THE QUANTITATIVE SEPARATION AND ESTIMATION BY THIN-LAYER CHROMATOGRAPHY OF LIPIDS IN NERVOUS TISSUE

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During the last four years there has been a great increase in the use of thin-layer chromatography for the separation of lipids but for brain lipids reports have been concerned mainly with the procedure as a qualitative rather than as a quantitative technique.

JATZKEWITZ[1] has determined the eight sphingolipids in brain, quantitatively, by scraping off the silicic acid containing the separated fractions from the plate and estimating the lipids colorimetrically. In 1962, HONEGGER[2] published a semi-quantitative method of estimation of brain lipids by visual comparison of spot densities on thin-layer chromatoplates. DAVISON AND GRAHAM-WOLFAARD[3] have also determined brain lipids quantitatively by thin-layer chromatography, but their method also involves scraping off the silicic acid as well as the lipid and then transfering the "scraped off" material to columns for elution before hydrolysing the lipids for analysis.

The procedure to be described below is considered to be much simpler in that lipids are determined directly on the plate. It is an application of methods by ZÖLLNER, WOLFRAM AND AMIN[4] who estimated cholesterol esters, and PRIVETT, BLANK AND LUNDBERG[5] who estimated mono-, di- and triglycerides by spraying with sulphuric acid and scanning the separated spots with a densitometer.

Two methods for scanning thin-layer chromatograms have recently been published. CSALLANY AND DRAPER[6] coat the silicic acid layer with Neatan and remove it from the plate before scanning, SQUIBB[7] uses a clear plastic onto which the silicic acid is bonded; this can then be cut into strips prior to scanning in standard equipment.

MATERIALS AND EQUIPMENT

Merck's Kieselgel G spread on glass plates 20 cm × 20 cm, in layers about 250 μ thick. Plates are activated 1 h before use at 120° for 30 min. Chloroform, methanol G.P.R., *n*-propanol, distilled once and 12.5 % aqueous ammonia.

The samples were applied to the plate with an Agla micrometer syringe mounted vertically on the coarse adjustment of an old microscope. This enables the sample to be applied to the plate without scratching the surface of the silicic acid.

The chromatoplate is placed on a bed which moves over a scale so that samples are always applied in exactly the same positions.

The scanner is of the reflectance type (Fig. 1) and is manually operated. Rays from the light source strike the plate at right angles and are reflected onto photocells from which the signal passes through an amplifier and is registered on a voltmeter.

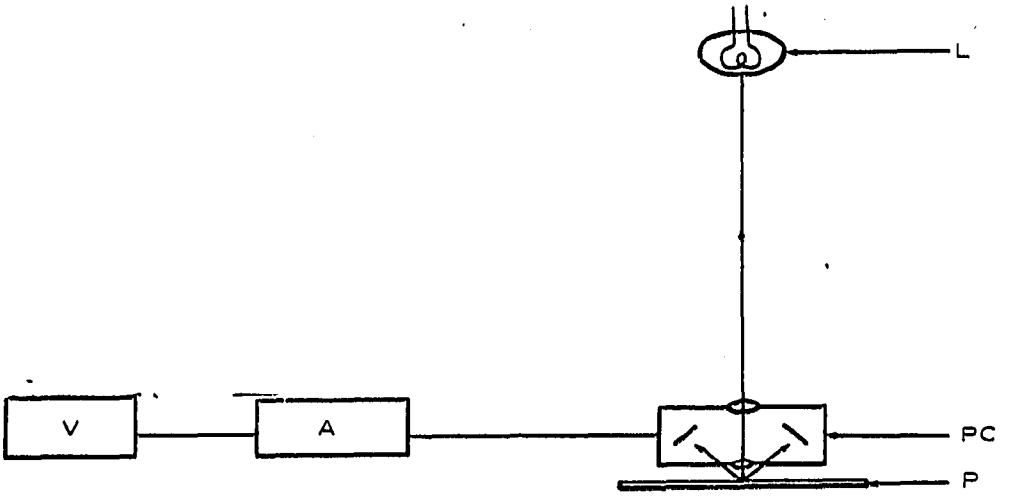

Fig. 1. Block diagram of scanner. A = amplifier; L = light source; P = plate; PC = photocell; V = voltmeter.

METHOD

Total lipids are extracted from freeze-dried brain with $CHCl_3$–MeOH (2:1, v/v) in the ratio of 20 ml of solvent to 1 g of the original wet tissue. An aliquot of the extract (equivalent to 150–200 μg of total lipid) is applied to the silicic acid as a narrow band (0.8 cm × 0.1 cm) under a stream of nitrogen, without prior concentration. Samples are run in duplicate on the same plate in parallel with standard lipids of varying concentration.

The prepared chromatoplates are run in small tanks, previously saturated with the solvents used by JATZKEWITZ[1], except that the volumes of solvents in the second mixture, *n*-PrOH–NH_4OH, are 39:11, which has been found to produce better separations.

After drying, the plates are sprayed with 50% sulphuric acid to which methyl orange has been added (5 mg %). The plates are then heated at 160° for 20 min when the lipids show up as carbonized spots on a white background. (Fig. 2).

The separated lipids are scanned directly on the plate by the reflectance scanner which measures the optical density at intervals of 0.3 mm along the length of the plate. The trace produced is shown in Fig. 3. A trace is also made along a blank strip of the sprayed, heated silicic acid.

The areas under the curves are measured with a planimeter and the mean value of 3 readings is taken. The area is found to be proportional to the weight of lipid in the sample (see Figs. 4–6). By comparison of the graphs of areas under the curves against weight of standard lipid applied with the area of a particular lipid in the sample, it is possible to calculate the amount of that lipid in the tissue.

Cholesterol is determined colorimetrically by a modified Liebermann-Burchard reaction on an aliquot of the original lipid extract.

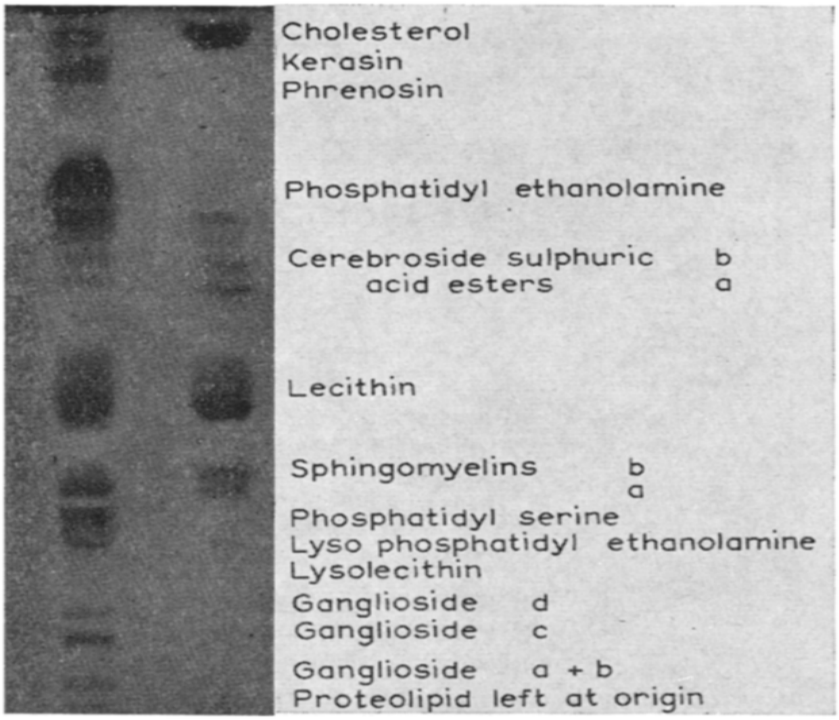

Fig. 2. Lipids in rat brain separated on a thin layer of silicic acid on a glass plate and run first in $CHCl_3$–MeOH–H_2O (14:6:1) to 15 cm and then in *n*-PrOH–NH_4OH (39:11) to 10 cm. Lipids in position A are those found in rat brain. Lipids in position B are "standards": the lecithin is ovolecithin, the cerebroside is synthetic glucocerebroside. The phosphatidyl ethanolamine shows up as two spots when the spot is not overloaded: the faster running is the plasmalogenic form of the phosphatide.

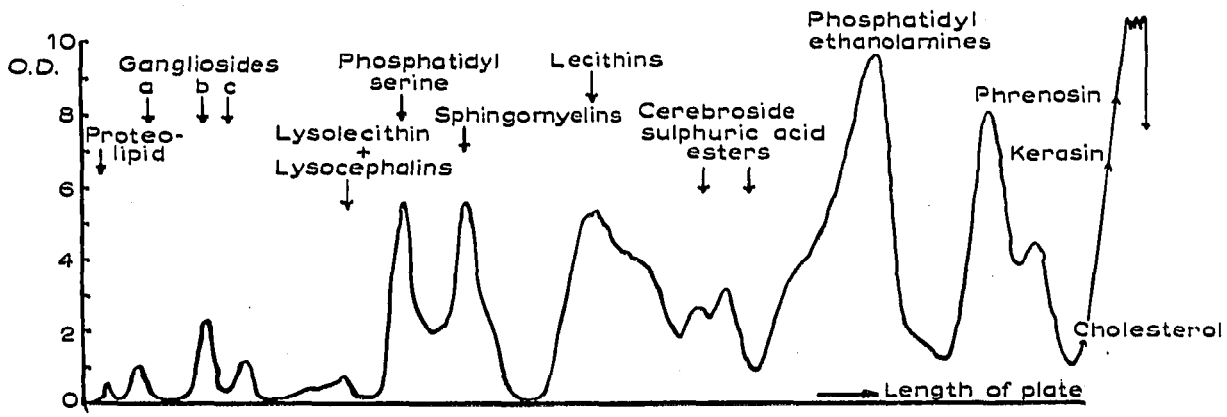

Fig. 3. Separation of lipids. 1.64 mg wet brain tissue ≡ 25 μl lipid extract of brain of stock rat 38 ≡ 151 μg lipid. Total length of plate = 20 cm.

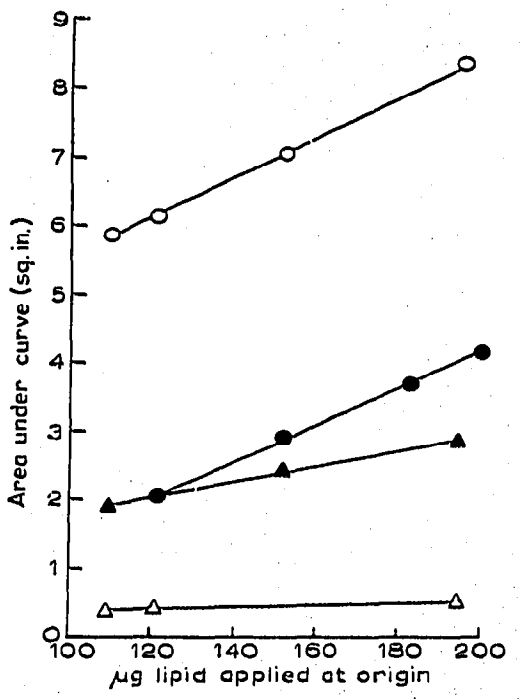

Fig. 4. Graphs showing relationship between area under the curve (sq. in.) and the quantities of different lipids found in varying amounts of total lipid. ● = sphingomyelins; ○ = cerebrosides; ▲ = cerebroside sulphuric acid esters; △ = gangliosides.

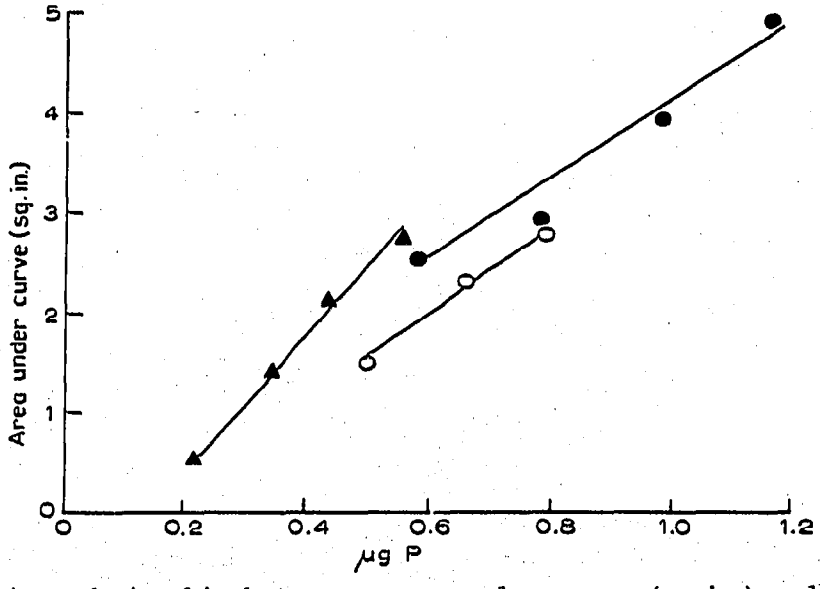

Fig. 5. Graphs showing relationship between areas under curves (sq. in.) and quantities of P of three phospholipids. ▲ = sphingomyelin; ● = phosphatidyl ethanolamine; ○ = ovolecithin.

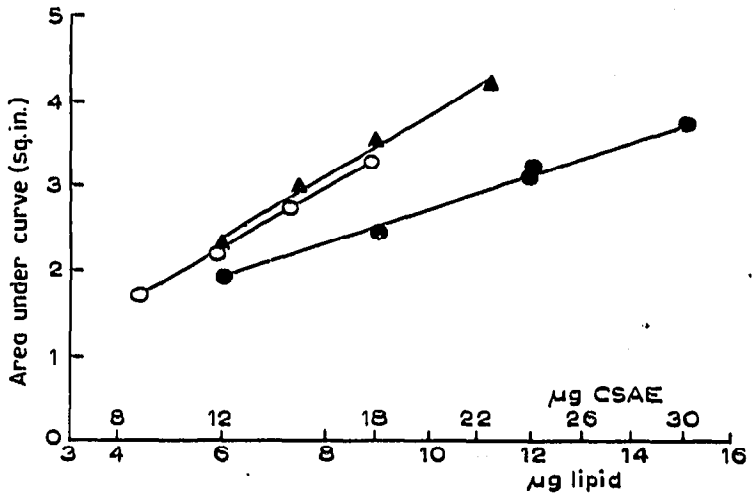

Fig. 6. Graphs showing relationship between area under the curve (sq. in.) and concentration of lipids. ○ = CSAE = cerebroside sulphuric acid esters; ● = sphingomyelins; △ = glucocerebrosides.

RESULTS AND DISCUSSION

Table I shows the reproducibility of the method. As would be expected, the standard errors of the smaller peaks are rather larger than could be desired, but that on the larger peaks is considered sufficiently small to make the method usable.

Methyl orange is added to the sulphuric acid spray so that even coverage of the plate is easier to obtain. Although this produces a slightly darker background due to carbonization of the methyl orange, the advantages greatly outweigh the disadvantages.

TABLE I

REPRODUCIBILITY OF THE METHOD

Lipid	*Amount of lipid applied (μg)*				
	194	*151*	*109**	*85*	*60*
	Area under curve as % of total area				
Ganglioside a + b	0.7	1.0	0.6 + 0.05	0.3	0.5
Ganglioside c	1.3	2.0	1.3 + 0.07	0.8	1.0
Ganglioside d	0.8	1.3	0.7 + 0.09	0.4	0.5
Lysocephalins and lysolecithin	0.8	1.3	1.4 + 0.02	1.4	1.6
Phosphatidyl serine	6.2	9.0	8.4 + 0.78	9.3	10.3
Sphingomyelin	9.0	6.3	7.3 + 0.34	4.1	3.8
Lecithin	19.4	18.9	18.9 + 0.41	18.3	18.9
Cerebroside sulphuric acid esters	7.5	6.6	6.0 + 0.41	4.8	4.8
Phosphatidyl ethanolamine	32.4	31.4	33.2 + 0.59	35.3	35.2
Degradation product of phosphatidyl ethanolamine	2.4	2.8	2.7 + 0.45	2.5	4.2
Cerebrosides	20.4	19.9	20.4 + 0.45	22.6	19.0
Total area under curve (sq. in.)	38.59	36.70	29.7 + 0.7	22.71	16.73
Wet weight of brain tissue (mg)	2.09	1.63	1.18	0.92	0.65

* All values given in this column are the means and standard errors of 5 separate determinations on 3 different days, on 3 different plates.

TABLE II

APPROXIMATE R_F VALUES FOR LIPIDS IN NERVOUS TISSUE RUN IN $CHCl_3$–MeOH–H_2O (14:6:1) TO 15 cm AND THEN IN *n*-PrOH–12.5% aq. NH_4OH (39:11) TO 10 cm

Lipid	R_F
Ganglioside a	0.022
Ganglioside b	0.027
Ganglioside c	0.09
Ganglioside d	0.13
Lysocephalin	0.22
Phosphatidyl serine	0.25
Lysolecithin	0.27
Sphingomyelin a	0.31
Sphingomyelin b	0.33
Lecithin	0.46
Cerebroside sulphuric acid esters a	0.61
Cerebroside sulphuric acid esters b	0.64
Phosphatidyl ethanolamine	0.67–0.80*
Phrenosin	0.93
Kerasin	0.96
Cholesterol	0.99
Free fatty acids	1.00

* The first figure is the R_F value measured from the end of the spot and the second figure is the R_F value measured from the front of the spot.

TABLE III

CONCENTRATION OF LIPIDS IN RAT BRAIN TISSUE AS DETERMINED BY THIN-LAYER CHROMATOGRAPHY (TLC) COMPARED WITH THE RESULTS OF OTHER AUTHORS

Lipid	*Results by TLC Lipid (mg) in total brain of 50 day old rat*	*Other authors' results Lipid (mg) in total brain*	*Reference*
Cerebrosides	19.3*	16.5 (40 day old rat) 14.6 (65 day old rat)	KOCH AND KOCH[8] KISHIMOTO AND RADIN[9]
Cerebroside sulphuric acid esters	11.0	7.2 (40 day old rat)	KOCH AND KOCH[8]
	Lipid P (moles/g) of wet brain tissue		
Sphingomyelin	9.1	8.1 (42-day-old rat) 11.0 (2-month-old rat)	BIETH, FREYSZ AND MANDEL[10] MANDEL AND BIETH[11]
Lecithin	32.2	28.2 (42-day-old rat)	BIETH, FREYSZ AND MANDEL[10]
Total choline phosphatides	41.3	29.4 (3–6 months old)	NIEMIRO AND PRZYJEMSKI[12]

* Using a synthetic glucocerebroside as standard.

Equal quantities of different lipids give varying areas under the densitometric curves (Figs. 5 and 6) so that although the figures given in Table I for the percentages of individual lipids present in the extract can be used for direct comparison of different tissues, they cannot be taken as an absolute indication of the amount of each component present. Therefore it is essential to have pure lipids for use as standards.

The R_F values of the lipid are not reproducible from day to day, but in relation to one another they are constant. Therefore it is advisable to run standards on each plate. The approximate R_F values of the lipids in the solvents used are given in Table II.

The two spots found for sphingomyelin and for the cerebroside sulphuric acid esters are probably due to the difference in fatty acid composition. The gangliosides have differing proportions of hexosamine to neuraminic acid in the molecule.

Not many figures are available in the literature for quantities of lipids in rat brains, especially for cerebrosides, since most of the figures for "cerebroside" given in the literature before 1955 include values for ganglioside as it was the sugar moiety of both which was estimated. Table III shows the results obtained by this method compared with those for rat brains reported in the literature.

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SUMMARY

Lipids from brain tissue have been estimated densitometrically after separation on thin-layer chromatograms.

Standard error on five determinations on three different days for lipids present in large amounts is 2 %, but on lipids present in smaller amounts the standard error is sometimes as much as 10 %.

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