A PREPARATIVE METHOD FOR THE ISOLATION OF BRAIN CEREBROSIDE, SULFATIDE AND SPHINGOMYELIN

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INTRODUCTION

Methods for the separation and purification of one or at the most two of the three major sphingolipids of brain on a preparative scale are available but no specific method has been published by which all three can be prepared from a single lipid extract. This paper describes a procedure specifically designed for the preparative fractionation and purification of sphingomyelin, cerebroside and sulfatide from ox brain.

Among the earlier methods available are the solvent fractionations of the ether insoluble lipid of brain by which BLIX[1] prepared pure sulfatide and KLENK and his co-workers[2,3] prepared cerebroside and sphingomyelin. Chromatographic preparations previously reported include the separation of cerebroside and sphingomyelin from sphingolipid preparations of brain[4,5] and the separation and partial purification of cerebroside and sulfatide from a chloroform–methanol extract of brain[6]. All of these procedures are based on chromatography on silicic acid columns eluted with mixtures of chloroform and methanol. LONG AND STAPLES[6] also report a fractionation on alumina columns. SVENNERHOLM AND THORIN[7] purified sulfatide from a combined cerebroside–sulfatide fraction obtained by chromatography of brain lipids on silicic acid. Final purification was carried out on a column of diethylaminoethyl cellulose. RADIN and co-workers prepared cerebroside from a chloroform–methanol extract of brain[8] and an ether insoluble lipid fraction from spinal cord[9] by chromatography on a magnesium silicate column followed by purification with mixed bed ion exchange resin. Magnesium silicate has also been used to purify a sulfatide fraction obtained by solvent fractionation of brain lipids[10]. ROUSER *et al.*[11–14] have outlined at a number of symposia the separation of cerebroside, sulfatide and sphingomyelin consequent to the fractionation of total brain lipids on diethylaminoethyl cellulose, silicic acid, and magnesium silicate columns. Full technical details of these procedures or adequate characterization of the fractions obtained have not been reported. An adaptation of the procedure using chromatography on magnesium silicate and diethylaminoethyl cellulose has been used for the preparation of sulfatide from human brain[15]. Preparations of sphingolipids from sources other than nervous tissue include the purification of sphingomyelin from red blood cells[16], plasma[17], and from a commercial beef heart sphingomyelin preparation[18].

EXPERIMENTAL

Reagent and solvents

All solvents were reagent grade and used without further purification. Mallinckrodt silicic acid, 100 mesh, reagent grade; Merck reagent grade aluminum oxide, suitable for chromatographic adsorption; and Florisil, Floridin Co., Tallahassee, Florida; were used for chromatography adsorbents. Fines were removed from the Florisil by suspending 100 g in 500 ml of distilled water and decanting after 10 min. This is repeated until the supernatant is clear. The Florisil is then dried at 100° over night and stored in a closed container.

Analytical procedures

Phosphorus was determined by the method of FISKE AND SUBBAROW[19] after digesting with perchloric acid. Carbohydrates were assayed by a modification of the procedure of RADIN *et al.*[9] which is described in detail elsewhere[20]. Sulfate and sphingosine were assayed by the procedures described by LONG AND STAPLES[6]. Nitrogen was assayed by the direct Nesslerization procedure of LANG[21] except the digestion mixture was diluted with 3 ml of water and 1 ml aliquots reacted with 1 ml of Nessler's reagent. Total fatty acids were determined by titrating with 0.05 *N* methanolic KOH. Free fatty acids for the titration were obtained by first refluxing the sample with 2 *N* methanolic sulfuric acid for 8 h, extracting with petroleum ether and then saponifying the ether extract with 2 *N* KOH in 50 % ethanol for 2 h at 100°. The fatty acids were finally extracted into petroleum ether after reacidifying the hydrolysate with H_2SO_4. The petroleum ether extract was washed repeatedly with water until neutral and aliquots taken to dryness and dissolved in methanol before titrating. Total weight of the lipids was determined by drying aliquots under vacuum at 80° in tared, aluminum dishes. Choline was assayed by adapting features of several assays[22–25] with certain modifications to increase the level of sensitivity. The lipid to be assayed and containing up to 300 μg of choline is hydrolyzed with 2 ml of aqueous saturated $Ba(OH)_2$ in a closed, screw capped tube for a minimum of 10 h at 100°. The hydrolysate is filtered on Whatman No. 1 filter paper and 0.5 ml of the filtrate pipetted into a 12 ml conical centrifuge tube. 0.5 ml of an aqueous saturated ammonium reineckate solution is mixed with the sample and the mixture is allowed to stand 3 h at 4°. The remainder of the procedure is carried out at 4° except as indicated. The tube is centrifuged at approximately 2,000 r.p.m. for 10 min and the supernatant solution drawn off with a Pasteur pipet. The reineckate remaining in the tube is washed three times by suspending in 0.5 ml of ice cold *n*-propanol saturated with choline reineckate. The suspension is centrifuged and the supernatant solution discarded after each washing. The reineckate is then dissolved in 5 ml of acetone and recentrifuged to remove a small amount of suspended material. After closing the tube with a cork to avoid evaporation of the acetone, the solution is allowed to come to room temperature in the dark and then read at 327 mμ.

Paper and thin-layer chromatography

Hydrolysates of the lipids were chromatographed for the detection and identification of carbohydrates with the ethyl acetate–pyridine–water solvent system of JERMYN AND ISHERWOOD[26]. A 2 h, 100°, 3 N sulfuric acid hydrolysate which had been

passed through a column of AG-3 anion exchange resin to remove the sulfuric acid was used. Thin-layer chromatography of the lipids for identification purposes and as one criterion of purity was carried out as previously described[27].

Preparation of brain sphingolipids

Ox brains were obtained from 30 to 60 min after slaughter and carried in ice to the laboratory. The brains were freed of adhering membrane and blood clots and the lipids extracted with chloroform–methanol[28]. The sphingolipids were separated from this extract by first removing the solvents under vacuum on a rotary evaporator. The lipids were then suspended in diethyl ether, 50 ml of ether for every 21 g of lipid. The suspension is allowed to stand at 4° for 90 min, transferred to 250 ml stainless steel centrifuge bottles and centrifuged 20 min at 5,000 r.p.m. The supernatant solution is decanted and the insoluble residue resuspended in one half the volume of ether used in the initial extraction. This suspension is allowed to stand 90 min and centrifuged as before. The procedure is repeated once more using one quarter of the original volume of ether. The final residue or sphingolipid fraction is approximately 29 % of the original weight of lipid.

This crude sphingolipid preparation is freed of acyl lipids by treating essentially under the mild alkaline conditions described by MARINETTI[29]. The lipids are dissolved in chloroform, 25 ml/g of lipid, and 10 ml of methanol and 2.5 ml of 0.5 *N* sodium methoxide in methanol per g of lipid is added. The mixture is incubated at 37° for 30 min and neutralized with 1 ml of methyl formate for every 35 ml of the reaction mixture. Water is then added in an amount equal to 5 % of the total volume and the mixture passed through a Sephadex column as described by WELLS AND DITTMER[20]. 1 g of Sephadex is used for every 10 ml of the mixture. This latter procedure removed the water soluble reagents and reaction products. 94 % of the original weight of the crude sphingolipid preparation is recovered.

Alternatively, a sphingolipid preparation obtained by the method of CARTER *et al.*[30] was treated by the mild alkaline hydrolysis procedure and Sephadex as described above. A yield of 91 % of the crude sphingolipid preparation is obtained.

Chromatographic fractionation of brain sphingolipids

Columns containing from 1 to 150 g of silicic acid have been used. The silicic acid with one half its weight of Hyflo Super-Cel (Johns-Manville) is suspended in chloroform and the column packed as described by HANAHAN *et al.*[31]. The column is washed with $CHCl_3$, 10 ml/g of silicic acid, and the sphingolipid run on the column dissolved in $CHCl_3$. It is imperative that enough $CHCl_3$ to completely dissolve the sphingolipids be used at this point. Failure to do so causes greatly reduced flow rates and poor resolution. 20 ml of $CHCl_3$ for each gram of sphingolipid is satisfactory. Up to 20 mg of lipid per g of silicic acid can be chromatographed. The column is eluted in succession with the equivalent of 10 ml of $CHCl_3$; 25 ml of $CHCl_3$–CH_3OH (11:1) with 0.5 % H_2O (v/v/v); 30 ml of $CHCl_3$–CH_3OH (6:1) with 1.0 % H_2O (v/v/v) and 20 ml of $CHCl_3$–CH_3OH (1:4) (v/v) per g of silicic acid. The effluent is collected in the equivalent of 3.5 ml/g of silicic acid in each fraction and at a flow rate of 3.5 ml/h per g of silicic acid.

The location of the sphingolipids in the fractions collected is determined by thin-layer chromatography. 0.5 ml samples of each fraction is taken to dryness

in vacuo at 35°, dissolved in approximately 30 μl of $CHCl_3$–CH_3OH (2:1) (v/v) and spotted on a silica gel thin-layer plate. All 23 fractions are spotted on one 20 × 20 cm plate. Development is carried out in $CHCl_3$–CH_3OH (4:1) containing 2 % water and 2 % pyridine (v/v/v/v). A general detection spray, Rhodamine 6G, and a specific phosphate spray[27] are used in succession for detection of the lipids. A typical separation is shown in Fig. 1. On the basis of the thin-layer plate, the fractions containing each of the sphingolipids are combined and then taken to dryness under vacuum.

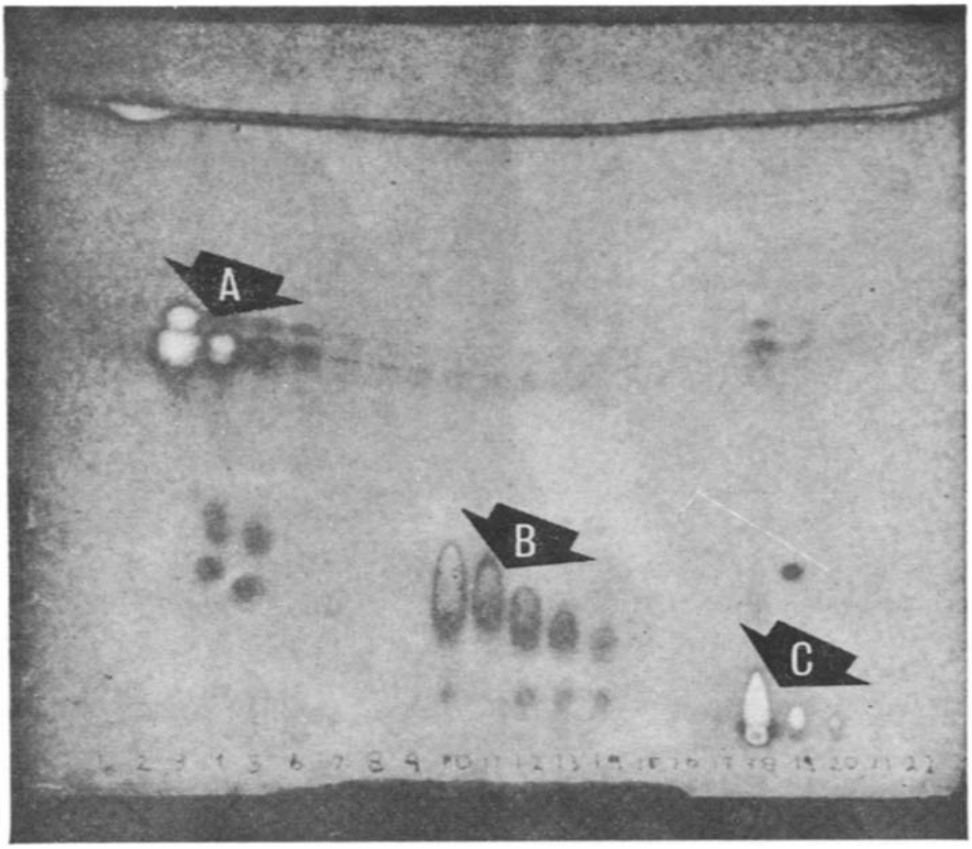

Fig. 1. Silica gel G thin-layer plate of sample from fractions of sphingolipids chromatographed on a 150 g silicic acid column. The three major fractions have been identified as A, cerebroside; B, sulfatide; C, sphingomyelin. The plate was developed in chloroform–methanol–water–pyridine (40:10:1:1) (v/v/v/v). Detection of the compounds was made with Rhodamine 6G[27].

Final purification

As can be seen on the thin-layer plate, Fig. 1, and as has been shown by chemical analysis, the fractions obtained by chromatography on silicic acid are not completely homogeneous and generally further purification is necessary. When the ether insoluble or sphingolipid fraction prepared from a $CHCl_3$–CH_3OH extract as described above is used, the cerebroside fraction obtained from silicic acid requires no further purification. With the sphingolipid preparation of CARTER *et al.*[30] the cerebroside is found to contain small amounts of phospholipid which may be removed by eluting the cerebroside from a Florisil column with $CHCl_3$–CH_3OH (2:1) as described by KISHIMOTO AND RADIN[8]. For our purposes, 1 g of Florisil was used for every 115 mg of cerebroside and the column was eluted with 6 ml of $CHCl_3$–CH_3OH per g of Florisil. The phospholipids present are retained on the column.

Regardless which sphingolipid preparation is used, it is found necessary to remove trace amounts of phosphorus containing material from the sulfatide. Purification is carried out by chromatography on Florisil as used for the cerebroside except no more than 35 mg of lipid per g of Florisil is used. Finally, a slight yellowish tint of the preparation is removed by dissolving the sulfatide in a minimum volume of $CHCl_3$ and precipitating with two volumes of acetone. The mixture is allowed to stand at 4° for 30 min and then centrifuged. Solutions of the sulfatide in $CHCl_3$ after this treatment are colorless.

Sphingomyelin as obtained from the silicic acid column is contaminated with traces of sulfatide and is also yellow in solution. Both the sulfatide and yellow color are removed by chromatography on alumina by a modification of the system described by RHODES AND LEA[36]. One gram of alumina is used for every 42 mg of crude sphingomyelin. The sphingomyelin in 1 ml of $CHCl_3$–CH_3OH (3:2) (v/v) is eluted through the column with the equivalent of 5 ml of the same solvent per g of alumina.

RESULTS AND DISCUSSION

Recovery of sphingolipids

Various criteria were used to determine the yield of purified sphingolipids including weight and analysis of galactose, phosphorus and sphingosine nitrogen. In a typical preparation using a 150 g silicic acid column, 2.08 g (90 %) of 2.32 g of sphingolipid prepared from a $CHCl_3$–CH_3OH extract was recovered in the three fractions isolated. This 2.08 g consisted of 1.226 g cerebroside, 0.310 g sulfatide and 0.548 g sphingomyelin. After final purification a yield of 0.286 g (95 %) sulfatide and 0.446 g (81 %) of sphingomyelin was obtained. Analysis of galactose and phosphorus before and after the final purification step showed that 5 % of the sulfatide and 2 % of the sphingomyelin is lost. The nature of the contaminant which accounts for the discrepancy between the weight loss and phosphorus loss in the sphingomyelin purification was not identified.

In a preparation using the ethanol insoluble or sphingolipid preparation of CARTER *et al.*[30] a closer check was made on the recovery by following the total sphingosine and galactose content as well as weight throughout the fractionation. Using 2.78 g of sphingolipid on a 150 g column, 2.41 g (86.5 %) was recovered in the three pooled fractions. This was distributed with 1.430 g of cerebroside, 0.442 g of sulfatide and 0.534 g of sphingomyelin. After the final purification 1.35 g (94.5 %) of cerebroside, 0.331 g (74.8 %) of sulfatide and 0.384 g (86.8 %) of sphingomyelin were obtained. On the basis of galactose and phosphorus, 94 % of the cerebroside, 90 % of the sulfatide and 95 % of the sphingomyelin were recovered in the final purification step. The weight of lipid recovered from the silicic acid column indicates that as much as 14 % of the sphingolipid may be lost during this step of the fractionation. Analysis of sphingosine and galactose indicates otherwise. In this preparation, a total of 37.5 mg of sphingosine nitrogen and 380 mg of galactose was placed on the column. The three fractions obtained from the column contained a total of 34.9 mg of sphingosine nitrogen and 376 mg of galactose. This corresponds to a recovery of 93.2 % of the total sphingolipid and 98.8 % of the cerebroside and sulfatide. After the final purification the three fractions contained a total of 34.7 mg of sphingosine nitrogen and 362 mg of galactose which corresponds to an over all recovery of 92.6 % of the total sphingo-

lipids and 95.3 % of the cerebroside and sulfatide combined. The higher recoveries indicated by the sphingosine and galactose assays as compared with those obtained on the basis of weight are consistent with the presence of lipids other than sphingolipids in the original sphingolipid preparation of which some are eluted from the silicic acid. Both fatty acids or fatty acid methyl esters and lyso plasmalogens would be expected to remain in this fraction after the mild alkaline methanolysis treatment used in the preparation. The exact nature of the contaminating material has not, however, been characterized. In summary, recoveries of 92–95 % of the sphingolipids can be expected.

Very few recoveries have been reported for chromatographic preparations of sphingolipids. The 5–10 % losses of lipid sulfate previously reported[6–7] for chromatographic preparations of sulfatide are in the range of the losses reported here. SCHWARZ *et al.*[5] give a fairly complete report on the recovery of cerebroside and sphingomyelin after silicic acid chromatography of the sphingolipids of human brain by a fractionation method similar to that reported here. They report recoveries of 100 % of

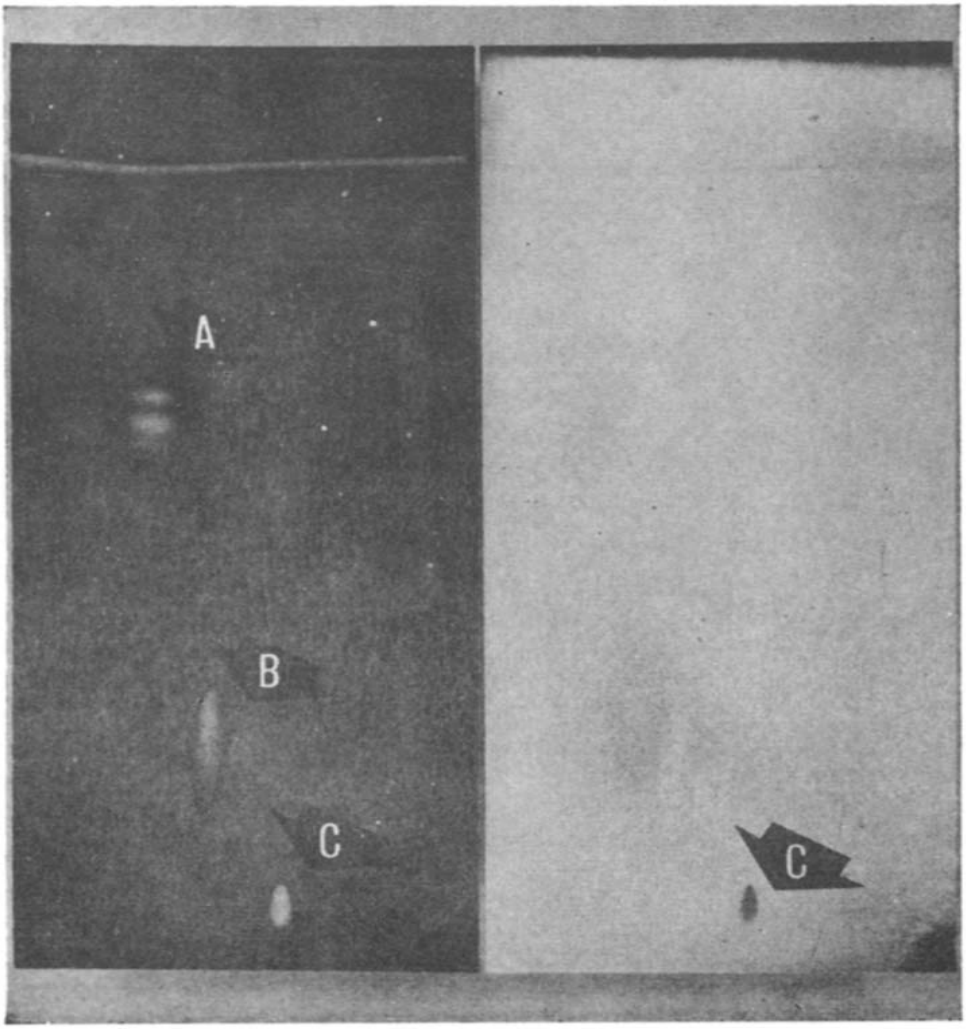

Fig. 2. Silica gel thin-layer plate of purified sphingolipid preparations. The three sphingolipids are: A, cerebroside; B, sulfatide; C, sphingomyelin. The photograph at the left was made after spraying with Rhodamine 6G and that at the right is of the same plate sprayed with a specific phosphate reagent[27].

TABLE I

CHEMICAL COMPOSITION OF PURIFIED SPHINGOLIPIDS

Compound	*Composition (% weight)*														
	Nitrogen			*Phosphorus*			*Galactose*			*Sulfur*			*Choline*		
	Found		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*
	I	*II*		*I*	*II*		*I*	*II*		*I*	*II*		*I*	*II*	
Cerebroside	1.69	1.70	1.71	<0.005	<0.005	0	21.6	21.8	21.9	—	—	0	—	—	0
Sulfatide	1.57	1.56	1.57	<0.005	<0.01	0	20.3	20.0	20.1	3.66	3.51	3.59	—	—	0
Sphingomyelin	3.49	3.50	3.48	3.88	3.86	3.84	0	0	0	—	—	0	14.6	—	15.0

Compound	*Molar ratios*														
	P/N			*Galactose/N*			*Sulfur/N*			*Choline/N*			*Fatty acid/N*		
	Found		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*	*Found*		*Theory*
	I	*II*		*I*	*II*		*I*	*II*		*I*	*II*		*I*	*II*	
Cerebroside	—	—		0.98	0.99	1.00	—	—		—	—		—	1.02	1.00
Sulfatide	—	—		1.01	0.99	1.00	1.03	0.99	1.00	—	—		—	1.01	1.00
Sphingomyelin	0.505	0.505	0.500	—	—	—	—	—		0.496	—	0.500	—	0.51	0.50

the weight, 92–100 % of hexose and 83–89 % of phosphate. No sulfatide is accounted for in any of their fractions. Taking into consideration the inconsistency between the recovery of total weight and hexose and phosphate, the recoveries are probably of the same order as that obtained here.

Characterization of products

Chromatography of the fractions on thin-layer plates and chemical analysis were used as criteria of the quality of the fractions obtained. Chromatograms on silica gel plates (Fig. 2) show no gross contamination in any of the preparations. The detection reagents used are sensitive to as little as 0.005 μmoles of lipid. Paper chromatography of acid hydrolysates of the cerebroside and sulfatide fractions show that the only carbohydrate present is galactose. In addition no glycerol could be detected on these chromatograms. A relatively complete chemical analysis of significant constituent groups of each compound obtained from sphingolipids prepared by two different extraction procedures is given in Table I. Theoretical values for lignoceryl cerebroside, lignoceryl sulfatide and behenyl sphingomyelin have also been tabulated as a basis of comparison. Both on the basis of the percent weight of the various constituents and the molar ratios of the constituents, all of the compounds prepared by this method are highly pure.

Further efforts to characterize the sulfatide obtained by this method supports recent reports[32,33] that the sulfate is not on the number 6 carbon of the galactose as originally reported by THANNHAUSER *et al.*[34]. Periodate oxidation under the conditions described by CARTER *et al.*[35] failed to show any oxidation of the sulfatide over a 12 h period. Under the same conditions, 2.08 moles of periodate per mole of cerebroside were reduced in 2 h. This observation confirms the periodate oxidation study of YAMAKAWA *et al.*[32] and supports their view that the sulfate is on the number 3 carbon.

Generally in the past preparations of the various sphingolipids have been characterized by analysis of various constituents and/or carbon, hydrogen and nitrogen analyses. Where this type of data has been given[1–3,7–10,15,35] the preparations obtained here compare very favorably. In conclusion this method permits the separation of all three of the major sphingolipids from a mixture in a yield of 92–95 % and of a quality equal or better than that obtained by previously reported methods for preparing one or at the most two of the sphingolipids from a mixture.

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SUMMARY

Ox brain sphingolipids are separated into cerebroside, sulfatide and sphingomyelin fractions by chromatography on a silicic acid–Hyflo Super-Cel column. Elution is carried out with mixtures of chloroform–methanol–water. Depending on the method by which the sphingolipids are prepared, it is necessary to free the cere-

broside of small amounts of contaminating lipid by chromatography on Florisil. Regardless of the source, the sulfatide and sphingomyelin require further purification. Final purification of the sulfatide is carried out by chromatography on Florisil followed by precipitation from chloroform with acetone. Final purification of sphingomyelin is accomplished by chromatography on alumina. Final yields of 92–95 % of the sphingolipids in a brain sphingolipid preparation are obtained. Chemical analysis of the products indicates purities of the order of 99 %. Choline of sphingomyelin is assayed by a new micro assay based on the formation of choline reineckate.

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