## Quantitative isolation of brain sulfatides*

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» In the course of studies on the fatty acid composition of brain sphingolipids, it was necessary to develop a procedure for the quantitative isolation of sulfatides. While studying the metabolism of galactolipids in rat brain, Radin, Lavin, and Brown (1) separated a sulfatide fraction from total lipids after their passage through Florisil (activated magnesium silicate) and mixed ion resins; no attempt was made to characterize the fraction obtained. In applying their procedure, we found that resinous material eluted with the sulfatides seriously interfered with the gas-liquid chromatographic analysis of the fatty acids of the sulfatides. Several other resins were tried, but we were unable to find any anion exchange resin stable to the mixtures of electrolytes and organic solvents used in the procedure. The simple and mild procedure devised by Lees, Folch, Sloane Stanley, and Carr (2), based on the distribution of lipids between the two phases of a series of related solvent systems, did not give quantitative yields with cerebral white matter and practically no yield at all with gray matter. Long and Staples (3) reported a complete separation of cerebrosides and sulfatides from rat brain by chromatography on silicic acid, but, though we made many attempts, we never succeeded in reaching a complete separation with human material. In the method to be described, cerebrosides and sulfatides are isolated together by chromatography on silicic acid and then separated on a column of diethylaminoethylcellulose.

*Materials.* Reagent grade methanol was dried with anhydrous sodium sulfate and NaOH-pellets and dis-

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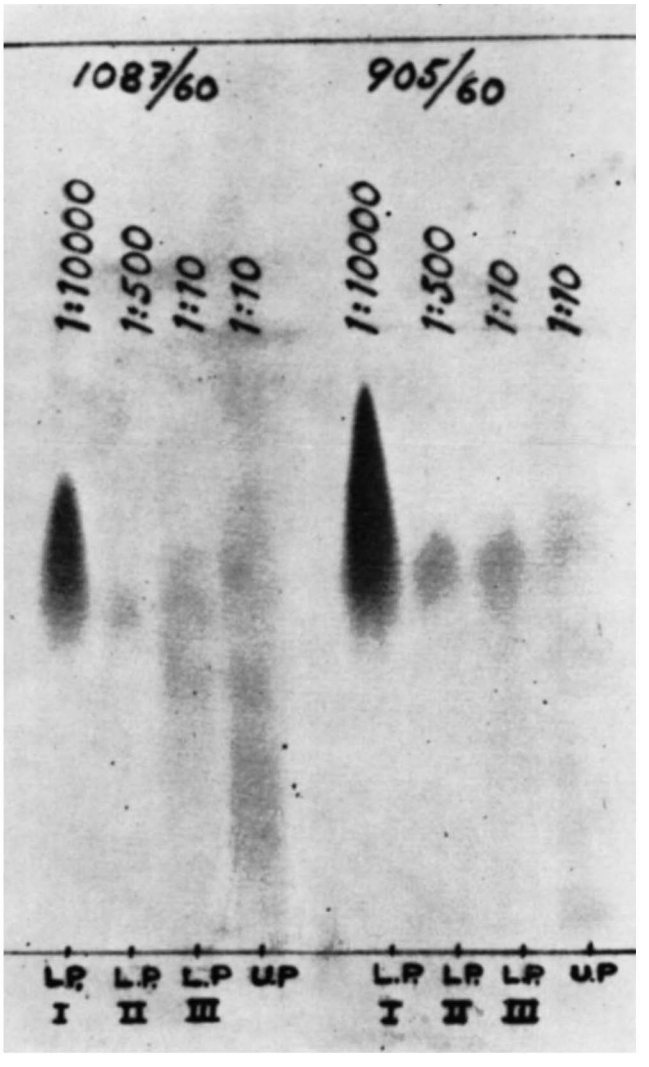


FIG. 1. Paper partition chromatogram of the three lower phases (L.P.) and the upper phase (U.P.) at the partition of the sulfatide extracts. The figure shows the part of the fraction spotted on the paper.

tilled. Chloroform was dried over calcium chloride and distilled. The solvents were stored in a brown bottle and used within a week. Tetrahydrofuran was redistilled and hydroquinone (1 g/liter) was added. All other solvents used were redistilled and stored without additives.

Silicic acid (Baker A. R., Lot No. 4680) passing through a 120 DIN sieve (German standard, equivalent to U. S. 280–300 mesh) was used. In order to remove the finest particles, the silicic acid was suspended in methanol; after 30 min, the methanol was decanted. This procedure was repeated. The remaining silicic acid was dried on a Büchner funnel with suction and activated at 120° for 18 hr. Diethylaminoethyl-(DEAE)cellulose (Whatman powder DE 50, W. & R. Balston, Ltd.) was used without any previous treatment. Paper partition chromatograms were run on Schleicher & Schüll 2045b papers.

TABLE 1. THE PARTITION OF THE LITHIUM CHLORIDE ELUATE FROM THE DEAE-COLUMN

| Sulfatides | Lower Phase I | Lower Phase II | Lower Phase III | Final Upper Phase | Recovery of Sulfatides |
|---|---|---|---|---|---|
| *Total brain 258/61* | | | | | % |
| Amount (mg) | 116 | 0.25 | 0.01 | 0.10 | .. |
| % of total | 99.7 | 0.2 | .. | 0.1 | 99.9 |
| *Cerebral cortex 1087/60* | | | | | |
| Amount (mg) | 86 | 0.55 | 0.02 | 0.40 | .. |
| % of total | 98.8 | 0.6 | .. | 0.5 | 99.5 |
| *Cerebral marrow 905/60* | | | | | |
| Amount (mg) | 360 | 1.5 | 0.03 | 0.60 | .. |
| % of total | 99.4 | 0.4 | .. | 0.2 | 99.8 |

Galactose was estimated by an orcinol method (4) after hydrolysis according to Radin *et al.* (1). Nitrogen, carbon, and hydrogen were analyzed at Mikroanalys-laboratoriet, Uppsala (W. Kirsten). Sulfate was assayed by a reducing method, developed by Dr. E. Mårtensson in our laboratory.[1] For the assay of very low amounts of sulfatides, a semiquantitative paper chromatographic method was used.[2]

*Preparation of a Crude Sphingolipid Extract.* About 50 g of human brain tissue (separated gray and white matter or part of a whole brain) was homogenized with 10 ml of chloroform–methanol 1:2 per gram of brain material. The homogenate was brought to a boil on a water bath and filtered through a sintered glass funnel. The residue was re-extracted twice with 5 ml chloroform–methanol 1:1 per gram of brain material. The combined filtrates were evaporated nearly to dryness. In order to hydrolyze glycerophospholipids, which interfere with the chromatographic separation, 12 g KOH-pellets and water were added to a final volume of 200 ml, and the mixture was left overnight at 35°. This saponification procedure did not give any measurable hydrolysis of pure sulfatides. The lipid extract was acidified to pH 4-5 with 4 N hydrochloric acid. Chloroform–methanol 2:1 (800 ml) was added, and the mixture was shaken thoroughly. After standing for 24 hr, the lower phase was evaporated to dryness under reduced pressure. The upper phase contained, besides salt and low molecular organic materials, most of the disialogangliosides, a small percentage of total sulfatides, and very small amounts of phospholipids. This phase was poured into a Visking dialysis tubing and dialyzed against running water for 24–48 hr. It was then taken to dryness in a rotating evaporator, the

[1] Erik Mårtensson, *Biochim. et Biophys. Acta*, in press.
[2] Lars Svennerholm, in preparation.

residue was extracted with chloroform–methanol 2:1, and the extract was added to the original lower phase.

*Chromatography on Silicic Acid.* Silicic acid (50 g) was slurried with chloroform and poured into a column 3 cm in diameter. The lipid extract dissolved in chloroform was pipetted onto the column. The following elution scheme was used: 250–500 ml chloroform followed by successive 500-ml volumes of chloroform–methanol 4:1, 1:1, and 1:4 (v/v).

Fractions of about 25 ml were collected with an automatic collector. The elution of the lipids was followed by paper partition chromatography with tetrahydrofuran–diisobutylketone–water 45:5:6 (v/v), as described by Beiss and Armbruster (5). Cerebrosides and sulfatides were eluted with chloroform–methanol 4:1 together with some auto-oxidized lipid material, cephalin B (6), and some partially hydrolyzed plasmalogens. All tubes containing cerebrosides and/or sulfatides were combined.

*Chromatography on DEAE-Cellulose.* DEAE-cellulose (5 g), freed from air in a vacuum desiccator, was slurried in chloroform–methanol 4:1 or 2:1, poured into a column 2 cm in diameter, and allowed to settle by gravity. The crude glycolipids, put directly onto the column or evaporated first to dryness and redissolved, were eluted with 125 ml of chloroform–methanol 2:1. The eluting solvent was changed to 125 ml chloroform–methanol 2:1 containing 5% glacial acetic acid, which eluted any altered lipid material, cephalin B, and nonlipid contaminants. The sulfatides were finally eluted with a mixture of 100 ml chloroform–methanol 2:1 and 5 ml 0.5 N lithium chloride in water.

The eluate containing the sulfatides was transferred to a separatory funnel, and 20 ml of water was added. After thorough shaking, the funnel was left for 24 hr. The lower phase contained 98–99% of the sulfatides. The upper phase was then extracted once with 20 ml chloroform and the new lower phase was added to the first lower phase. This second extraction increased the recovery of sulfatides in the partition step to better than 99.5%. Small amounts of acid lipids, mainly gangliosides, were left in the upper phase as shown by paper partition chromatography. The lower phase was dialyzed against water for 48 hr and then evaporated to dryness in a rotating evaporator to give the final product.

Analyses of the sulfatides from cerebral white matter in a typical run showed the following results: C, 64.7; H, 10.5; N, 1.56; S, 3.43; hexose (as galactose), 19.2. Ratio of nitrogen/hexose/sulfur = 1.04:1:1.02. In two separate experiments, the yield of sulfatides was 90 and 95% calculated from sulfate determinations on the original lipid extract and the isolated sulfatides.

The purpose of the present investigation was to develop a quantitative procedure for the isolation of sulfatides. The loss of 5–10% of sulfate does not necessarily mean that sulfatides were lost during the isolation. The loss of some sulfate may well be due to the presence of other sulfate-containing compounds in the original extracts. This assumption is supported by the data of Long and Staples (3), who recovered lipid galactose quantitatively but only 89% of lipid sulfate. We have therefore tested the recovery of sulfatides at each step of the isolation procedure.

Purified sulfatides have been reported to be rather unstable in an acid milieu, so there was reason to suspect some hydrolysis of sulfatides in connection with the silicic acid chromatography. Nevertheless, no cerebrosides were detected after chromatography of pure sulfatides on silicic acid. The sulfatides were also stable in acetic acid and could be left for several weeks at room temperature in the final eluate from the DEAE-column without any cerebroside being formed.

In a short preliminary note, the elution of sulfatides from DEAE-columns with ammonia in chloroform–methanol was described (7). We found that the sulfatides so isolated (as ammonium salts) were not stable even in the dry state, undergoing a gradual decomposition to cerebrosides. In the present method, the sulfatides are obtained as lithium salts and are stable in this form.

The recovery of sulfatides from the chromatography on DEAE-cellulose was quantitative, as determined by sulfate analyses. The capacity of the DEAE-cellulose was not exactly determined, but 300 mg of sulfatides in a crude glycolipid extract was quantitatively retained on a 5-g column. As the capacity of the cellulose will depend on the presence of the other lipids containing acidic groups, the value of testing the capacity of the column with pure sulfatides is limited.

In conclusion, the method described seems to give a quantitative yield of sulfatides in a rapid and simple way. At the same time, there is also a quantitative isolation and recovery of cerebrosides.

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