notes on methodology

The photometric determination of gangliosides with the sulfo-phospho-vanillin reaction

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SUMMARY A simple, quantitative method is described for the photometric determination of gangliosides. The procedure is based on the sulfo-phospho-vanillin reaction, and does not require prior hydrolysis. It has been shown that the reaction is probably due to oxidation by sulfuric acid of the sphingosine moiety which results in the formation of aldehydes or ketones or both which then react with the phosphoric acid–vanillin reagent to produce a rose-colored complex. The reaction permits the determination of the amount of ganglioside present in a sample; and, together with the resorcinol reaction to measure the NANA content, it can be used to determine whether a purified ganglioside is a mono-, di-, or trisialoganglioside.

SUPPLEMENTARY KEY WORDS sphingosine–NANA ratio · mono-, di-, trisialogangliosides

THE SULFO-PHOSPHO-VANILLIN reaction was first applied to the analysis of lipids present in biological fluids and tissues by Chabrol and his coworkers (1–3). These investigators demonstrated that the rose color produced with a lipid mixture was due mainly to its content of unsaturated fatty acids and cholesterol. More recently the procedure has been applied to the analysis of the total lipids present in mammalian sera by Drevon and Schmit (4) and by Frings and Dunn (5); the latter investigators used an olive oil solution as a standard.

Gangliosides are high molecular weight, water-soluble lipids which accumulate markedly in brain tissue in Tay-Sachs disease, Landing's disease, and related genetic disorders of lipid metabolism which have been classified under the general heading of the "gangliosidoses" (6). Gangliosides generally consist of a structure containing a saturated fatty acid, sphingosine, glucose, galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (NANA). The different gangliosides isolated from mammalian tissues differ only in the number and/or the point of attachment of the carbohydrate or NANA residues (7–9). Most commonly employed methods for ganglioside analysis are based either on the resorcinol reaction for NANA (10, 11) or the orcinol reaction for carbohydrates (12). The only two constituents present in unimolar quantities in all gangliosides are the fatty acids, e.g., stearic acid, and the organic base sphingosine. Kishimoto and Radin (13) have published an involved method for the analysis of gangliosides based on their stearic acid content as determined by methanolysis followed by GLC.

The present report describes a simple, sensitive, colorimetric procedure for gangliosides which does not require prior hydrolysis. It is based on the fact that only the sphingosine residue contributes to the color produced with the sulfo-phospho-vanillin reaction.

Materials. Vanillin (No. V-9) was purchased from Fisher Scientific Co., Springfield, N.J. Sulfuric and phosphoric acids were reagent grade chemicals. Sphingosine and the mono-, di-, and trisialoganglioside preparations were purchased from Supelco, Inc., Bellefonte, Pa. Beef, normal human, and Tay-Sachs gangliosides were isolated from the chloroform–methanol 2:1 extracts of cerebral cortex grey matter by means of Sephadex column chromatography as described by Rouser, Kritchevsky, and Yamamoto (14). The mono-, di-, and trisialoganglioside and Tay-Sachs ganglioside preparations were purified by removing the minor components by preparative TLC on Silica Gel H plates with the technique of Wherrett, Lowden, and Wolfe (15).

The phospho-vanillin reagent was prepared by adding 800 ml of 85% phosphoric acid to 200 ml of 0.6% (w/v) vanillin in water. Gangliosides and sphingosine were dissolved in chloroform–methanol 2:1 containing 1.0% water to give concentrations of 1.0 mg/ml and 250 μg/ml, respectively.

Procedure. Aliquots of solutions of ganglioside (50–500 μg), and of sphingosine (25 and 50 μg), used as standards, were pipetted into 15-ml test tubes (fitted with screw caps), and the solutions were evaporated to dryness on a 37°C water bath under a stream of nitrogen. The blank consisted of 0.1 ml of solvent (chloroform–methanol 2:1 containing 1% water) which was similarly evaporated to dryness. To each tube 0.05 ml of water and 0.75 ml of concentrated sulfuric acid were added. The contents of each tube were thoroughly dispersed with a Vortex mixer and the tubes were closed with a Teflon-lined screw cap. The tubes were heated in a boiling water bath for 40 min and then cooled to room temperature. 5 ml of phospho-vanillin reagent was added to each tube,

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Abbreviations: S-P-V, sulfo-phospho-vanillin; NANA, *N*-acetylneuraminic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

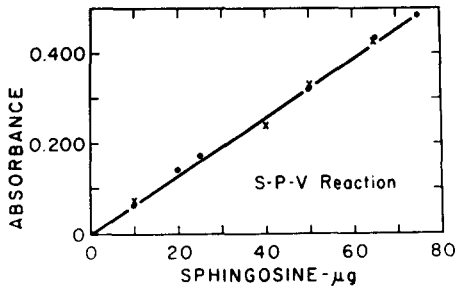

FIG. 1. Relationship between absorbance and sphingosine concentration (10–75 μg). × and ● indicate 2 separate preparations of sphingosine.

the contents were mixed with the Vortex mixer, and the color was developed in a 37°C bath for 15 min. The contents were cooled to room temperature and the absorbance of each unknown and standard was read at 525 nm in a Beckman DU spectrophotometer using 1.0 cm quartz cells against the reagent blank set at zero. The sphingosine content of each ganglioside sample was calculated according to Beer's law or from a calibration curve as is illustrated in Fig. 1. Alternatively, a purified preparation of a ganglioside of known composition, e.g., the Tay-Sachs ganglioside (G_{M2}), can also be utilized as a standard.

Studies of Factors Which May Influence Color Development. In order to determine the optimal conditions of the S-P-V reaction for gangliosides, a number of experimental factors were studied which could influence the color yield. These included first the amount of concentrated sulfuric acid required in the initial digestion of the ganglioside. The data obtained in these experiments are shown in Fig. 2. They indicate that for a fixed amount of water (0.05 ml) a plateau is reached at about 0.7 ml of acid. Thus 0.75 ml of concentrated sulfuric acid was utilized as an optimal amount for the ganglioside analysis. The next factor investigated was the time of heating at 100°C for the acid hydrolysis step. The data obtained in these studies show that a plateau was reached in about 40 min (Fig. 3). This time was therefore utilized as the optimal heating time. The time required for color development after the addition of the phospho-vanillin reagent was the last factor investigated and the results obtained (not shown) revealed that complete color development occurs within 5 min at 37°C with no further change over a 1-hr period. The colors may be read at any time during this time interval.

Using the optimal conditions for the S-P-V reaction, a spectral curve for the color obtained with a ganglioside preparation was performed in a Beckman DU spectrophotometer. There was maximum absorbance between 520 and 530 nm (Fig. 4) so that any value on the plateau could be utilized for the absorbance measurements.

Ganglioside Analysis. The procedure was employed to analyze 50–650 μg of a normal human ganglioside preparation. The results obtained are shown in Fig. 5 and demonstrate a stoichiometric relationship between the amount of ganglioside and absorbance over this range of values. Purified samples of mono-, di-, and trisialogangliosides were run both with the S-P-V reaction and with the resorcinol reaction for NANA (11). The results are given in Table 1.

Results and Discussion. The inadequacies of methods for the quantitative determination of gangliosides, based upon the reactivity of NANA or other carbohydrate residues, have been thoroughly discussed by Kishi-

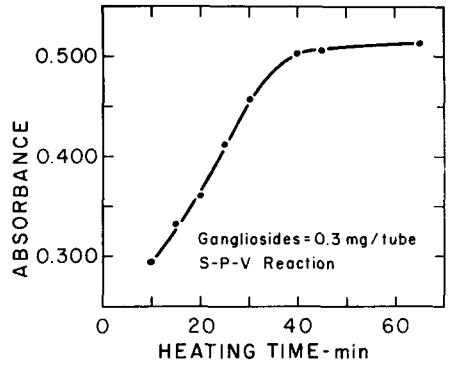

FIG. 3. Effect of heating time at 100°C (acid hydrolysis) on the color yield with the S-P-V reaction for gangliosides.

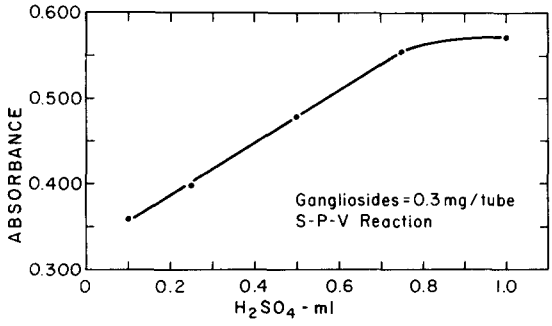

FIG. 2. Effect of variation of the amount of concentrated sulfuric acid on the color yield with the S-P-V reaction for gangliosides.

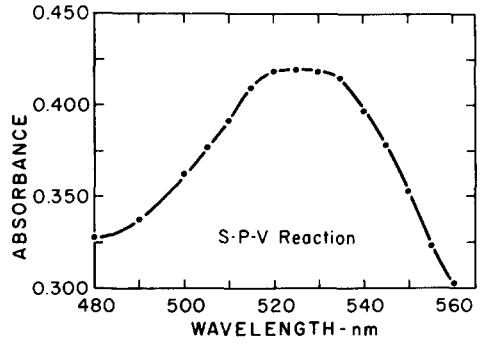

FIG. 4. Spectral curve of color obtained with the S-P-V reaction for gangliosides.

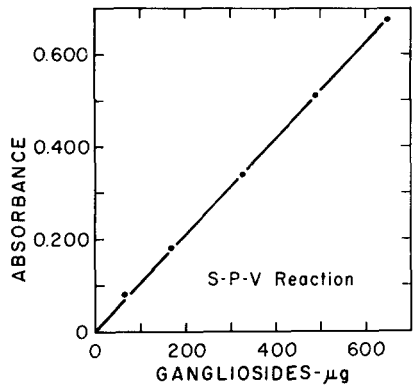

FIG. 5. Relationship between absorbance and ganglioside concentration (50–650 μg).

moto and Radin (13). Their fatty acid method is based upon the measurement of the stearic acid content of purified gangliosides by means of methanolysis and GLC analysis of the resultant fatty acid methyl esters. However, stearic acid constitutes only 80% of the total fatty acids of brain gangliosides, and gangliosides obtained from brain tissue of different species (16), from different regions of normal human brain (13), and from different tissues (8) differ appreciably in their fatty acid composition. It is evident that a simple, colorimetric procedure based upon the sphingosine content of purified gangliosides, as is presented here, suffers from none of these drawbacks and would be the method of choice.

When the S-P-V reaction was applied to various components which make up the structure of the gangliosides (7–9), no appreciable amount of color was formed with stearic acid, glucose, galactose, *N*-acetylgalactosamine, and NANA. Significant color yields were obtained with oleic acid, sphingosine, dihydrosphingosine, and ceramide, and their molar color yields are listed in Table 2. It can, therefore, be concluded from these experiments that the color obtained with gangliosides in the S-P-V reaction results from the sphingosine residue, provided the gangliosides contain little or no unsaturated fatty acids. This conclusion is supported by the data obtained on the fatty acid composition of Tay-Sachs and normal brain gangliosides, as determined by GLC by Ledeen, Salsman, and Cabrera (17). Their results show that Tay-Sachs gangliosides contain about 1.2% of unsaturated fatty acids, whereas normal gangliosides contain about 2.5%. Results reported by Kishimoto and Radin (13) and by Svennerholm (16), as well as data obtained in this laboratory, indicate even lower levels of unsaturated fatty acids in purified gangliosides.

One possible explanation for the positive S-P-V reaction for sphingosine, both as a salt and a free base or as a component of gangliosides, is the presence of a double bond. However, the fact that dihydrosphingosine also gives a positive S-P-V reaction casts some doubt on the hypothesis. An alternative explanation is that acid hydrolysis of a ganglioside yields sphingosine sulfate, as occurs with sphingomyelin (18). The long chain amino-alcohol is then oxidized to an aldehyde which reacts with the phospho-vanillin reagent (19).

If the absorbance value obtained for a given amount of normal human ganglioside (Fig. 5) is compared with the same absorbance value for sphingosine (Fig. 1), there is a 6:1 (approximately) weight relationship between these substances. This value checks well with a mol wt of 300 for sphingosine and an estimated value of 1800 for a mixture of normal human gangliosides (20). When the reaction was applied to the samples of mono-, di-, and trisialogangliosides of known composition in conjunction with the resorcinol reaction for NANA (11) and the experimental values calculated, there was a close correlation between the experimental and theoretical values for these gangliosides (Table 1). The S-P-V reaction, therefore, permits the determination of the amount of ganglioside present in a sample independently of its NANA or carbohydrate content. When utilized together with the resorcinol reaction, it allows one to determine whether a purified ganglioside is a mono-, di-, or trisialoganglioside.

TABLE 1 ANALYSIS OF PURIFIED GANGLIOSIDES OF KNOWN COMPOSITION

Gangliosides*	NANA/Sphingosine	
	Theor.	Exper.
Monosialoganglioside (G_{M2})	1.03	0.88
Disialoganglioside (G_{D1b})	2.06	2.02
Trisialoganglioside (G_{T1})	3.09	3.01

* For structures of these gangliosides see Ref. 8.

TABLE 2 MOLAR COLOR YIELDS OF GANGLIOSIDES AND OF SOME OF THEIR STRUCTURAL COMPONENTS WITH THE S-P-V REACTION

Compound	Mol wt	Molar Extinction Coefficient
Monosialoganglioside (G_{M2})*	1490	11,200
Monosialoganglioside (G_{M1})†	1870	10,000
Disialoganglioside (G_{D1a})†	1980	10,400
Trisialoganglioside (G_{T1})†	2280	12,300
Sphingosine	298	14,500
Dihydrosphingosine	300	19,200
Ceramide	666	7,500
Oleic Acid	282	15,900

* This sample was prepared by the technique of Folch, Lees, and Sloane Stanley (21) and contains about 15% of gangliosides other than the Tay-Sachs ganglioside (G_{M2}).

† These gangliosides were purchased from Supelco, Inc., and each sample contained between 15 and 30% of other gangliosides.

The data in Table 2 show that the S-P-V reaction gives essentially the same molar color yield regardless of the type of ganglioside involved. It is evident from the oleic acid and sphingosine values that the method cannot be applied directly to tissue extracts but can be applied to relatively crude ganglioside preparations which have been washed free of other lipids. A highly purified sample (G_{D1a}) gave a value of 10,400, which is within the experimental error of that found (11,200) for a more crude preparation (G_{M2}). No explanation can be offered at present for the somewhat higher color yield obtained with dihydrosphingosine as compared with sphingosine, nor for the lower value obtained with the ceramide preparation. The application of the method for the analysis of gangliosides in brain and other tissues is presently under investigation.

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