Preparation and Properties of Sialomucopolysaccharides Obtained from Rat Brain

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Sialomucopolysaccharides were released from the defatted protein residue by the proteolytic action of papain after extraction of rat whole brains with chloroform-methanol (2:1, v/v). Further purification is achieved by dialysis to remove low-molecular-weight fragments and by precipitation of nucleic acids and glucuronic acid-containing mucopolysaccharides by treatment with cetylpyridinium chloride. Gel filtration of the sialomucopolysaccharides through Sephadex G-200 removes the major portion of the impurities that absorb light in the ultraviolet region. The sialomucopolysaccharides were fractionated on DEAE-Sephadex to yield a population of sialomucopolysaccharides that show an increase in *N*-acetylneuraminic acid content and a decrease in fucose content as the concentration of chloride required to elute the individual components is increased. On gel filtration on Sephadex G-200, those sialomucopolysaccharide molecules rich in *N*-acetylneuraminic acid and poor in fucose appear to be larger molecules than those rich in fucose and poor in *N*-acetylneuraminic acid. A structure is proposed in which all sialomucopolysaccharide molecules are assumed to possess the same repeating unit consisting of hexosamine and hexose. The molecules differ from each other in the number of fucose and *N*-acetylneuraminic acid residues attached to the basic structure. Most of the hexosamine is present as glucosamine, although one fraction was obtained that appeared to contain galactosamine. Most of the hexose present is accounted for as galactose and mannose, although small amounts of glucose were found in some fractions. Methods of analysis for the *N*-acetylneuraminic acid and hexosamine components of the sialomucopolysaccharides were defined.

The major neuraminic acid-containing material of rat brain, after removal of the gangliosides by organic solvents, is the protein-linked sialomucopolysaccharides (Brunngraber & Brown, 1964a). It has been shown that the sialomucopolysaccharides isolated from the defatted protein residue by proteolytic digestion are a mixture of sialomucopolysaccharides that differ in the molar ratios of their constituent sugars (Brunngraber & Brown, 1964b). The present work advances the purification procedure used to isolate the sialomucopolysaccharides from brain, defines optimum conditions for the determination of NANA* and hexosamine present in purified samples of sialomucopolysaccharide mixtures, and provides some information on the structure of the sialomucopolysaccharide molecule.

MATERIALS AND METHODS

Isolation of sialomucopolysaccharides. Rat whole brains were homogenized in 19 vol. of chloroform-methanol (2:1,

v/v) and centrifuged. The organic phase was decanted and shaken with one-fifth its volume of 0.1 M-KCl according to the method of Folch, Lees & Sloane-Stanley (1957). The methanol-rich upper phase was separated. This material was dialysed and concentrated to provide the ganglioside preparation used in this study.

The defatted precipitate obtained from one rat brain was dried and suspended in 6 ml. of 0.1 M-sodium acetate buffer, pH 5.5, containing 5 mM-cysteine and 0.5 mM-EDTA (sodium salt). After incubation for 24 hr. at 60° with crystalline papain (0.035 mg./ml. of suspension) the mixture was centrifuged. The precipitate obtained was washed with water and the water wash combined with the supernatant. The washed precipitate (the 'papain-resistant fraction') was analysed for NANA. The supernatant was dialysed against 1 l. of water and the water changed three times over a 24 hr. period. The diffusate was concentrated and analysed for NANA. This material represents components of low molecular weight released by the proteolytic activity of papain.

The non-diffusible components of high molecular weight, including the sialomucopolysaccharides, were treated with cetyl pyridinium chloride to remove by precipitation the insoluble glucuronic acid-containing mucopolysaccharides and the nucleic acids. The procedure is that of Scott (1960).

* Abbreviation: NANA, *N*-acetylneuraminic acid.

The E_{260} value of the solution was determined. To each ml. of the solution, 1 ml. of 0.01 M- Na_2SO_4 and 1 ml. of cetylpyridinium chloride solution were added. The concentration of the cetylpyridinium chloride solution used was varied with the extinction at 260 m μ ; a freshly prepared cetylpyridinium chloride solution at a concentration of 0.034%/ E_{260} unit was employed. For example, for a solution with an E_{260} value of 11.8, the concentration of the cetylpyridinium chloride reagent was $11.8 \times 0.034\%$ or 0.4% cetylpyridinium chloride. The mixture was incubated at 30° in a water bath for 30 min. The precipitate that formed was removed by centrifugation. The supernatant was treated with pentan-1-ol to remove the cetylpyridinium chloride. For each mg. of cetylpyridinium chloride calculated to be present in the solution, 0.2 ml. of pentan-1-ol was added with stirring at 0° in an ice bath. The two phases were separated in a separatory funnel in the cold room; the upper pentan-1-ol phase was discarded. This step was repeated. The last traces of pentan-1-ol were removed by dialysis. The final solution contained the sialomucopolysaccharides. This preparation was used in the studies described below.

Chromatography on DEAE-Sephadex. DEAE-Sephadex was prepared for use by swelling in an excess of water and removal of fines by decantation. It was briefly washed with 0.5 N-HCl, followed with water, 0.5 N-NaOH and again with water. It was washed and equilibrated with the starting buffer (5 mM-sodium phosphate, pH 7.0). Sialomucopolysaccharides dissolved in a small volume of 5 mM-sodium phosphate buffer, pH 7.0, were applied to a DEAE-Sephadex A-50 (medium grade) column (1 cm. $\times$ 22 cm.). After the column was washed with 5 mM-phosphate buffer to elute unadsorbed polysaccharides, elution was carried out with a linear gradient of NaCl in 5 mM-sodium phosphate buffer from 0–0.3 M-NaCl. The linear gradient was achieved by the use of the Varigrad device described by Peterson & Sober (1959). The flow rate was 3–5 ml./hr.

Gel filtration on Sephadex G-200. Sephadex G-200 (140–400 mesh) gel-filtration medium (lot no. To56) was added to distilled water and allowed to swell for at least 5 days. Columns (34 cm. $\times$ 4 cm., bed volume 420 ml.), with a flow rate of approx. 12 ml./hr., were operated under a hydrostatic head that never exceeded 15 cm. The gel had been deaerated under reduced pressure before packing into the column. Samples of sialomucopolysaccharide (5 ml.) dissolved in water were applied to the top of the column by layering under solution already present; 8 ml. fractions were collected.

Cleavage of NANA with neuraminidase. Sialomucopolysaccharides were incubated in a medium containing 0.05 M-sodium acetate buffer, pH 5.5, which contained 0.9 g. of NaCl and 0.1 g. of $\text{CaCl}_2/100$ ml. in the presence of 25 units of *Vibrio cholerae* neuraminidase (Svennerholm, 1963).

Analytical methods. NANA was determined by the use of the thiobarbituric acid method of Warren (1959), the orcinol method (Bogoch, 1958) and the resorcinol method (Svennerholm, 1956a,b). Hexosamine was determined by the Elson-Morgan method as modified by Boas (1953). The procedure for the differential determination of glucosamine and galactosamine was that described by Radhakrishnamurthy & Berenson (1964). Hexose and fucose were determined directly on the unhydrolysed solution of the sialomucopolysaccharide by utilizing the primary cysteine reaction of Dische (1955); hexoses were

also determined by the secondary cysteine reaction of Dische (1955). In the former reaction, the ratios of the extinction coefficients for mannose and galactose to that of the glucose are 0.4 and 0.5 respectively. In the latter reaction, the ratios of extinction coefficients of mannose and galactose to that of glucose are 0.23 and 2.50 respectively. Therefore, in the absence of glucose, these two reactions permit the determination of mannose and galactose in mixtures (Dische, Shettles & Osnos, 1949). The two reactions were also employed in the presence of glucose by correcting extinctions obtained for this sugar. An independent measure of glucose determination was available through the use of an enzymic method (Glucostat kit purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.) employing glucose oxidase. Glucose was also determined by the enzymic method based on the reduction of NADP measured at 340 m μ utilizing Calsul kit no. 869 004 (Calbiochem, Los Angeles, Calif., U.S.A.).

Materials. NANA and papain (twice recrystallized, sodium acetate suspension) were both obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Glucosamine, galactosamine, mannose and fucose were obtained from Calbiochem. *Vibrio cholerae* neuraminidase was obtained from General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. The rats used were of the Wistar strain obtained from Abrams Small Stock Breeder Inc., Chicago, Ill., U.S.A.

RESULTS

Isolation of sialomucopolysaccharides. A single treatment of rat brain with chloroform-methanol (2:1, v/v) extracts approx. 58% of its content of NANA. The remainder is bound to the denatured protein in the defatted residue. Digestion of this residue with papain at pH 5.5 for 24 hr. liberates 25% of the total NANA present in the original tissue. Further treatment with papain will solubilize about half of the remaining insoluble 'papain-resistant' NANA. However, the present work is confined to that fraction which is released by the single 24 hr. proteolytic digestion. The NANA liberated by the papain digestion consists of a diffusible NANA-containing material (6%) and the non-diffusible sialomucopolysaccharides (19%). The sialomucopolysaccharides isolated by this purification sequence were treated with cetylpyridinium chloride to remove the glucuronic acid-containing mucopolysaccharides and nucleic acids to provide the starting material for the following experiments.

Analysis of NANA. Three spectrophotometric methods for NANA were applied to the sialomucopolysaccharides and gangliosides (Table 1). The thiobarbituric acid method, which requires hydrolysis for 1 hr. in 0.1 N-sulphuric acid at 80° for maximal NANA values, gave values that were 83% of the value obtained by both the orcinol and resorcinol methods when these were applied to the sialomucopolysaccharide. We confirmed the work of Hess & Rolde (1964), who reported that NANA

Table 1. *Comparison of three spectrophotometric methods for the analysis of NANA in gangliosides and sialomucopolysaccharides*

The thiobarbituric acid assay was preceded by hydrolysis in 0.1N-H₂SO₄ at 80° for the times indicated. Values are expressed in terms of μ moles of NANA present/g. fresh wt. of brain.

Method of analysis	Ganglioside NANA (μ moles/g.)	Sialomuco- polysaccharide NANA (μ moles/g.)
Orcinol	1.46	0.60
Resorcinol	1.62	0.60
Thiobarbituric acid and 2 hr. hydrolysis	1.25	—
Thiobarbituric acid and 1 hr. hydrolysis	—	0.50
Thiobarbituric acid and 1 hr. hydrolysis, corrected for destruction of NANA	—	0.55
Thiobarbituric acid and 2 hr. hydrolysis, corrected for destruction of NANA	1.62	—

is destroyed at an initial rate of 9%/hr. during acid hydrolysis in 0.1N-sulphuric acid at 80°. Correction for this destruction increased the value obtained by the thiobarbituric acid method to 90% of the value obtained by the other methods. The same value as that of the corrected value was obtained if the standard NANA was subjected to exposure to sulphuric acid under the same conditions as the sialomucopolysaccharide.

As was reported by Hess & Rolde (1964) and Booth (1962), the value for gangliosidic NANA, as determined by the thiobarbituric acid method, provides values below those obtained by the orcinol and resorcinol methods. Multiplication of the value for gangliosidic NANA obtained by the thiobarbituric acid method after a 2 hr. hydrolysis by 1.3, as Hess & Rolde (1964) suggested, gave a value corresponding to that obtained by the resorcinol procedure. Exposure of the sialomucopolysaccharides and gangliosides to acid hydrolysis for up to 2 hr. had no effect on the values obtained by the resorcinol or orcinol reactions. Booth (1962) reported a slight increase in extinction obtained by the orcinol method for periods of hydrolysis up to 2 hr.

Liberation of NANA by acid hydrolysis and neuraminidase action. To obtain the maximum value for sialomucopolysaccharide NANA, it is necessary to hydrolyse the samples for 1 hr. in 0.1N-sulphuric acid at 80° (Table 2). Maximum values for gangliosidic NANA requires a minimum of 90 min. Comparison of the data shows that the NANA present in sialomucopolysaccharides is

Table 2. *Rate of acid hydrolysis of sialic acid from gangliosides and sialomucopolysaccharides*

Samples were hydrolysed in 0.1N-H₂SO₄ at 80° for the times indicated. Values are expressed in terms of percentages of the maximum values for NANA obtained by analysis with the thiobarbituric acid method. Values were not corrected for degradation of NANA during hydrolysis.

Time of hydrolysis (min.)	Extent of hydrolysis (% of maximum value)	
	Sialomuco- polysaccharide	Ganglioside
0	0	3
20	77	66
40	95	73
60	100	85
90	100	100
120	100	100
180	97	100

somewhat more readily cleaved by acid than is the NANA moiety of the gangliosides.

An 18 hr. incubation of sialomucopolysaccharides with *Vibrio cholerae* neuraminidase (40 units/ml.) liberated all of its neuraminic acid. The neuraminic acid residues in the sialomucopolysaccharide are not equally susceptible to the cleaving action of this enzyme: 60% of its NANA was released within 40 min. of incubation; prolonged incubation was required to liberate the rest.

Liberation of hexosamine by acid hydrolysis. A 1 hr. hydrolysis in 1.5N-hydrochloric acid at 100° sufficed to obtain maximal values for the hexosamine content of sialomucopolysaccharides. No further change in this value was obtained if the hydrolysis was prolonged to 8 hr. Between 2 and 4 hr. was required for maximal values when the hydrolysis was conducted in 0.75N-hydrochloric acid.

Chromatography of sialomucopolysaccharides on DEAE-Sephadex. A sialomucopolysaccharide preparation from ten rat whole brains was chromatographed on DEAE-Sephadex (Fig. 1). A part of the material was not retained by the anion-exchanger; the remainder was eluted throughout the range (0–0.25M) of a linearly increasing sodium chloride concentration gradient. It was not possible to separate individual components, but it was evident that there was a wide distribution of sialomucopolysaccharides. Contents of test tubes containing eluates corresponding to fractions I, II, III and IV respectively were combined, dialysed to remove sodium chloride and concentrated. These were analysed for various carbohydrate constituents (Table 3). Changes in composition are correlated

Fig. 1. Chromatogram obtained when a sialomucopolysaccharide preparation from ten rat whole brains is subjected to column chromatography on DEAE-Sephadex A-50 (medium grade). Column dimensions were 1 cm. $\times$ 22 cm. All solutions of NaCl were in 5 mM-sodium phosphate, pH 7. Flow rate was 3–5 ml./hr. —, Concentration of NANA (μ g./ml.) as a function of effluent volume. ----, NaCl concentration gradient. Sialomucopolysaccharides not retained by the anion-exchanger are designated as fraction I, sialomucopolysaccharides eluted between 0 and 0.06 M-NaCl as fraction II, sialomucopolysaccharides eluted between 0.06 M- and 0.13 M-NaCl as fraction III, and sialomucopolysaccharides eluted between 0.13 M- and 0.25 M-NaCl as fraction IV. Contents of test tubes containing the indicated fractions were combined, and the fractions were dialysed and concentrated. The carbohydrate composition of these fractions is given in Table 2.

with the strength of binding of the sialomucopolysaccharide to the anion-exchanger, as judged by the concentration of sodium chloride required to elute it. This concentration increased in the order: I, II, III and IV. The NANA/fucose, hexosamine/fucose and hexose/fucose ratios (Table 3) also show an increase in the order: I, II, III and IV. The percentage of NANA in the material of each fraction increases in the same sequence. This is evident when the fucose/NANA, hexosamine/NANA and hexose/NANA ratios are computed. These all decrease in the order: I, II, III and IV.

All of the hexosamine in fractions I, II and IV is glucosamine. However, 15% of the hexosamine of fraction III appears to be galactosamine. The presence of glucose in fraction I is consistent with previous findings (Brunngraber & Brown, 1964a). The presence of glucose in other fractions has not previously been observed. Previous experiments (Brunngraber & Brown, 1964a,b) have shown that glucose is absent from those sialomucopolysaccharide fractions that are retained by Dowex 1 (Cl⁻ form) or DEAE-Sephadex. Fraction IV, which contains glucose, does not contain galactosamine and this would appear to eliminate the possibility that this fraction is contaminated with gangliosides. The fact that glucose was absent as judged by the Glucostat reaction and paper chromatography in previous work suggests that

Table 3. Carbohydrate composition of sialomucopolysaccharide fractions isolated by chromatography on DEAE-Sephadex

Eluates corresponding to fractions I, II, III and IV (see Fig. 1) were combined, dialysed, concentrated and analysed for fucose, hexosamine, hexose and NANA. Sialomucopolysaccharides isolated from ten rat whole brains had been applied to the column.

Fraction	Composition (μ moles)						Total hexose	Total hexose*
	Fucose	NANA	Hexosamine	Galactose	Mannose	Glucose		
I	0.69	0.55	1.71	1.08	2.15	0.43	3.66	2.83
II	0.46	0.71	1.36	0.94	1.57	0.11	2.62	2.17
III	0.66	1.71	3.22	1.89	2.04	0.09	4.02	3.52
IV	0.56	2.43	3.23	2.13	2.65	0.51	5.29	4.47
Total†	2.37	5.44	9.52	6.04	8.41	1.14	15.59	12.99

Fraction	Molar ratios						Hexose*/fucose
	NANA/fucose	Hexosamine/fucose	Galactose/fucose	Mannose/fucose	Glucose/fucose	Hexose/fucose	
I	0.8	2.48	1.66	3.12	0.62	5.40	4.11
II	1.54	2.96	2.04	3.41	0.24	5.69	4.72
III	2.44	4.83	2.86	3.09	0.14	6.09	5.33
IV	4.34	5.77	3.80	4.73	0.91	9.44	8.00
Total†	2.29	4.01	2.54	3.54	0.48	6.57	5.48

* Total hexose determined by the use of the primary cysteine reaction of Dische (1955) with galactose as a standard.

† Carbohydrate composition and molar ratios for sum of fractions I, II, III and IV recovered on chromatography.

Fig. 2. Chromatogram obtained when a sialomucopolysaccharide preparation from about nine rat whole brains was subjected to gel filtration on a column of Sephadex G-200, with a bed volume of 420ml. The gel had been equilibrated in distilled water, and the applied sialomucopolysaccharides were eluted with distilled water: 8ml. fractions were collected. —, Concentration of NANA ($\mu\text{g./ml.}$) as a function of effluent volume., E_{280} . Blue Dextran (DB), which is totally excluded from the gel matrix, is eluted between 112 and 136ml. Sialomucopolysaccharides are eluted between 128 and 440ml. The fraction corresponding to that portion of the sialomucopolysaccharides eluted between 128 and 288ml. (high-molecular-weight fraction) was combined and designated fraction I. The fraction corresponding to the portion of sialomucopolysaccharides eluted between 288 and 440ml. (low-molecular-weight fraction) is designated fraction II. These fractions were subsequently chromatographed on DEAE-Sephadex (Fig. 3).

this sugar may appear as a contaminant rather than as an integral part of the sialomucopolysaccharide molecule.

Gel filtration on Sephadex G-200. A sialomucopolysaccharide preparation from nine rat whole brains was subjected to gel filtration on Sephadex G-200 (Fig. 2). The substance begins to appear in the effluent immediately after the elution of Blue Dextran 2000. Blue Dextran has an average molecular weight of 2000000 and is completely excluded from the gel matrix. Sialomucopolysaccharides are eluted over a broad range, suggesting that this material is characterized by a range of molecular sizes. No two or more components appear to possess a molecular size sufficiently different to permit separation into discrete peaks. The chromatographic profile in experiments with Sephadex G-200, Sephadex G-100 and Sephadex G-75 suggests the presence of a population of sialomucopolysaccharides that differ in molecular size.

Sephadex G-200, as well as Sephadex G-100 and G-75, were effective in separating the sialomucopolysaccharides from the bulk of the material that absorbs in the ultraviolet region. In most experiments it was possible to achieve complete separation of this impurity from the sialomucopolysaccharides. Some overlap occurred in the experiment of Fig. 2. These experiments showed that the materials that absorb in the ultraviolet light are impurities rather than constituents associated with the sialomucopolysaccharides.

Determination of the ultraviolet extinction at wavelengths of 226.5 and 260m μ yielded the same

Table 4. Carbohydrate composition of sialomucopolysaccharide fractions isolated by gel filtration on Sephadex G-200

Eluates corresponding to fractions I and II (see Fig. 2) were combined, dialysed, concentrated and analysed for fucose, hexosamine, hexose and NANA. Total hexose was determined by the use of the primary cysteine reaction of Dische (1955) with galactose as standard.

Fraction	Composition (μmoles)			
	NANA	Hexosamine	Fucose	Hexose
I	3.94	5.08	1.42	9.29
II	3.13	7.09	2.32	11.60
Total*	7.07	12.17	3.74	20.89

	Molar ratios		
	NANA/fucose	Hexosamine/fucose	Hexose/fucose
I	2.77	3.57	6.54
II	1.34	3.05	5.00
Total*	1.89	3.25	5.58

* Carbohydrate composition and molar ratios for the sum of fractions I and II recovered on gel filtration.

Fig. 3. (a) Chromatogram obtained when fraction II (low-molecular-weight fraction) obtained by gel filtration of sialomucopolysaccharides from rat brain (Fig. 2) was subjected to column chromatography on DEAE-Sephadex. Column dimensions were 1 cm. $\times$ 22 cm. All solutions of NaCl were in 5 mM-sodium phosphate, pH 7. (b) Chromatogram obtained when fraction I (high-molecular-weight fraction) obtained by gel filtration of sialomucopolysaccharides from rat brain was subjected to column chromatography on DEAE-Sephadex. —, Concentration of NANA (μ g./ml.) as a function of effluent volume; ----, NaCl concentration gradient.

chromatographic profile as that obtained at 280 μ (Fig. 2).

The fraction corresponding to that portion of the sialomucopolysaccharides eluted between 128 and 288 ml. (high-molecular-weight fraction) was designated fraction I (Fig. 2). Material eluted between 288 and 440 ml. (low-molecular-weight fraction) is designated fraction II. The chemical data (Table 4) reveal that the high-molecular-weight fraction represents the group of sialomucopolysaccharides that contain a greater percentage of NANA. The hexosamine/NANA, fucose/NANA and hexose/NANA molar ratios are greater for fraction II than for fraction I. The NANA/fucose, hexosamine/fucose and hexose/fucose molar ratios are greater for fraction I than for fraction II.

These analytical values are confirmed by chromatography of fractions I and II on DEAE-Sephadex (Fig. 3). The major part of fraction I is eluted between 0.08 M- and 0.24 M-sodium chloride. Fraction II includes those sialomucopolysaccharides that are not adsorbed by DEAE-Sephadex, as well as those eluted between 0 and 0.10 M-sodium chloride.

DISCUSSION

The present results have extended the purification procedure of brain sialomucopolysaccharides by demonstrating that gel filtration will remove most of the ultraviolet-absorbing impurities in the

sialomucopolysaccharides prepared by digesting defatted protein residues with papain, dialysing and removing impurities by cetylpyridinium chloride. The data on amino nitrogen content of the sialomucopolysaccharides obtained previously (Brunngraber & Brown, 1963, 1964a) are therefore invalidated, since most of this material is not an integral part of the sialomucopolysaccharide molecule.

The use of distilled water as solvent in gel filtration causes the sialomucopolysaccharides to aggregate and they are eluted as a high-molecular-weight component. The elution volume of the sialomucopolysaccharides corresponds to that expected for a molecular weight somewhat less than 10000 when the gel filtration is carried out with 0.05 M-tris-hydrochloric acid buffer, pH 7.5, containing 0.1 M-potassium chloride. This buffer was used by Andrews (1965) in estimating the molecular weight of proteins by gel-filtration procedures. The ultraviolet-absorbing impurities cannot be removed from the sialomucopolysaccharides if this buffer is used.

Rat brain was extracted only once with chloroform-methanol (2:1, v/v), since Folch, Ascoli, Lees, Meath & LeBaron (1951) reported that the amount of solids extracted by a second extraction amounted to no more than 0.5% of the amount of solids present in the first extraction. Similar results were obtained with both grey and white matter.

The sialomucopolysaccharides investigated in the present work represent approx. 19% of the brain NANA. It should be noted that about 17% of the brain content of NANA remains in the defatted brain residue after papain digestion for 24 hr. A considerable amount of this material is probably similar in nature to the sialomucopolysaccharides, as judged by its content of fucose and glucosamine. However, it may contain some gangliosides that are not extracted with chloroform-methanol (2:1, v/v) (Suzuki, 1965). Suzuki (1965) found that the extraction of the more polar gangliosides was not complete unless the chloroform-methanol mixture used as solvent contained at least 60% of methanol. We have found that appreciable amounts of galactosamine are present in the 'papain-resistant' fraction.

Pronase, trypsin and pepsin do not release more sialomucopolysaccharide than papain. Some success has been achieved by resuspending the protein residue in fresh buffer and papain after removal of the proteolytic digestion products. About half of its NANA content is released if the papain digestion is repeated two or three times in this manner. The present work was limited to that sialomucopolysaccharide fraction which is released during the initial 24 hr. hydrolysis. It remains to be deter-

mined whether the sialomucopolysaccharides present in the 'papain-resistant' fraction after a 24hr. digestion resembles those that are released.

Although papain solubilizes about 60–65% of the NANA present in the defatted brain residue after digestion for 24hr., 80–85% of its hexosamine content is released. A large part of this hexosamine is diffusible, whereas only a small percentage of the NANA that is released is diffusible. The present experiments with gel filtration suggest that the compounds containing 'diffusible' NANA differs qualitatively from the sialomucopolysaccharide studied here. Their molecular weight (or size) is so much smaller than that of the sialomucopolysaccharides that they cannot be considered to represent the lowest-molecular-weight fraction of the sialomucopolysaccharide population. The chromatogram of Fig. 2 indicates that the smallest molecular size of the sialomucopolysaccharide population is sufficiently large to prevent their dialysis and is larger than a non-diffusible protein residue that follows it in the elution sequence.

The contamination of the sialomucopolysaccharide preparation with gangliosides must be considered. The failure to find galactosamine and glucose in sialomucopolysaccharide preparations (Brunngraber & Brown, 1963, 1964a,b) suggests that gangliosides are absent. Although small amounts of glucose and galactosamine have been detected in some fractions of the sialomucopolysaccharide preparation obtained by chromato-

graphy on DEAE-Sephadex, the two sugars were not present in the same fraction. Fraction IV, which contained glucose, did not contain galactosamine. Fraction III, which contained galactosamine, yielded a negligible amount of glucose (Table 3).

It has been instructive to compare molar ratios of sugars in fractions obtained from sialomucopolysaccharide preparations by means of anion-exchange chromatography on DEAE-Sephadex or Dowex 1 (Cl⁻ form), as well as gel filtration on Sephadex G-200 (Table 5). Fractions obtained from the sialomucopolysaccharide preparation are listed in order of increasing NANA/fucose ratio. As this ratio increases, those of hexosamine/NANA and hexose/NANA decrease. The rate of decrease in the last two ratios is not as marked as the rate of increase in the NANA/fucose ratio. Further, the hexose/fucose and hexosamine/fucose ratios increase as the NANA/fucose ratio increases. The rate of increase of the first two ratios is somewhat less than that of the NANA/fucose ratio. By contrast, the hexose/hexosamine ratio has an average value for the 14 fractions listed of 1.67 ± 0.26 (16%), and shows no consistent correlation with the NANA/fucose ratio.

All of these observations can be explained by postulating that both NANA and fucose are linked to a basic hexosamine-hexose repeating structure that is common to all of the sialomucopolysaccharide fractions isolated. Variable amounts of NANA and fucose are linked to this residue.

Table 5. *Molar ratios of saccharide components present in different sialomucopolysaccharide subfractions isolated by chromatography*

Data for Expts. 1 and 2 were obtained by fractionating sialomucopolysaccharides from rat brain on DEAE-Sephadex. Data for Expt. 3 were obtained by fractionating sialomucopolysaccharides from rat brain by gel filtration on Sephadex G-200. Data for Expts. 4 and 5 were obtained by fractionating sialomucopolysaccharides from mitochondrial and microsomal preparations from rat brain on Dowex 1 (Cl⁻ form). [Data for Expts. 1, 4 and 5 were taken from previously published work (Brunngraber & Brown, 1964a,b).]

Expt. no.	Fraction	Molar ratio					
		NANA/ fucose	Hexosamine/ fucose	Hexose/ fucose	Hexose/ hexosamine	Hexosamine/ NANA	Hexose/ NANA
1	1	1.4	2.3	3.4	1.51	1.64	2.49
	2	2.4	3.5	3.8	1.06	1.46	1.56
	3	4.2	2.9	5.1	1.75	0.69	1.21
	4	6.8	3.0	6.8	2.27	0.44	1.01
2	1	0.80	2.48	4.11	1.65	3.10	5.14
	2	1.54	2.96	4.72	1.59	1.91	3.05
	3	2.44	4.83	5.33	1.09	1.88	2.05
	4	4.34	5.77	8.00	1.38	1.32	1.83
3	2	1.34	3.05	5.00	1.82	2.26	3.70
	1	2.77	3.57	6.54	1.63	1.28	2.35
4	1	1.03	1.69	2.90	1.72	1.63	2.81
	2	3.33	3.12	6.66	2.12	0.93	2.00
5	1	1.28	2.50	5.00	2.00	1.94	3.88
	2	4.35	3.83	7.08	1.84	0.90	1.66

Molecules rich in NANA are relatively poorer in fucose; molecules rich in fucose are relatively poorer in NANA. The present interpretation is consistent with the observations of Dische (1965), who pointed out that the carbohydrate moiety of almost all glycoproteins investigated so far have a fundamental repeating unit consisting of a hexose- and hexosamine-containing residue. Fucose and NANA are attached to this basic repeating unit.

In conclusion, it should be pointed out that the findings of McIlwain and his collaborators (McIlwain, 1963) with regard to the gangliosides and the effects of basic proteins on membrane function should be considered in relation to the protein-linked sialomucopolysaccharides and their possible role in membrane function.

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