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A silicic acid adsorption method for the determination of ganglioside sialic acid*

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SUMMARY

A semimicro method for the analysis of ganglioside sialic acid is presented. The method is based upon the fact that brain gangliosides can be adsorbed on a small amount of silicic acid from 92% *n*-butanol and eluted with methanol. Recovery of added gangliosides was found to be 93.5%, and the coefficient of variation for the analysis of 5 to 50 mg of tissue was found to be ±4.4%.

A cerebroside-like material, which gave a characteristic purple color with Bial's orcinol reagent, was isolated from brain tissue in 1942 by Klenk (1). The term ganglioside was applied to this material because of its abundance in the ganglion cells of the nervous system. Fatty acids, glucose, galactose, galactosamine, sphingosine, and the material responsible for the characteristic color reaction, sialic acid, have been isolated as hydrolysis products of gangliosides (2, 3, 4, 5, 6). Sphingolipids containing sialic acid have also been isolated from horse erythrocytes (7), bovine erythrocytes (8), ox spleen (9), and human spinal cord (10). Strandin, isolated from gray matter of brain by Folch *et al.* (11), is a sphingolipid containing sialic acid (12, 13), as is the mucolipid isolated from ox brain by Rosenberg and Chargaff (14, 15). Strandin preparations, homogeneous by many criteria, when subjected to three-phase counter-current distribution (16) showed the presence of several distinct fractions. Svennerholm (17), using a cellulose column, isolated two gangliosides from human brain tissue varying in sialic acid and hexosamine content. Kuhn *et al.* (18) have reported the isolation of two ganglioside fractions from beef brain. The term gangliosides, as applied by Klenk (19), refers to a group of closely related sphingolipids characterized by their content of sialic acid. Thus, all the materials cited above fall within this class and are distinguished from the glycosphingolipids [polycerebrosides (20), globosides (21), and cytosides (22)], which do not contain sialic acid.

If Klenk's definition of gangliosides is accepted, then methods for their quantitative determination that depend on the characteristic component, sialic acid, should meet certain criteria. The assay of sialic acid must be specific or be preceded by preliminary purification that eliminates interference from materials such as other carbohydrates and unsaturated fatty acids. Because sialic acid is also a constituent of many proteins and oligosaccharides (23), a preliminary separation of nonganglioside-bound sialic acid is important. Purification should not be selective for differences in ganglioside composition, such as fatty acid and carbohydrate residues.

In 1941, Klenk and Langerbeins (24) first described a method for the quantitative determination of gangliosides based on selective solvent extractions and determination of sialic acid by the orcinol reaction. Svennerholm (25) reported that this method was inadequate since a considerable loss of gangliosides occurred in the ether fraction. Svennerholm described a method, using an improved orcinol reaction, which removed interfering unsaturated fatty acids by selective hydrolysis and petroleum ether extraction. Solvent distribution methods (26, 27) based upon Folch's washing procedure have also been proposed. Several improved methods for the determination of sialic acid have been reported (26, 28, 29). Smits and Edgar (30) have suggested a method for calculating ganglioside content

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from lipid hexosamine levels but this is not strictly acceptable in view of Klenk's definition and the report of a hexosamine-free ganglioside in brain (31, 32).

In the present study, the adsorption of brain gangliosides on silicic acid was investigated as a rapid purification step preliminary to the specific measurement of ganglioside-bound sialic acid. Since gangliosides vary in their sialic acid content, all results will be expressed as ganglioside-N-acetylneuraminic acid (G-NANA).

MATERIALS AND METHODS

Silicic Acid. Silicic-acid powder (Mallinckrodt Chemical Works, St. Louis, Missouri, 100 mesh, suitable for chromatographic analysis) was washed with absolute methanol (2 ml per g), air dried at room temperature, and then dried at 70° overnight. Drying at higher temperatures led to low recoveries of gangliosides.

N-Acetylneuraminic Acid (NANA). NANA was prepared from human blood plasma (33). Crystallization from glacial acetic acid yielded crystals of pure NANA melting with decomposition at 184–185°. The material moved as a single spot in the following solvents: butanol—acetic acid—water 4:1:5 v/v,[1] R_f 0.32; butanol—propanol—0.1N HCl 1:2:1, R_f 0.46. This material was used as a standard in all photometric assays.

Ganglioside Standard. Since gangliosides constitute a group of related compounds that may vary in their solubility properties, the isolation procedure for these materials was quantified as far as possible in order to detect any selective losses. All extracts and fractions obtained during the isolation were analyzed for total sialic acid by the resorcinol procedure, and the results were expressed as percentage of total NANA detected.

(*a*) *Extraction.* Dry gray matter, 1.2433 g, was placed in a large fritted-glass filter and twice extracted for 5 minutes with 120 ml of acetone—water 95:5. The acetone extract was analyzed for sialic acid. The tissue residue was extracted with eight successive 100-ml portions of hot (60°) methanol—chloroform 2:1 for 5 minutes. The successive methanol—chloroform extracts were collected and analyzed separately. Ninety-eight per cent of the total extractable NANA was found in the first four methanol—chloroform extracts.

(*b*) *Distribution with Folch Solvents.* The first four methanol–chloroform extracts were pooled and evaporated to dryness *in vacuo*. The residue was subjected to a six-transfer double-withdrawal distribution between the phases obtained by mixing chloroform, methanol, and water (8:4:3). On completion of the distribution, the six phases were diluted to volume and analyzed. Ninety-eight per cent of the total NANA found was in upper phases No. 1 and No. 2 (91% and 7%, respectively).

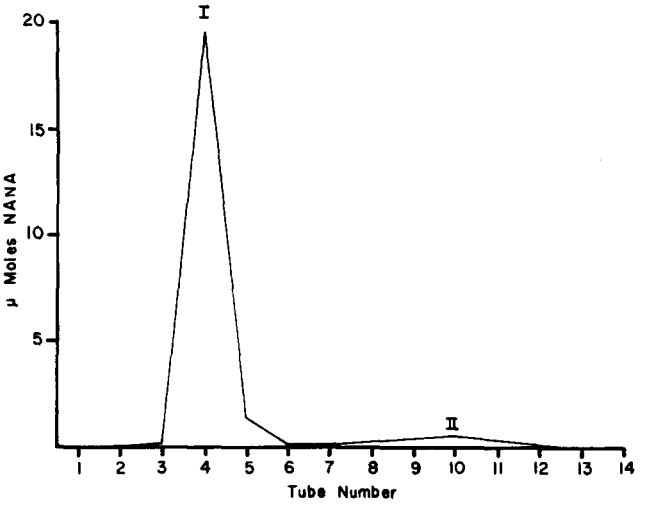

FIG. 1. Fractionation of crude ganglioside on Sephadex G-50 column (3 x 30 cm). Column was developed with distilled water and 10-ml fractions were collected.

(*c*) *Sephadex Chromatography.* Upper phases No. 1 and No. 2 were pooled and evaporated to dryness under a stream of nitrogen, and the crude ganglioside residue was dissolved in 3 ml of water and placed on a Sephadex G-50[2] water column (3 × 30 cm). The elution pattern from the column is shown in Figure 1. It was found that 94% of the NANA behaved as "high-molecular-weight material" (Peak I) and the balance behaved as "low-molecular-weight material" (Peak II). The tubes comprising Peak I were pooled and diluted to volume, and aliquots were taken for the various analyses. This preparation ("gangliosides A") was found to contain 21.8% sialic acid (as NANA), 6.9% hexosamine, 26.5% hexose (as galactose), and 12.7% sphingosine.[3] The complete absence of non-gangliosidic material in this preparation was not proven. As 26.0 mg of "gangliosides A" was obtained from a portion of the extract equivalent to 1.04 g of dry

[1] All solvent ratios are v/v.

[2] Sephadex (Pharmacia, Uppsala, Sweden) is available from Pharmacia Laboratories, Inc., 501 Fifth Avenue, New York 17, New York. Sephadex consists of small grains of cross-linked dextran. These particles exclude molecules with molecular weights above approximately ten thousand and therefore allow the chromatographic separation of low- and high-molecular-weight materials.

[3] Svennerholm (25) has reported 25.0% sialic acid, 6.8% hexosamine, and 29.2% hexose content for pooled gangliosides prepared from senile human brains.

tissue, this material is present at a concentration of 2.5% (dry weight) in human cerebral cortex.

Chemical Analyses. Sphingosine was determined according to the method of Robins *et al.* (34) as modified by Meltzer (16). For the determination of hexose, the anthrone method of Roe (35) was followed using galactose as a standard. Hexosamine was determined according to Method B of Svennerholm (36).

ANALYTICAL METHOD

Preparation of Dry Tissue. Tissue is homogenized in approximately four volumes of water, lyophilized, carefully ground in a mortar, and dried *in vacuo* over phosphorus pentoxide to a constant weight.

Silicic Acid Method. Dry tissue, 5 to 50 mg, is placed in a medium porosity fritted-glass filter. Five milliliters of acetone—water 95:5 at room temperature is added to the filter to wash down the walls. The tissue is extracted for 5 minutes with occasional suspension of the tissue in the solvent with the aid of a glass rod. The solvent is then forced through the filter with the aid of positive air pressure and discarded.

Two milliliters of methanol—chloroform 2:1 at 60° is added, and the tissue is extracted, with occasional stirring, for 5 minutes. Pressure is then used to force the extract into the flask. The 2-ml extraction is repeated four times, then followed with a 5-ml extraction for 5 minutes. The total extract (15 ml) is taken to dryness at 60° under a stream of nitrogen. The residue is carefully suspended in 1.0 ml of methanol —water 1:1. Absolute *n*-butanol (11.5 ml) is added and thoroughly mixed until one clear phase results.

Silicic acid (1.5 g) is placed in a fritted-glass filter (20-mm diameter, medium porosity) and packed by gentle tapping. A 10-ml aliquot of the butanol solution is added to the silicic acid, and pressure is applied until the level of the solution just reaches the surface of the silicic acid. This is followed with two 5-ml portions of butanol—methanol—water 92:4:4 and the butanol effluent is discarded. The gangliosides, which have been adsorbed on the silicic acid, are eluted with two 5-ml portions of absolute methanol into a 15-ml glass-stoppered centrifuge tube. The methanol is evaporated at 60° under a stream of nitrogen. The residue is dissolved in water and analyzed directly for NANA or appropriate aliquots may be taken for analysis.

Resorcinol Analysis for NANA. The resorcinol method of Svennerholm (37) was used to measure NANA. Heating was carried out at 110°, and color was extracted with butyl acetate (tech)—*n*-butanol 85:15 as recommended by Miettinen and Takki-Luukkainen (29). Dichromatic readings were made at 450 and 580 mμ, and NANA was calculated assuming that any excess absorption at 450 mμ was due to galactose (37). The molecular extinction coefficients ($\epsilon \times 10^{-3}$) of NANA at 450 and 580 mμ were found to be 2.63 and 10.58, respectively. The $\epsilon \times 10^{-3}$ of galactose at 450 and 580 mμ were found to be 0.386 and 0.130, respectively. Other sialic acids have slightly different extinction coefficients.

TABLE 1. REPRODUCIBILITY OF ANALYSIS OF NORMAL ADULT HUMAN CEREBRAL CORTEX

Tissue	G-NANA
mg dry wt	*μmoles/g*
5.03	16.3
5.04	17.9
10.01	18.4
10.04	18.9
10.08	18.7
20.00	17.5
20.07	17.4
24.9	18.2
25.0	19.0
25.0	18.4
49.7	18.6
49.9	17.4
Mean	18.1
Standard Deviation	±0.79
Coefficient of Variation	4.4%

EXPERIMENTAL

Reproducibility and Recovery. The coefficient of variation obtained with the silicic-acid method for the analysis of 5 to 50 mg of tissue was found to be ±4.4%. These data are presented in Table 1. Recovery was measured by adding "gangliosides A" (see above) to the methanol—chloroform extracts. The amount of added gangliosides was approximately 50% and 100% of the ganglioside content of the tissue. The average recovery in four experiments was 93.5% with a coefficient of variation of ±2.4%.

Acetone Extraction. A preliminary extraction of the tissue with acetone is desirable because it removes cholesterol and a portion of phospholipids and cerebrosides as well as substances of low molecular weight (25). To test the effect of acetone extraction on the amount of ganglioside found in the tissue, a series of samples was analyzed for gangliosides after varying numbers of acetone extractions. These data are presented in Table 2. As the number of extractions is increased, more gangliosides are lost. Repeated acetone extraction was demonstrated to extract additional lipid hexose and to decrease the 450 mμ/580 mμ resor-

TABLE 2. THE EFFECT OF ACETONE EXTRACTION ON GANGLIOSIDE ANALYSIS IN NORMAL ADULT HUMAN CEREBRAL CORTEX

Number of Acetone Extractions*	Ganglioside-N-acetylneuraminic Acid,	Loss
	μmoles/g	%
0	17.7	
1	17.2	2.8
3	15.5	12.3
5	13.3	24.8

* Each extraction consisted of 5 ml of acetone—water 95:5 for 5 minutes at room temperature.

cinol-absorption ratio of the methanol eluate. A single acetone extraction causes a minimal loss of gangliosides while removing a substantial quantity of other materials and has therefore been incorporated into the silicic acid method.

Methanol—Chloroform Extraction. Re-extraction of a 20-mg sample of gray matter with methanol–chloroform 2:1 according to our regular procedure yielded an extract free of G-NANA, suggesting that the methanol—chloroform extraction procedure is adequate.

Specificity. The direct analysis of sialic acid in lipid extracts by the resorcinol procedure is complicated by interfering chromogens. Figure 2 illustrates the extent to which interfering chromogens of white matter are removed by the silicic-acid method. The resorcinol spectra of the methanol eluates obtained in the analysis of several tissues are shown in Figure 3.

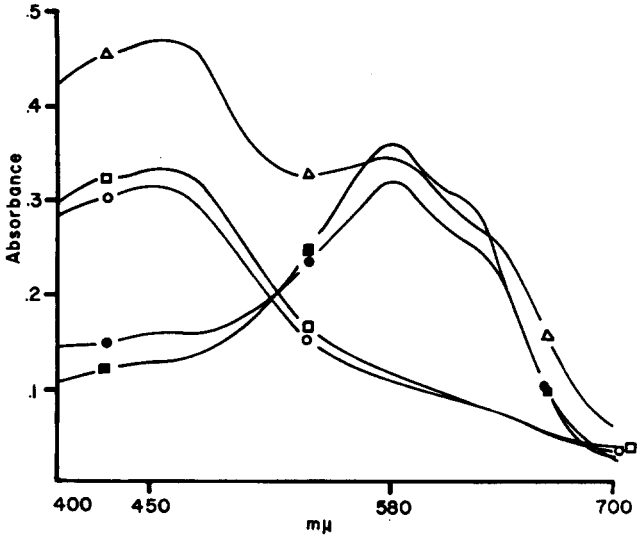

FIG. 2. Absorption spectra of the resorcinol chromogens of 0.207 mg of ganglioside (■) and of fractions obtained during analysis of human white matter by the silicic acid method. Quantities represent milligrams of tissue. ○, acetone extract (50.0 mg); △, methanol–chloroform extract (26.7 mg); □, butanol–methanol–water wash (26.7 mg); ●, methanol eluate (40.0 mg).

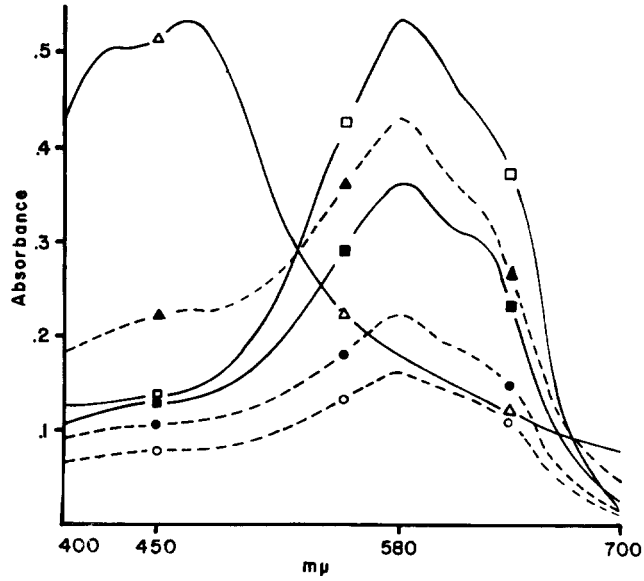

FIG. 3. Absorption spectra of the resorcinol chromogens of 0.250 μmoles of NANA (□); 5.59 μmoles of galactose (△); 0.207 mg of ganglioside (■); and methanol eluates from silicic acid of 20 mg of human white matter (○), 6.8 mg of human cerebral cortex (●), and 15.0 mg of whole, 12-day-old, rat brain (▲).

It is assumed that galactose (of cerebrosides, cerebroside sulfate, and gangliosides) is the only interfering material in the methanol eluate that produces significant absorbance with the resorcinol assay. The interference from galactose is eliminated by the use of dichromatic readings (37).

The interfering effect of any remaining unsaturated fatty acids, caused by "self" color, can be detected by running a tissue blank (without resorcinol) as suggested by Svennerholm (25). Normally there is no color in tissue blanks; however, when old or poorly preserved tissue samples are analyzed, the resorcinol-absorption spectra show the presence of interfering chromogens and tissue blanks will appear brown. It seems probable that this effect results from the presence of autoxidized lipids that are adsorbed by the silicic acid and eluted with the gangliosides.

The method also eliminates chromogens that interfere with the analysis of sialic acid by the thiobarbituric acid assay (28), as shown in Figure 4. This assay gave values for G-NANA and sialic-acid content of ganglioside preparations that were approximately 80% of those obtained with the resorcinol assay. Although the reason for the difference was not investigated, it may be that there was incomplete liberation of sialic acid during the hydrolysis step.

Svennerholm (13) has presented evidence that lipid extracts of wet tissue contain a nondialyzable sialoprotein. Thus, methods utilizing a Folch extraction followed by solvent partition and dialysis may deter-

mine some nonlipid-bound sialic acid. Although preliminary acetone extraction of dry tissue and subsequent extraction with methanol—chloroform 2:1 as recommended by Svennerholm to minimize extraction of nonlipid material is used in the silicic-acid method, the possibility remained that protein or peptide-bound sialic acid was extracted and subsequently eluted from the silicic acid to be measured as G-NANA. In order to demonstrate the presence or absence of protein or peptide-bound sialic acid, the methanol eluate derived from 50 mg of cerebral cortex was dissolved in water and passed over a Dowex 50 W-X1 [H^+] column. Ninety-six per cent of the sialic acid was recovered in the water wash from the column. It was also observed that 97% of the NANA in the methanol eluate was soluble in methanol—chloroform 1:2.

Assay of G-NANA in Tissue. The silicic-acid method was applied to various normal and pathological tissue samples. Several of these samples were also analyzed by Svennerholm's method (25) using the resorcinol assay as described above. These data are presented in Table 3. The lower values obtained with Svennerholm's method may result from loss of gangliosides in the acetone fraction. This procedure involves a 1-hour and 2-hour Soxhlet acetone extraction for gray matter and white matter, respectively. Since formalin storage is known to destroy gangliosides (38), the values obtained with formalin-fixed tissue are probably low.

DISCUSSION

The criteria set forth in the introduction for the determination of ganglioside sialic acid are largely satisfied by the silicic-acid method. Most of the materials that interfere with the assay of sialic acid in lipid extracts are removed, as illustrated by the absorption spectra obtained with the methanol eluate. Correction for interference from a small amount of remaining galactose is included in the method. Absorbance caused by unsaturated fatty acids, detectable by use of a tissue blank, is normally absent. The absence of nonlipid-bound and cation-bound sialic acid in the methanol eluate was indicated, as no significant quantity of sialic acid expressed as G-NANA was retained on a cation exchange column of low cross-linkage and none was insoluble in methanol—chloroform 1:2. Evidence that the method is not selective for any of the ganglioside fractions that occur in human brain is supplied by the recovery data obtained with the "gangliosides A" preparation. This preparation contains practically all of the lipid-soluble sialic acid except for the small amount of uncharacterized material observed as Peak II of the Sephadex column.

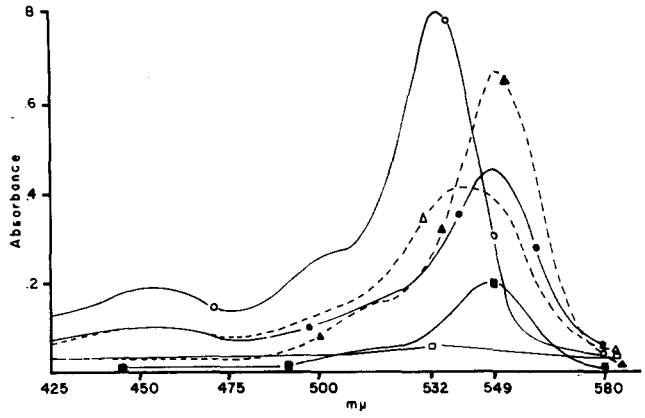

FIG. 4. Absorption spectra of the thiobarbituric acid reaction products of 0.065 μmoles of NANA (▲), 0.032 mg of ganglioside (■), and of fractions obtained during analysis of human cerebral cortex by the silicic acid method. Quantities represent milligrams of tissue. ○, acetone extract (12.0 mg); △, methanol–chloroform extract (1.92 mg); □, butanol–methanol–water wash (1.59 mg); ●, methanol eluate (2.4 mg).

The adsorption and elution of brain gangliosides on silicic acid as outlined is a convenient purification step for the measurement of G-NANA.

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TABLE 3. GANGLIOSIDE-N-ACETYLNEURAMINIC ACID LEVELS IN VARIOUS TISSUE SAMPLES

Tissues	G-NANA (μmoles/g dry wt)	
	Silicic Acid Method	Svennerholm Method
Normal cerebral cortex	18.1	14.6
Normal cerebral white matter	4.3	2.4
Tay-Sachs cerebral cortex*	48.2	33.3
Tay-Sachs white matter*	28.6	26.2
Gargoylism cerebral cortex*	11.4	
Atrophic cerebral cortex*	2.9	
Whole human pituitary gland	2.6	
Whole human adrenal gland	1.4	
Whole rat brain (12 days old)	15.0	

* Formalin fixed.

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