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Chromatographic Separation of Brain Lipids

CEREBROSIDE AND SULPHATIDE

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In recent years chromatography on silicic acid columns has been widely used for the resolution of the lipid mixtures extractable from living cells [egg yolk (Lea, Rhodes & Stoll, 1955; Rhodes & Lea, 1957); liver (Hanahan, Dittmer & Warashina, 1957); plasma (Garton & Duncan, 1957); spermatozoa (Lovern, Olley, Hartree & Mann, 1957); heart (Gray & Macfarlane, 1958; Gray, 1958)]. Alumina columns have also been employed in some cases [yeast (Hanahan & Jayko, 1952); egg yolk (Rhodes & Lea, 1957)]. The lipids of brain and spinal cord, which contain both cerebroside and sulphatide, have not been investigated by chromatography on alumina, and silicic acid chromatography has been applied only to enriched material, after the removal of the bulk of the other lipids by solvent fractionation (Weiss, 1956; Payne & Platt, 1958).

The purpose of the present work was to examine the chromatographic behaviour of the cerebroside and sulphatide, present in the lipid mixture extractable by chloroform–methanol from rat brain, with both alumina and silicic acid columns. Some of the early findings have already been reported in preliminary form (Long & Staples, 1959*a*).

EXPERIMENTAL

Materials

Lipids. Partially purified samples of ox-brain cerebroside (prepared by Dr R. Rodnight, Institute of Psychiatry, Maudsley Hospital, London) and of ox-brain sulphatide (Lees, Folch, Sloane-Stanley & Carr, 1959) were given by Dr G. H. Sloane-Stanley of this Department. The cerebroside sample was free from sulphatide; the sulphatide specimen had a sulphate : galactose molar ratio of 0·89. Lecithin and phosphatidylethanolamine were obtained from egg yolk by the method of Rhodes & Lea (1957). Lysolecithin was prepared according to Long & Penny (1957). Samples of phosphatidylserine and sphingomyelin were given by Dr J. N. Hawthorne and Dr June Olley respectively. Synthetic dihydrocerebroside (palmitoyl) was a gift from Dr D. Shapiro, Weizmann Institute, Rehovoth, Israel.

Solvents. Methanol was refluxed for 30 min. with NaOH pellets and granulated zinc, and distilled through a fractionating column. Chloroform was shaken five times with water, and the lower phase was distilled through a fractionating column; the water present distilled over first as a $CHCl_3$–water azeotrope and was discarded. The freshly distilled $CHCl_3$ was immediately treated with methanol, to give $CHCl_3$–methanol (98:2, v/v).

Chromatographic materials. Silicic acid (A.R. 100-mesh powder, Mallinckrodt Chemical Works; purchased from Bell and Croyden Ltd., 50 Wigmore Street, London, W. 1) was sieved and the material passing a 240 British Standards sieve was discarded. The silicic acid was then activated by heating at 120° for 24 hr. Alumina (Brockmann, grade II) was obtained from Savory and Moore Ltd., London. Two batches were used, *A* and *B*; these appeared to be similar in chromatographic properties, except that type *B* had a greater adsorptive affinity for sulphatide. The cellulose powder used was Whatman no. 1.

All other chemicals were the best available commercially.

Methods

Extracts of rat-brain lipid. The whole brains of one to four adult Wistar albino rats, of either sex, were homogenized with $CHCl_3$–methanol (2:1, v/v), 19 ml. of solvent/g. of brain being used. The homogenate was filtered through a sintered-glass funnel (porosity 2), and the residue was washed with 5 ml. of solvent/g. of brain. The combined filtrates were freed from water-soluble substances by solvent partition with 0·1 M-KCl, according to the method of Folch, Lees & Sloane-Stanley (1957), as described for ox

brain by Long & Staples (1959*b*). The $CHCl_3$-rich phase was evaporated to dryness under reduced pressure, and the residue was dissolved in $CHCl_3$–methanol–water (64:32:4, by vol.) and again evaporated to dryness. This process was repeated once more in order to decompose the proteolipid (Folch & Lees, 1951). The residue was finally suspended in $CHCl_3$–methanol (50:50 or 98:2, v/v). Protein was removed by filtration through a sintered-glass funnel (porosity 3). The filtrate, diluted to 25 ml., was stored overnight at 2° and again filtered to give a clear solution of brain lipids.

Chromatography. Alumina (13 g.) or silicic acid (7 g.) was slurried with $CHCl_3$–methanol (50:50 or 98:2, v/v, respectively) and columns (18 cm. × 1 cm. diam.) were prepared in the usual way. The lipid solution, in the appropriate solvent, was pipetted on to the column.

For gradient elution, the mixing vessel had a volume of either 125 or 200 ml., and was fitted with a mechanical stirrer. The reservoir contained the second solvent and this was arranged to run into the mixing vessel at such a rate that the volume of eluting solvent in the latter remained constant. The composition of solvent running on to the column was calculated from the exponential equation of Drake (1955). Fractions (15 or 25 ml.) were collected and transferred to glass-stoppered reagent bottles.

Analytical procedures

Galactose. The lipid solution, containing not more than 0·3 μmole of galactose, was pipetted into a 10 ml. tapered graduated centrifuge tube, and evaporated to dryness on a warm-water bath in a current of air or N_2. The residue was dissolved with warming in a mixture of ethanol (0·5 ml.) and water (0·1 ml.); 3N-H_2SO_4 (2 ml.) was then added. At this stage most lipids, including cerebroside, produce an opalescence, but sulphatide remains in solution. The tube was covered with a glass bulb and hydrolysis was carried out for 2 hr. on a boiling-water bath (Svennerholm, 1956). After cooling, the hydrolysate was partially neutralized by addition of 11N-NaOH (0·5 ml.). The tube was centrifuged briefly to detach water droplets from the upper part, and water was added to give a total volume of 3 ml. Powdered $BaCO_3$ (about 30–40 mg.) was then added, and the contents of the tube were well mixed and allowed to stand overnight.

After centrifuging down the $BaSO_4$ and excess of $BaCO_3$, a 2 ml. sample of clear supernatant was pipetted into a test tube (125 mm. × 16 mm. diam.) and 0·5 ml. of water was added. The tube was cooled in ice–water and 5 ml. of conc. H_2SO_4, containing 0·2 % of orcinol, was added. After the contents were mixed with a glass plunger, the tube was heated on a boiling-water bath for exactly 2 min. (Brückner, 1955). The extinction of the cooled solution was read in a photoelectric colorimeter (Evans Electroselenium Ltd.), with the 624 Ilford filter (maximum transmission at 520 mμ). The same procedure was applied to duplicate galactose standards (0·3 μmole) and a water blank.

When 0·3 μmole of various lipids was tested by this procedure, the galactose value was zero for cholesterol, cholesteryl oleate, ovolecithin, phosphatidylserine, sphingomyelin and for lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid and behenic acid. A mixture of phosphatidylethanolamine and ethanolamine plasmalogen (0·3 μmole), however, gave a final extinction equal to that obtainable from 0·02 μmole of galactose. Thus it was necessary to apply a small correction to galactose determinations on fractions in which appreciable quantities of ethanolamine-containing lipids were also present.

Sulphate. The lipid solution, containing not more than about 0·3 μmole of sulphatide, was evaporated to dryness in a 3 ml. tapered centrifuge tube. To the residue water (0·15 ml.) and conc. HCl (0·15 ml.) were added and the tube, capped by a glass bulb, was heated on a boiling-water bath for 1 hr. To the cooled hydrolysate 1·5 ml. of $CHCl_3$–methanol (2:1, v/v) was added and the contents were mixed thoroughly with a Pasteur pipette. The lower $CHCl_3$-rich phase, containing fatty acids, long-chain aldehydes, sphingosine and cholesterol, was withdrawn and discarded. The aqueous methanolic phase was washed twice in the same way with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The final aqueous acidic methanolic phase was then evaporated to dryness in a current of air or N_2 on a warm-water bath. The above procedure is a modification of the method used by Lees & Folch (1959).

The residue was dissolved in water (0·15 ml.) and a solution in 0·1N-HCl of 4-amino-4′-chlorodiphenyl hydrochloride (0·224 %, containing 0·1 % of cetyltrimethylammonium bromide; 0·15 ml.) was added. The tube was allowed to stand at room temperature for not less than 2 hr. (Jones & Letham, 1956). The microcrystalline ppt. of the amine sulphate was centrifuged down, and 0·1 ml. of the clear supernatant was diluted with 10 ml. of water before determination of the extinction at 254 mμ in a Unicam SP. 500 spectrophotometer. Potassium sulphate standards (0·3 and 0·15 μmole) and duplicate water blanks were subjected to the same procedure.

The 4-amino-4′-chlorodiphenyl hydrochloride was prepared according to Gelmo (1906). It has been reported by Jones & Letham (1956) that orthophosphate reacts with the reagent, but we have observed no interference from 2 μmoles of orthophosphate under the conditions used. Glycerophosphate, derived from 2 μmoles of egg phosphatidylethanolamine, also did not interfere with the determination of sulphate.

Total nitrogen. The lipid solution, containing 0·2–1·0 μg.atom of total N, was evaporated to dryness in a test tube and 0·12 ml. of 72 % perchloric acid was added. Ashing was conducted on a Gallenkamp micro-Kjeldahl digestion apparatus at 170° for 2 hr. Water (2·5 ml.) was added followed by Nessler's reagent (King & Wootton, 1956; 1·5 ml.). The extinction was read in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter no. 621. Duplicate standards (1 μmole of $NH_4H_2PO_4$) and a water blank were run through the same procedure.

Sphingosine. The lipid solution, containing not more than 1 μmole of sphingosine, was evaporated to dryness; the residue was hydrolysed by heating for 1 hr. on a boiling-water bath with 0·5 ml. of 6N-HCl (Robins, Lowry, Eydt & McCaman, 1956). The cooled hydrolysate was treated with 2·5 ml. of $CHCl_3$–methanol (2:1, v/v) and, after being mixed thoroughly with a Pasteur pipette, the lower $CHCl_3$-rich phase was transferred into a second tube. The aqueous methanolic phase was extracted twice more with 1 ml. portions of $CHCl_3$–methanol (17:3, v/v). The combined $CHCl_3$-rich extracts were evaporated to dryness and ashed for total N, as already described.

Amino nitrogen. The lipid sample, containing not more than about 0·2 μg.atom of amino N, was evaporated to dryness, treated with 0·1 ml. of water and 2·0 ml. of the ninhydrin reagent of Moore & Stein (1948), and heated on

a boiling-water bath for 20 min. After dilution of the solution to 10 ml. with ethanol–water (80:20, v/v), the extinction was determined in a photoelectric colorimeter (Evans Electroselenium Ltd.) with Ilford filter no. 626. Standard $NH_4H_2PO_4$ (0·2 μmole) and a water blank were simultaneously run through the same procedure.

Total phosphorus. The lipid sample, containing not more than about 0·2 μg.atom of total P, was evaporated to dryness and ashed with 0·25 ml. of 72% perchloric acid at 170° for 2 hr. After dilution with water to 2 ml., the tube was placed in a boiling-water bath for 20 min. The cooled solution was analysed for orthophosphate by the method of Berenblum & Chain (1938), as modified by Long (1943).

Acyl ester. Determinations were made according to Shapiro (1953).

Cholesterol. This was determined by the Liebermann–Burchard procedure (King, 1946).

RESULTS

Removal of water-soluble contaminants and proteolipid protein from lipid extracts of rat brain

Since the aim of the present work was to study the chromatographic behaviour of cerebroside and sulphatide, it was necessary to ensure that the lipid extracts under examination were free from other carbohydrate-containing substances. The crude lipids prepared by extraction of brain tissue with $CHCl_3$–methanol (2:1, v/v) are well known to contain ganglioside (Folch, Arsove & Meath, 1951), and may contain traces of free monosaccharides and their phosphate esters. These water-soluble materials may be removed by solvent partition (Folch *et al.* 1957; Long & Staples, 1959*b*). As further experiments were planned to study the chromatographic behaviour of the ethanolamine- and serine-containing phospholipids, it was also considered desirable to remove the proteolipid protein. The crude lipid extract of brain (stage 1) was therefore subjected to the following successive treatments: (*a*) removal of water-soluble components by solvent partition (stage 2); (*b*) precipitation of proteolipid protein by heat treatment (stage 3); (*c*) passage through a cellulose column (stage 4), according to Lea & Rhodes (1953). At each stage, analyses were made for galactose, sphingosine, total N, amino N, total P, ester and cholesterol. The results, shown in Fig. 1, are in terms of μmoles or μg.atoms of constituent/g. of fresh rat brain.

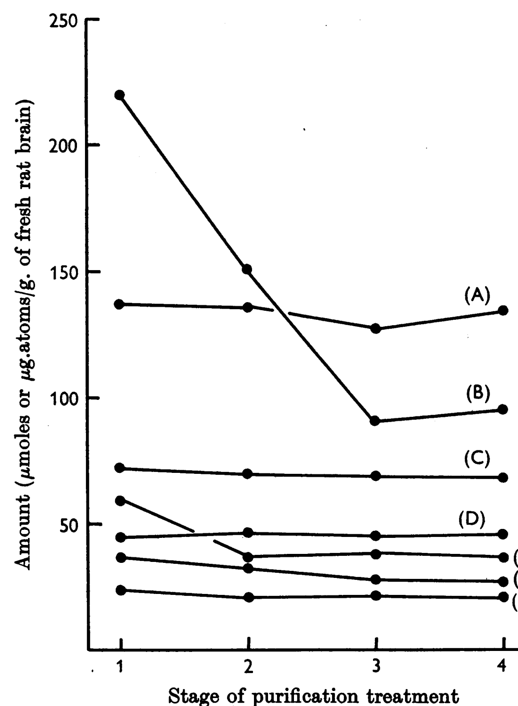

Fig. 1. Analytical values, in μmoles or μg.atoms/g. of fresh tissue, for a $CHCl_3$–methanol (2:1, v/v) extract of rat brain (stage 1), and after the following successive treatments: washing out of water-soluble substances (stage 2), removal of proteolipid protein (stage 3) and passage through a cellulose column (stage 4). (A) Acyl ester; (B) total N; (C) total P; (D) cholesterol; (E) amino N; (F) sphingosine; (G) galactose.

It will be seen from Fig. 1 that the cholesterol remains constant throughout the series of treatments. Thus analyses for this typical lipid constituent indicated that no manipulative losses occurred. Acyl ester and total P contents also varied very little, as would be expected. Total N and amino N fell significantly after the solvent-partition procedure (stage 2), reflecting the loss of free amino acids, and total N showed a further substantial decrease after removal of proteolipid protein (stage 3). There was a slight fall in galactose and sphingosine contents after the solvent-partition process, which largely represents the removal of ganglioside. There also seems to be a further slight fall in sphingosine after removal of proteolipid protein. However, this may be due to a slight solubility in the $CHCl_3$-rich phase of the less polar amino acids formed on hydrolysis of the proteolipid protein in stage 2; such behaviour would have contributed to the apparent sphingosine content.

Passage through a cellulose column (stage 4) did not lead to the removal of any further components for which analyses were made. This suggests that the solvent-partition procedure is adequate to remove water-soluble material. Stage 4 was omitted in subsequent experiments.

Chromatography on alumina

Samples of the cerebroside and sulphatide preparations were chromatographed on alumina in the following way. The material was dissolved in $CHCl_3$–methanol (80:20, v/v) and 2 ml. of the

solution was applied to an alumina column, equilibrated with the same solvent. Type *A* alumina was used. The column was washed with 25 ml. of $CHCl_3$–methanol (80:20, v/v) followed by 50 ml. of $CHCl_3$–methanol (50:50, v/v), the eluate being collected in 25 ml. fractions. Gradient elution was then applied. The mixing vessel contained 125 ml. of $CHCl_3$–methanol (50:50, v/v), and the reservoir 150 ml. of $CHCl_3$–methanol–water (45:45:10, by vol.). Eleven more 25 ml. fractions were collected and galactose was determined on each fraction. The results are shown in Fig. 2.

It will be observed that the peak derived from the cerebroside sample occurred in fraction 8, whereas that from the sulphatide was in fraction 11. The curve for the cerebroside sample also exhibited a shoulder in fraction 11, although the material was free from sulphatide.

Similar types of experiments with type *A* alumina were conducted on the washed deproteinized lipids of rat brain (stage 3 of the previous section), except that the mixed lipids were loaded on to the column in $CHCl_3$–methanol (50:50, v/v). The results are shown in Fig. 3.

The fractions collected before gradient elution was begun contained all the cholesterol of the lipid applied to the column, as well as the choline-containing phospholipids (Rhodes & Lea, 1957). When gradient elution was started, galactose-containing fractions were eluted to give a curve which was similar in appearance to that obtained for the cerebroside sample shown in Fig. 2. The galactose peak appeared in fraction 8, but the shoulder was rather more prominent. In this experiment, the recovery of galactose in the seven fractions (nos. 6–12) was 22·3 μmoles/g. of brain, compared with 21·3 μmoles applied to the column. Thus both cerebroside and sulphatide must have been completely eluted, the latter probably contributing to the shoulder (fraction 10). Total N and sphingosine analyses were also carried out on the combined galactose-containing fractions, and the molar ratio (total N:sphingosine N:galactose) was 1·00:1·01:1·01. These fractions usually contained a trace of acyl ester, unaccompanied by either phosphorus or amino N; it is thought that this acyl ester is derived from the breakdown of aminophospholipids, which occurs on alumina columns (Long & Staples, 1960; Long, Shapiro & Staples, 1960).

In fraction 13, aminophospholipids began to be eluted, as indicated by determinations of amino N, phosphorus and ester; this material has been shown to be ethanolamine-containing lipid (Long & Staples, 1960). However, these amino-containing fractions are well separated from those which contain galactose. It is clear that the order of elution is: cerebroside, sulphatide, ethanolamine-

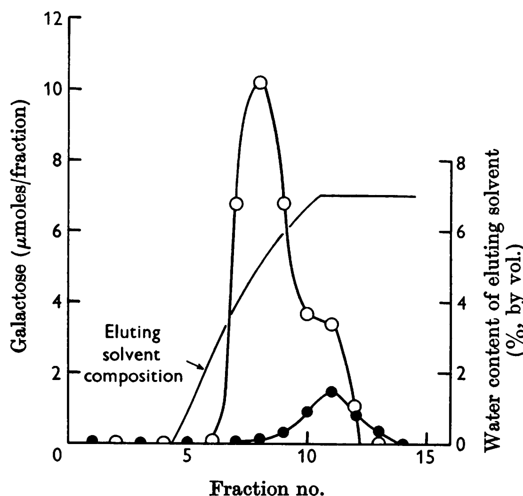

Fig. 2. Chromatographic behaviour of samples of cerebroside (45·7 mg.; ○) and sulphatide (5·20 mg.; ●) on an alumina (type *A*) column. The lipids were applied to the column in $CHCl_3$–methanol (80:20, v/v). The solvent was then changed to $CHCl_3$–methanol (50:50, v/v), and this was followed by gradient elution (composition shown), whereby the water content of the latter solvent was increased from 0 to 7% (by vol.). Fractions, 25 ml.

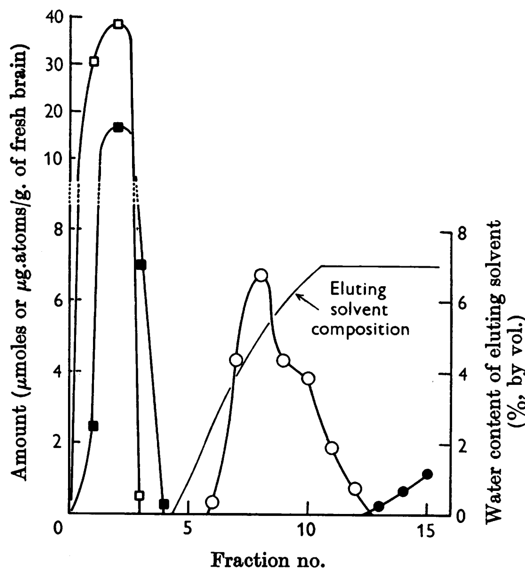

Fig. 3. Chromatography of washed, deproteinized, rat-brain lipids on alumina (type *A*). The lipids were applied to the column in $CHCl_3$–methanol (50:50, v/v); other conditions were as in Fig. 2. Cholesterol (□), total phosphorus (■), galactose (○), amino nitrogen (●). Composition of eluting solvent is shown.

containing phospholipid. These experiments were carried out before a reliable method of determining sulphate had been established.

When the sample of type *A* alumina, which was used in the foregoing work, had been used up, subsequent samples (designated type *B*) were found to possess the same affinity for cerebroside and ethanolamine-containing phospholipid, but to be rather more retentive with respect to sulphatide. In these experiments, which were carried out exactly as previously described, sulphate determinations were also made. A typical result is shown in Fig. 4.

After the application of gradient elution, galactose and sphingosine contents were in close agreement, the main peak again being found in fraction 8. Sulphatide, which began to be eluted in fraction 10, reached its peak in fraction 12, and beyond this point, sulphate, galactose and sphingosine values were almost identical, indicating that sulphatide free from cerebroside was being eluted. If the cerebroside content is taken to be equal to the difference between galactose and sulphate, the trailing edge of its elution curve is shown by the discontinuous line in Fig. 4. From this, it will be seen that with alumina type *B*, cerebroside and aminophospholipid are completely separated, whereas sulphatide overlaps the aminophospholipid as well as the cerebroside. The order of elution is again: cerebroside, sulphatide, aminophospholipid.

Chromatography on silicic acid

In these experiments, a sample of the washed, deproteinized, mixed lipids of rat brain (stage 3), dissolved in $CHCl_3$–methanol (98:2, v/v), was loaded on to a silicic acid column, equilibrated with the same solvent. After washing in the lipid, gradient elution was applied. The mixing vessel contained 200 ml. of $CHCl_3$–methanol (98:2, v/v), with 200 ml. of $CHCl_3$–methanol (90:10, v/v) in the reservoir; 15 ml. fractions were collected (Fig. 5). The cholesterol was completely eluted in the first three fractions. It was accompanied by a trace of ester, but was free from nitrogen and phosphorus. Immediately afterwards, a small galactose-containing peak was eluted (fractions 4–7); this accounted for 5 % of the total galactose applied to the column. The major peak followed in fractions 10–18, representing 82 % of the galactose applied. Both these peaks were free from sulphate and amino N. Beginning with fractions 20, however, aminophospholipid began to be eluted. After collection of 24 fractions, the eluting solvent was changed to $CHCl_3$–methanol (80:20, v/v). This first eluted a sharp peak of aminophospholipid, and later a third galactose-containing peak. In the latter, approximately equivalent amounts of galactose and sulphate were present. The galactose content accounted for 13 % of the amount applied, and the sulphate was equivalent to 89 % of that present in the original lipid. Thus the whole of the galactose and almost all the sulphate were recovered.

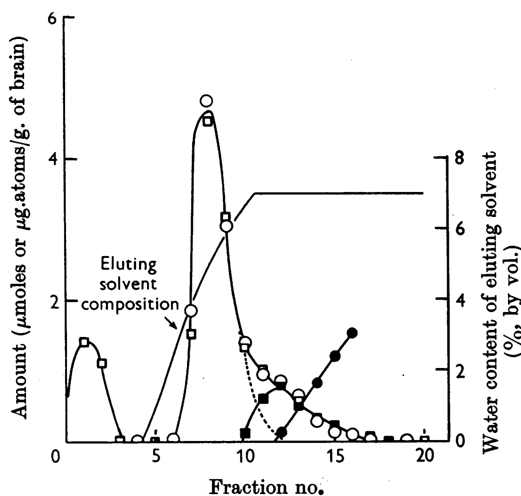

Fig. 4. Chromatography of washed, deproteinized, rat-brain lipids on alumina (type *B*). Experimental conditions were as in Fig. 3. Sphingosine (□), galactose (○), sulphate (■), amino nitrogen (●). Composition of eluting solvent is shown.

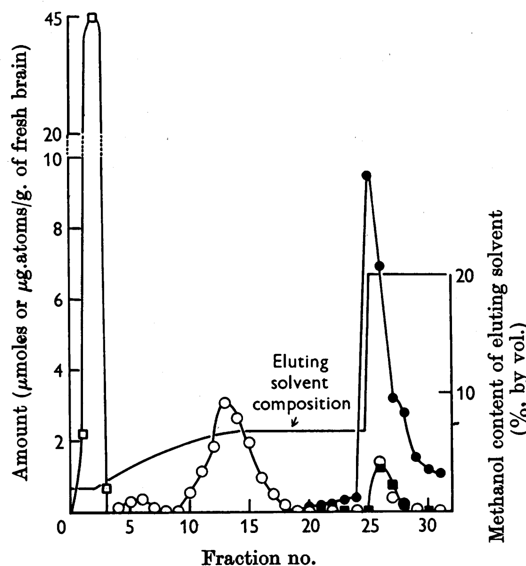

Fig. 5. Chromatography of washed, deproteinized, rat-brain lipids on silicic acid. The lipids were applied in $CHCl_3$–methanol (98:2, v/v). Cholesterol (□), galactose (○), amino nitrogen (●), sulphate (■). Composition of eluting solvent is shown.

Chromatography on silicic acid thus separated the galactose-containing lipids into three discrete peaks. The sulphatide is eluted much later than the main cerebroside peak, in contrast with its behaviour on alumina, where overlapping is observed (Fig. 4). Furthermore, the aminophospholipid precedes sulphatide on silicic acid, whereas the order on alumina is the reverse of this. In the main peak, galactose, total N and sphingosine N were equivalent to one another.

DISCUSSION

Previous investigations on the chromatographic behaviour of cerebroside and sulphatide have invariably been carried out on starting material, either brain or spinal cord, which had already been subjected to some solvent-fractionation technique in order to remove the bulk of the accompanying lipids. Such treatment normally leads to some loss of galactose-containing lipids. Radin, Brown & Lavin (1956) (see also Radin & Brown, 1960) extracted spinal-cord lipids with ether to remove cholesterol and other ether-soluble material, and chromatographed the residual lipids on Florisil, which retained the phospholipids. Sulphatide and cerebroside were then separated by adsorbing the former on an anion-exchange resin. Weiss (1956) extracted brain or spinal cord with acetone followed by light petroleum before subjecting the residual lipids to silicic acid chromatography.

In the experiments described here, lipid extracts of brain were chromatographed without previous treatment, other than removal of water-soluble substances and proteolipid protein. The results of chromatography on alumina are complicated by the slightly different properties of the two batches used, type *B* alumina having a greater affinity for sulphatide than had type *A*. It is clear that with the latter, a quantitative separation of cerebroside plus sulphatide from other brain lipids has been achieved (Fig. 3), whereas with type *B* alumina the sulphatide and ethanolamine-containing lipids overlap (Fig. 4). This observation is of some interest, since we have already noted the ion-exchange properties of alumina (Long *et al.* 1960). Different batches of alumina might therefore be expected to exhibit greatest variations with respect to electrically charged lipids. With type *B* alumina, the eluted sulphatide was found to contain no K^+ ions, as shown by flame-photometric determinations, even though the mixed lipids had earlier been washed with 0·1 M-potassium chloride. This suggests that type *B* alumina removes K^+ ions from their association with sulphatide. We have no parallel data for type *A* alumina.

It should be mentioned that the cerebroside and sulphatide components of rat brain exhibited similar chromatographic behaviour on alumina, independently of whether the proteolipid protein had been removed or not. This observation indicates that brain proteolipids (Folch & Lees, 1951) either do not contain cerebroside and sulphatide, or that the association between these lipids and protein is readily broken in contact with alumina.

Although the best separation of galactose-containing lipids from ethanolamine-containing lipids on alumina columns is achieved by the use of gradient elution, a fair separation can also be obtained by stepwise elution. Such a procedure was first developed in the early stages of this work and was later adopted by Davison, Dobbing, Morgan, Wajda & Payling-Wright (1960). However, under these conditions, we found that the early galactose-containing fractions were usually contaminated with a small amount of aminophospholipid, a feature which is absent when the gradient-elution technique is used.

The results obtained with rat-brain lipids on silicic acid columns (Fig. 5) may be compared with those reported for monkey-brain lipids by Weiss (1956), although the latter did not specify the exact composition of the chloroform–methanol mixtures used in his gradient-elution experiments. At low methanol concentrations, Weiss's peak I (containing 4·4 % of the total galactose applied to the column) may be taken to correspond with our first peak (5 %). His peaks II and III taken together (62·6 %) may be similar to our main peak (82 %), although his peak III had twice the expected content of total nitrogen; this latter finding could result from contamination with ethanolamine-containing lipid due to the use of too steep a gradient. Peak IV (19·3 %) is almost certainly the same as our sulphatide peak (13 %), and bears a rather similar relation to the elution pattern of phospholipid noted by us. The essential difference between the observations of Weiss (1956) and ourselves is that we do not find any separation of the main cerebroside peak into two fractions. However, this may be related to possible differences in the nature of the component fatty acid radicals, caused by the use of different animal species.

SUMMARY

1. The material extracted from rat brain by means of chloroform–methanol (2:1, v/v) has been analysed for galactose, sphingosine, total nitrogen, amino nitrogen, total phosphorus, cholesterol and acyl ester. Similar analyses were carried out on the extract after removal of water-soluble substances and proteolipid protein. The results indicate that no lipids, other than ganglioside, are lost in the course of this purification procedure.

2. Chromatography of the washed, deproteinized, lipid extract on alumina with a gradient-elution technique, in which the water content of the solvent was slowly increased, led to the successive elution of cerebroside, sulphatide and aminophospholipid. The cerebroside and sulphatide peaks always overlapped. With one batch of alumina the galactose-containing lipids were completely separated from the aminophospholipid.

3. Gradient-elution chromatography on silicic acid, whereby the methanol content of the solvent was increased, led to the complete separation of the cerebroside from the sulphatide. The former was also separated from the aminophospholipid, whereas there was overlapping between the sulphatide and aminophospholipid.

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The Phospholipids of Butter and their Effect on Blood Coagulation

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The suggestion by Macfarlane (1955) that dietary fats may play an important role in the aetiology of atheroma and that different lipids may have widely different effects on blood thromboplastin formation led us to investigate the effect of a number of dietary fats on blood coagulation *in vitro*. At the start of this work it was generally believed that the lecithin group of phospholipids were strong potentiators of blood coagulation (Macfarlane, Trevan & Attwood, 1941; Fullerton, Davie & Anastasopoulos, 1953) but later work by a number of workers showed that phosphatidylethanolamine present in the crude lecithin was the active material, and that pure lecithin was completely inactive (Poole, Robinson & Macfarlane, 1955; Lea, Rhodes & Stoll, 1955; Lea, 1956; O'Brien, 1956*a*, *b*; Robinson & Poole, 1956). Poole & Robinson (1956) have since demonstrated that synthetic specimens of