CHROM. 5314

Purification of ganglioside fractions by column chromatography on Sephadex G-100*

Gangliosides are generally extracted from brain tissue with chloroform–methanol (2:1) according to FOLCH-PI *et al.*[1]. After partitioning with water, the aqueous upper phase contains the gangliosides together with free amino acids, peptides, carbohydrates and a small quantity of lipids from the lower phase. The contamination of ganglioside fractions has long been recognized as a problem in radioactive and metabolic studies and various methods have been described for their purification[2,3].

This paper presents a simple and rapid technique for the purification of gangliosides by gel filtration on Sephadex G-100.

Experimental

Chemicals. Analytical grade reagents and redistilled organic solvents were used throughout.

Preparation of the lipid extracts. The experiments were carried out on Wistar rats, 30–40 days old. They were killed by decapitation and the whole brain quickly removed. The tissue was homogenized with chloroform–methanol (2:1) according to FOLCH-PI *et al.*[1]. The total lipid extract was washed once with water and twice with the "theoretical" upper phase (chloroform–methanol–water, 3:48:47), and the resulting upper phase plus the washing were taken to dryness, dissolved in 10 ml of chloroform–methanol (2:1) and "re-partitioned" with 2 ml of water according to SUZUKI AND CHEN[2].

The "crude ganglioside fraction" (CGF) obtained was kept for further experiments.

Purification of the CGF. The CGF was purified using two different procedures: (A) Sephadex G-100 chromatography or (B) dialysis.

(A) Sephadex G-100 was suspended in distilled water and poured into a 2 cm I.D. and 35 cm long column fitted with a Teflon stopcock and a porous glass plate. The final column height was adjusted to 25 cm by aspiration of the excess gel. Samples of the CGF taken to dryness and dissolved in 0.6 ml water containing between 300–1800 μg NANA were applied to the top of the column and eluted with distilled water at a flow rate of 8 ml/h. 2-ml fractions were collected in each tube and aliquots of these were used for further analysis. The effluent fractions were also monitored at 260 and 280 nm.

(B) In parallel experiments, a sample of CGF was exhaustively dialyzed against frequent changes of water, for four days, and used to check the results obtained by the Sephadex procedure.

Chemical procedures. The following analyses were carried out on the effluent from the column and on the dialyzed CGF: Ganglioside NANA was determined by the method of SVENNERHOLM[4] as modified by SUZUKI[5]. Protein determination was done according to LOWRY *et al.*[6]. The modified technique of ROSEN[7] was used for the estimation of amino acids, and lipid phosphorus was determined by the procedure of CHEN *et al.*[8].

* Supported by Grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (1003b) and the Instituto Nacional de Bromatología y Farmacología (Argentina).

Analysis of the purified ganglioside fraction. The fractions eluted from the Sephadex G-100 column which contained gangliosides were pooled. Aliquots were withdrawn from the pool for: (a) total hydrolysis, followed by quantitative analysis of amino acids; (b) separation of the amino acids by descending paper chromatography; (c) separation of the gangliosides by thin-layer chromatography (TLC).

Hydrolysis was carried out in 6 *N* HCl at 105° *in vacuo* and qualitative analysis of amino acids was done by descending chromatography on 3 MM Whatman paper using butanol–acetic acid–water (60:15:25) for development. The solvent front reached the bottom of the paper after a 20 h period, at 37°. The amino acids were detected by spraying with 0.2 % ninhydrin in 95 % ethanol, drying and heating at 70° for 5 min.

TLC of gangliosides was done on 20 × 20 cm glass plates coated with Silica Gel G (0.5 mm thickness). The plates were developed in a horizontal Desaga BN-chamber with propanol–butanol–water (65:10:25) at 20–25°. The solvent reached the front of the plate in about 3 h, and the chromatography was continued for 2 more h. Detection of the spots and quantitation of the individual gangliosides were carried out according to MACMILLAN AND WHERRET[9].

Radioactive assay. In some cases, rats were injected subcutaneously with 1 μCi/g body weight of U-[^{14}C]glucose. 1 h after injection, the animals were killed by decapitation and their brains processed as described above. The radioactive samples from each fraction collected from the column were placed in vials containing 12 ml of BRAY's solution[10] and counted in a Packard TriCarb Spectrometer (model 3003). Correction for quenching was done by the channel's ratio method[11,12].

Results

The pattern obtained with the CGF eluted through a column of Sephadex G-100 is shown in Fig. 1. Two peaks of absorbing material were resolved on this column. Peak I was eluted with the exclusion volume and contained the gangliosides and a small amount of contaminants. The bulk of amino acids and peptides, as well as other contaminating material was eluted with the total volume of the column (Peak II). Both peaks were clearly separated without overlapping.

A small amount of material absorbing at 260 nm was detected in Peak I. However most of the contaminants absorbing at this wavelength, possible nucleotide-sugars, were eluted together with Peak II.

Chromatography of labelled CGF showed that 99 % of the radioactivity was eluted with Peak II and only 1 % with Peak I.

In agreement with observations made by other investigators[13,14] the CGF still contained peptides and amino acids which were detectable by both the ninhydrin and LOWRY procedures. Fig. 1 shows that even after purification on Sephadex G-100, our ganglioside fraction (Peak I) contained small amounts of peptides. Table I shows the results obtained by hydrolysis of this peak; there was fourteen-fold increase in free amino acids after hydrolysis. Paper chromatography did not show positive ninhydrin material, but after hydrolysis five well defined spots appeared (Fig. 2). No attempt was made to characterize these spots. A few TLC plates of gangliosides were sprayed with ninhydrin reagent, and purple spots appeared at the individual ganglioside positions.

The ganglioside NANA recovered in Peak I was 88 %, and TLC of this fraction

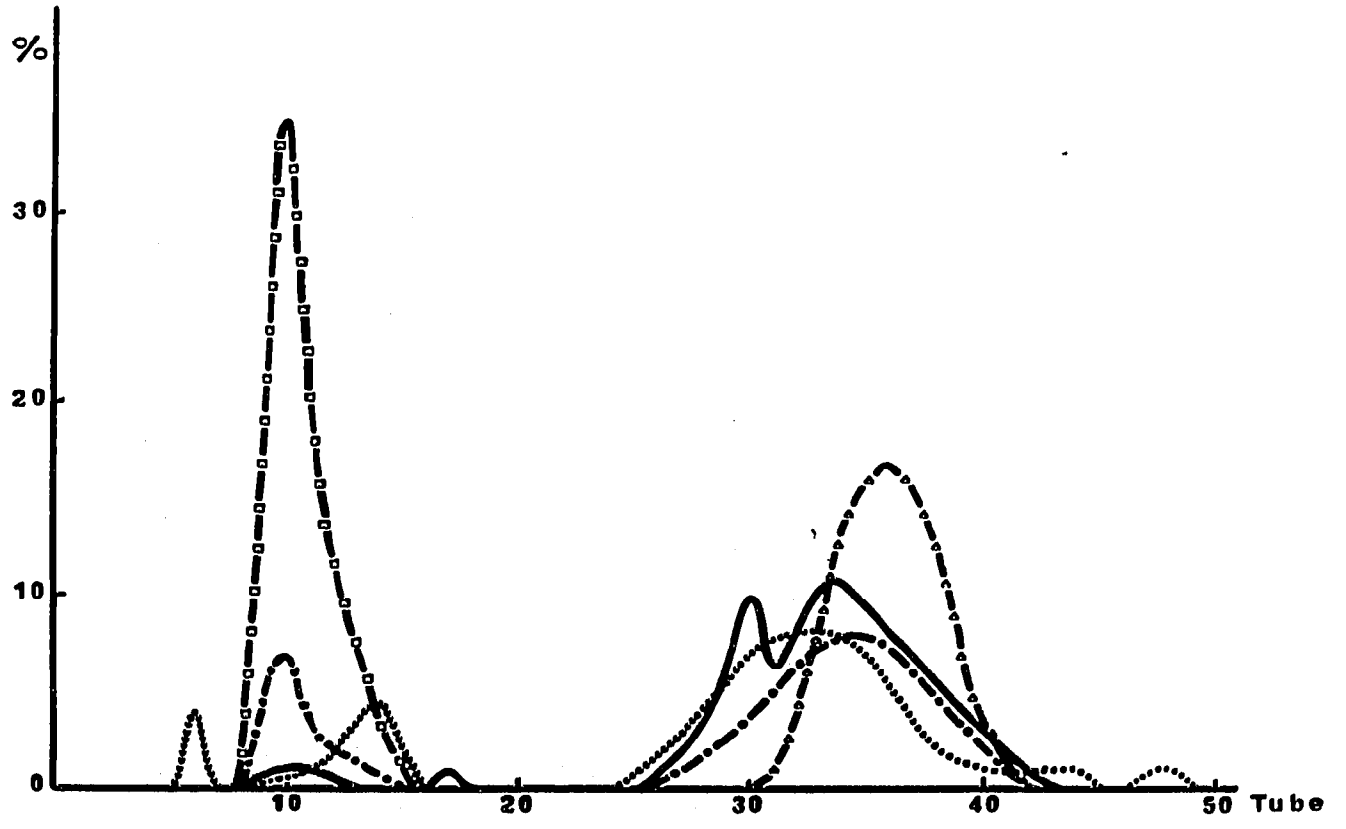

Fig. 1. Chromatographic pattern of the crude ganglioside fraction after elution from the Sephadex G-100 column. 2-ml fractions were collected in each tube as described under *Experimental*. Results are expressed as a percentage of the total amount eluted from the column. (□—□) gangliosides; (———) amino acids; (· · · · · ·) protein; (●—●) absorbance at 260 nm; (△—△) radioactivity.

TABLE I

ACID HYDROLYSIS OF PEAK I

Each figure represents the mean value of two separate experiments. For technical details see text.

	μmoles amino acid/g fresh tissue
Before hydrolysis	0.97
After hydrolysis	14.50

showed that the percentage distribution of the individual gangliosides was similar to that found in dialyzed CGF (Table II). It is clear (Table III) that our purified ganglioside fraction contained a much smaller amount of all the contaminants.

Discussion

Various methods have been described for the purification of the ganglioside fraction. Gangliosides are soluble in water and form high molecular weight micelles (mol. wt. 250000–450000) which do not dialyze through cellulose membranes[17]. This

property has been used for the purification of ganglioside fractions, but the long time required for dialysis (four days) is, however, its major limitation.

To ensure the complete elimination of contaminant lipids, SUZUKI[2] developed the "re-partition" technique. A rather exhaustive dialysis was necessary, anyway,

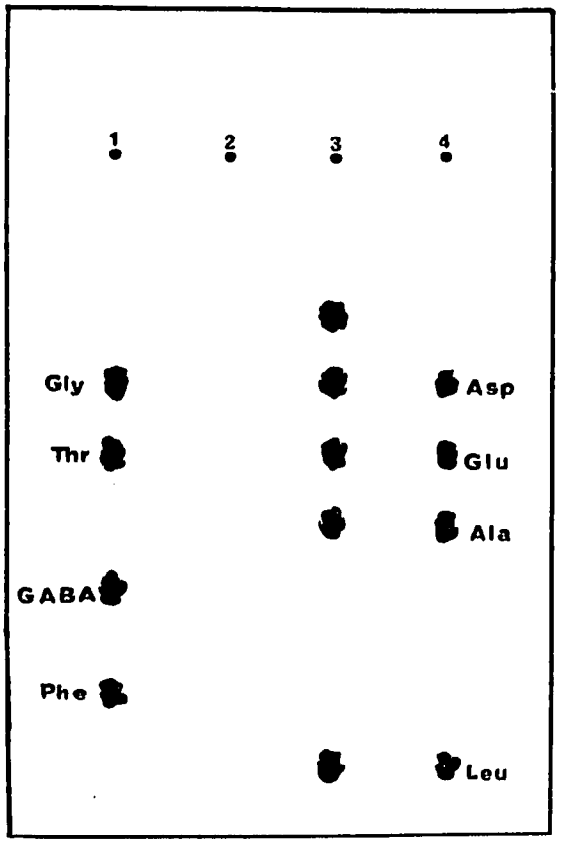

Fig. 2. Tracing of a one-way descending paper chromatogram of amino acids. Chromatographic conditions: 3 MM Whatman paper; solvent, butanol–acetic acid–water (60:15:25); development time, 20 h; development temperature, 37°; spray reagent, 0.2% ninhydrin in 95% ethanol. Samples: 1 and 4 = reference mixtures of amino acids; 2 = Peak I; 3 = Peak I after hydrolysis; 0 = origin.

TABLE II

COMPARISON OF THE INDIVIDUAL GANGLIOSIDE DISTRIBUTION ON TLC

Nomenclature of gangliosides is that of KOREY AND GONATAS[15] with the corresponding SVENNERHOLM[16] nomenclature in parentheses. Results are expressed as a percentage of the total gangliosides recovered from the plate. A = CGF purified on the Sephadex G-100 column; B = CGF purified by dialysis. For technical details see text.

Ganglioside	*A*	*B*
G_0	11.0	7.1
G_1 (G_{T1})	16.1	19.5
G_2 (G_{D1b})	13.8	16.9
G_3 (G_{D1a})	32.3	30.1
G_4 (G_{M1})	23.2	21.4
G_5 (G_{M2})	3.5	4.9

TABLE III

COMPARISON OF THE TWO PURIFICATION METHODS

A = CGF purified on the Sephadex G-100 column; B = CGF purified by dialysis; % = percentage of the total amount found in CGF. Experimental details, see text.

	CGF	*A*	*B*
Gangliosides			
μg NANA/g fresh tissue	374	329	380
%	100	88	103
Amino acids			
μmoles leucine/g fresh tissue	34.10	0.51	5.06
%	100	1.5	14.9
Protein			
μmoles albumin/g fresh tissue	320	120	230
%	100	37	72
Phospholipids			
μg P/g fresh tissue	69	16	33
%	100	23	48

to eliminate the trace amounts of free amino acids and other low molecular weight substances.

WELLS AND DITTMER[18] used Sephadex G-25 columns to exclude water soluble precursors and metabolites from the total lipid extract, but these authors did not remove amino acids from extracts which had been previously partitioned with water or salt solutions, and this procedure did not give quantitative separation of brain lipids. SIAKOTOS AND ROUSER[19] used a Sephadex G-25 column for the complete separation of the major lipid classes from water soluble non-lipids; they eluted with four different solvent systems and obtained gangliosides as a separate fraction. ROUKEMA AND HEIJLMAN[3] combined both purification methods: 24 h dialysis and Sephadex G-25 columns.

Our chromatographic procedure on Sephadex G-100 columns, as outlined, requires a minimum of working time and yields a consistently good recovery. The purified ganglioside fraction does not show any modification with regard to the pattern of migration on TLC, and it is available for further analytical and metabolic studies. The contamination of our purified fraction by phospholipids and specially by peptides, is considerably lower than the contamination obtained by dialysis.

An association of gangliosides and peptides has been demonstrated by ROSENBERG AND CHARGAFF[13] and other authors[14,20]. TRAMS AND LAUTER[21] obtained a ganglioside fraction which was peptide free, after a tedious and time consuming procedure. The presence of small amounts of peptides in our purified ganglioside fraction is consistent with BOOTH's hypothesis[22] of some association between gangliosides and peptides in aqueous systems, by ionic interaction.

The authors are grateful to Dr. CARLOS J. GÓMEZ for his interest and encouragement in this investigation and to Dr. EDUARDO F. SOTO for revision of the manuscript

and discussion of the results. They also thank Miss MARÍA I. RIGO ORDÓÑEZ for her technical assistance.

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1 J. FOLCH-PI, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
2 K. SUZUKI AND G. CHEN, *J. Neurochem.*, 14 (1967) 911.
3 P. A. ROUKEMA AND J. HEIJLMAN, *J. Neurochem.*, 17 (1970) 773.
4 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604.
5 K. SUZUKI, *Life Sci.*, 3 (1964) 1227.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. S. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 H. ROSEN, *Arch. Biochem. Biophys.*, 67 (1957) 10.
8 P. S. CHEN, Jr., T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
9 V. H. MACMILLAN AND J. R. WHERRETT, *J. Neurochem.*, 16 (1969) 1621.
10 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
11 L. A. BAILLIE, *Int. J. Appl. Rad. Isotopes*, 8 (1960) 1.
12 G. A. BRUNO AND J. E. CHRISTIAN, *Anal. Chem.*, 33 (1961) 650.
13 A. ROSENBERG AND E. CHARGAFF, *Biochim. Biophys. Acta*, 21 (1956) 588.
14 J. FOLCH-PI AND M. LEES, *Amer. J. Dis. Child.*, 97 (1959) 730.
15 S. R. KOREY AND J. GONATAS, *Life Sci.*, 2 (1963) 296.
16 L. SVENNERHOLM, *J. Neurochem.*, 10 (1963) 613.
17 D. B. GAMMACK, *Biochem. J.*, 88 (1963) 373.
18 M. A. WELLS AND J. C. DITTMER, *Biochemistry*, 2 (1963) 1259.
19 A. N. SIAKOTOS AND G. ROUSER, *J. Amer. Oil Chem. Soc.*, 42 (1965) 913.
20 S. BOGOCH, in S. M. ARONSON AND B. W. VOLK (Editors), *Cerebral Sphingolipidoses*, Academic Press, New York, 1962, p. 249.
21 E. TRAMS AND C. LAUTER, *Biochim. Biophys. Acta*, 60 (1962) 350.
22 D. A. BOOTH, *J. Neurochem.*, 9 (1962) 265.

Received January 22nd, 1971