere with periodate oxidation, the reaction was performed with colominic acid that had been saponified. The results were the same.

The methyl ester methyl glycoside of NAN was a convenient model compound for the periodate studies. The results shown in Table I are in agreement with those expected and those reported by Blix *et al.* (1956). This is evidence that periodate oxidation proceeded normally with colominic acid.

TABLE II

EFFECT OF PRETREATMENT OF COLOMINIC ACID WITH NaOH ON RELEASE OF NAN BY NEURAMINIDASE[a]

Substrate (μg colominic acid)	Enzyme (ml)	Buffer pH 5.0 (ml)	NAN Released (μg)	NAN Released (%)
120	—	0.3	7.0	5.5
60	0.1	0.3	10.0	15.8
120	0.1	0.2	12.0	9.6
Saponified				
115.6	—	0.3	5.0	4.0
57.8	0.1	0.3	55.0	91.0
115.6	0.1	0.2	110.0	91.0

[a] Incubation of colominic acid and saponified polymer with 0.1 ml neuraminidase for 1 hour at 37°. A final volume of 0.5 ml was obtained with 0.1 M acetate buffer (pH 5.0). The incubation was stopped and assayed for free NAN by the removal of a 0.2-ml aliquot. A Warren assay was performed immediately on this material. Yield is based on a monomer weight of 291.

In order to verify these results further, a 384-mg sample of colominic was oxidized under the same conditions with periodate. NAN was isolated in a yield of 43% following hydrolysis of the product with Dowex-50 and chromatography on Dowex-1 × 4 as described under Methods.

Action of Neuraminidase on Colominic Acid.—Table II shows the results obtained when colominic acid was treated with neuraminidase. Clearly the enzyme gave very rapid and complete splitting of the polymer when all the carboxyl groups were free but was much less active on colominic acid which had not been saponified. The enzyme has been used to prepare *N*-acetylneuraminic acid from colominic acid in good yields (Methods). The K_m for this neuraminidase preparation was 4×10^{-5} M colominic acid. A polymer of ten units was assumed for making the calculations.

DISCUSSION

The high yield of *N*-acetylneuraminic acid obtained by both acid hydrolysis and enzymic hydrolysis of colominic acid, and the absence of other carbohydrates, protein, and phosphorus, constitute good evidence that colominic acid is a homopolymer. These results are in agreement with those of Barry (1958).

The molecular weight of colominic acid prepared by the procedure used in this work was 3000–4000. The observed variation in the molecular weight and the polydisperse nature of a single preparation is to be expected for a bacterial exopolymer and for a substance that is sensitive to acid when an acidification step is used in the purification procedure. By selecting the proper growth conditions and modifying the purification procedure, one should be able to obtain preparations with substantially higher molecular weights.

If *N*-acetylneuraminic acid retains the pyranose ring structure in the polymer, which is likely, then the uptake of 0.2 μmole periodate per NAN unit in colominic acid would be best explained by a 2–8 ketosidic linkage between the NAN units. Any other linkage would require the uptake of at least 1 μmole periodate per NAN unit. Table III shows the results which would be expected from the periodate oxidation of the four most likely structures for a polymer containing ten NAN units. Clearly a 2–8 linkage is indicated. Examples of compounds are known which do not undergo

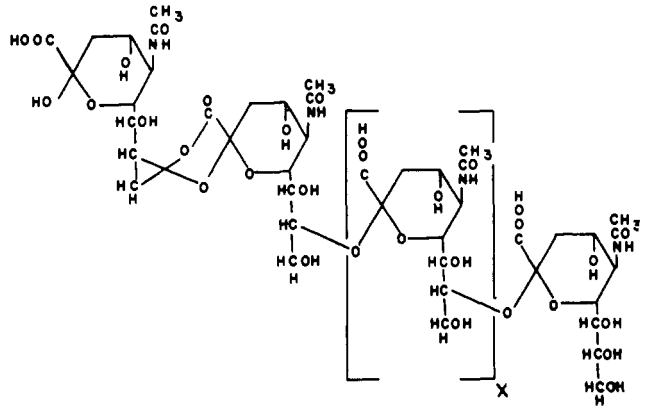
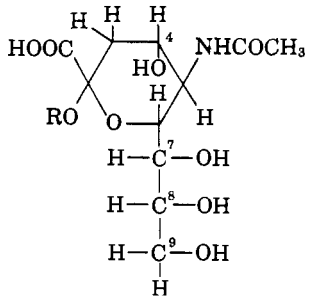


FIG. 1.—Structure of colominic acid; X = 8 (average).

TABLE III

RESULTS WHICH WOULD BE EXPECTED FOR THE PERIODATE OXIDATION OF A TEN-UNIT POLYMER HAVING THE LINKAGES INDICATED

	Linkage	μmoles Periodate per μmole NAN	μmole Formic Acid per μmole NAN
	2–4	2.0	1.0
	2–7	1.1	0.1
	2–8	0.2	0.1
	2–9	1.1	0.1

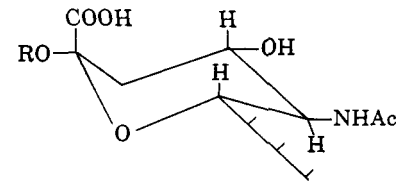
oxidation with periodate even though oxidizable groups are present. There is a possibility that colominic acid is giving anomalous behavior but the good agreement with the expected results for a 2–8 linkage and with the model compound give assurance that the oxidation is proceeding normally.

The consumption of alkali above *p*H 8.0 and the presence of groups reacting with hydroxylamine could be explained by (a) the presence of *O*-acetyl groups, (b) the presence of lactone linkages, or (c) ester linkages between the carboxyl group of one unit and a hydroxyl group of a neighboring unit.

The presence of acetyl groups was ruled out on the basis of (1) quantitative analysis for acetyl groups which showed only one *N*-acetyl group per NAN unit, (2) the failure to find acetic acid in colominic acid preparations which had been treated at *p*H 11.0, (3) the neutralization equivalent which would be much lower if acetyl groups were removed, and (4) the probability that the bacterial neuraminidase would split the *O*-acetylated compound.

The choice between an ester and a lactone structure is more difficult. No one has reported evidence for lactone formation in NAN and the lactone obtained by Kuhn and Baschang (1962) must be opened to obtain the *N*-acetylneuraminic acid. Further, *N*-acetylneuraminic acid would exist in the 1-C conformation in which all the large groups are equatorial.

Lactone formation in such a molecule is improbable. An ester linkage from the carboxyl to the hydroxyl in either the 7 or 9 position (Fig. 1) is more likely. Although we prefer esterification to the 9-position, there is no evidence to support this choice.



α-Glycoside in 1-C Conformation

If Gottschalk's (1958) proposal that neuraminidase is an α-ketosidase is correct then the monomer units are linked by an α-2,8-linkage. There is no evidence that the enzyme is truly an α-ketosidase. We prepared the methyl glycoside of *N*-acetylneuraminic acid and found that 3% of the product was split with neuraminidase. Although the product of the enzymic reaction was identified as *N*-acetylneuraminic acid and the reaction had all the characteristics of an enzymic process, we have not prepared pure α- and β-methylglycosides. Based on the 1-C conformation we would expect 97–98% of the methyl glycoside to be in the β configuration and 2% or 3% in the α form. This information agrees with the proposal that the enzyme is an α-ketosidase but further study is required before the specificity of the enzyme can be stated with certainty.

It has been customary to assume that sialic acid liberated from large molecules by neuraminidase occupies an end position in a chain and sialic acid not liberated is situated within a chain. In view of these results sialic acid units involved in ester linkages may not be released even if in a terminal position.

At this time we do not know whether the ester linkages are present in colominic which is biologically formed or are artifacts caused by the acid precipitation carried out during isolation, or are produced by self-catalysis since the molecule is quite acidic. The ease with which sialic acid is esterified and the lability of this ester may be chemical clues to the biological function of this ubiquitous sugar.

ACKNOWLEDGMENTS

The authors wish to thank Mr. H. Trahan for his enthusiastic aid in this work. The authors would also like to express their appreciation to Dr. S. Roseman for a culture of *Clostridium perfringens* and Dr. G. T. Barry for a culture of *Escherichia coli* K235 L+O.

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Immunochemical Studies with Gangliosides. II. Investigations of the Structure of Gangliosides by the Hapten-Inhibition Technique

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Received July 22, 1963

The hapten-inhibition technique has been used to obtain further information about the chemical structure of gangliosides. The most striking findings were obtained with antibodies produced against a ganglioside derivative (asialoganglioside) from which *N*-acetylneuraminic acid had been cleaved by mild acid hydrolysis. The results obtained in the present study are consistent with the sequence of hexoses and *N*-acetylgalactosamine proposed by Svennerholm (1962), and by Kuhn and Wiegandt (1963). Studies with a variety of sugars and sugar derivatives indicated that inhibition is encountered only with a glycosidically bound β-D-galactopyranose or 2-acetamido-2-deoxy-β-D-galactopyranose moiety.

Although there is at present general agreement about the number and type of carbohydrate residues in brain gangliosides (Svennerholm, 1962; Kuhn and Wiegandt, 1963; Wagner *et al.*, 1963), the exact order and configuration of the linkages of these molecules has remained a controversial subject. Conflicting results have been obtained by acid hydrolysis (Klenk and Gielen, 1961), permethylation (Klenk and Gielen, 1960; Egge, 1960; Karkas and Chargaff, 1960), oxidation with periodate (Klenk *et al.*, 1962), or reduction with borohydride (Kanfer and Brady, 1963). A reproducible method was developed in this laboratory (Yokoyama *et al.*, 1963) for the production of antibodies against gangliosides and asialoganglioside, the glycosphingolipid residue of gangliosides from which the *N*-acetylneuraminic acid had been removed by mild acid hydrolysis (Trams and Lauter, 1962). Because of the availability of these specific antibodies, it was thought desirable to utilize the hapten-inhibition technique (Landsteiner, 1945; Kabat, 1958) in an attempt to obtain further insight into the structure and configuration of gangliosides. This procedure is based on the observation that the presence of a low molecular weight compound which resembles the structure of a particular antigen is able to block the reaction between the antigen and its specific antibody. The validity of the procedure rests upon the assumption that in a comparison of a series of inhibitors the substance exhibiting inhibition at the lowest concentration has a structure most similar to the determining group on the antigen (Kabat and Mayer, 1961). The method has been used with considerable success to elucidate the structure of the antigenic group of dextran (Kabat, 1957) and blood group substance A antigen (Schiffman *et al.*, 1962).

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Experimental Procedure

Materials.—Gangliosides were prepared from beef brain by the method of Trams and Lauter (1962). Other samples were purchased from the Sigma Chemical Co. (lot 81B-623). When gangliosides were hydrolyzed under mildly acid conditions, two major glycolipid products were obtained. Analyses of these materials revealed that both contained 1 molecule each of sphingosine, fatty acid, glucose, and *N*-acetylgalactosamine. The compound designated as asialoganglioside contained, in addition to these components, 2 molecules of galactose. The other material, referred to as aminoglycolipid (Bogoch, 1961), had only 1 additional molecule of galactose.

A sample of Tay-Sachs' ganglioside was generously furnished to us by Dr. Joel A. Dain of Rhode Island University. Red-cell stroma globoside was prepared by Mr. Joseph V. Formica in this laboratory by the method of Makita and Yamakawa (1962). This material cochromatographed with authentic globoside kindly supplied by Dr. Yamakawa. Cytolipin H was a gift from Dr. Maurice M. Rapport. The following materials were the gifts of the respective persons mentioned: samples of *N*-acetylglucosamine, Dr. Roger W. Jeanloz; samples of anhydro derivatives of galactose, Dr. Nelson K. Richtmyer; various β-galactosides, Dr. Elizabeth Neufeld; samples of raffinose and stachyose, Dr. Dexter French; galactobiose, Dr. Roy L. Whistler; 4-*O*-β-D-galactosyl-*N*-acetylglucosamine, Professor Richard Kuhn, a sample of colominic acid, Dr. G. T. Barry; and samples of methyl galactosides, Dr. Hewitt G. Fletcher. Other sugars and oligosaccharides were purchased from commercial sources.

Methods.—Antiganglioside and antiasialoganglioside antibodies were produced in rabbits by the method of Yokoyama *et al.*, (1963). The rabbit antiserum ob-