# Analysis of Lipids from Fresh and Preserved Adult Human Brains

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Although values for the lipid content of human cerebral grey and white matter have been compiled by several authors (e.g. LeBaron & Folch, 1957; McIlwain, 1959; Deuel, 1951) much of this information has been obtained by older methods and only a few workers have reported lipid analyses on the same brain samples (Brante, 1949; Johnson, McNabb & Rossiter, 1949; Cumings, 1953, 1955). The purpose of this paper is to describe a simple technique for the complete analysis of the major brain lipids and to record the results obtained by this method for fresh and formalin-fixed human grey and white matter.

The present method is based on a preliminary separation of a washed protein-free chloroform–methanol extract of nervous tissue on an alumina column (Long & Staples, 1959, 1960, 1961; Rhodes & Lea, 1957; Davison & Wajda, 1959) with subsequent analysis of the original extract and each separated fraction by simple chemical procedures or by chromatography on paper. The method has been used for the analysis of samples of 50 mg. wet wt. or more of nervous tissue and also for the estimation of brain lipids labelled with radioactive isotopes.

## MATERIALS AND METHODS

The frontal areas of the cerebral hemispheres of adult patients dying without any neurological complications were removed at autopsy and dissected into white and grey matter. Samples were homogenized in $CHCl_3$–methanol (2:1, v/v), 19 ml. of solvent/g. of brain being used. The remaining brain was placed in neutralized formalin-0·9% NaCl solution (1:9, v/v). The moist $CHCl_3$–methanol extract was filtered, evaporated to dryness several times to decompose proteolipid A and B and much of proteolipid C (Folch & Lees, 1951) and freed from water-soluble substances by solvent partition with water (Folch, Lees & Sloane-Stanley, 1957).

### *Method for the separation and analysis of lipids*

Samples of 1–3 g. wet wt. of nervous tissue have been extracted with $CHCl_3$–methanol (2:1, v/v) and treated as described above. The washed lower phase, after solvent partition with water, was dried, and the lipid residue dissolved in 5 ml. of $CHCl_3$–methanol (98:2, v/v). The lipid extract was transferred to a column (20 cm. × 2 cm.) containing 50 g. of alumina (chromatography grade; British Drug Houses Ltd., Poole, Dorset) and then eluted successively with (1) 55 ml. of $CHCl_3$–methanol (98:2, v/v), (2) 600 ml. of $CHCl_3$–methanol (1:1, v/v), (3) 700 ml. of $CHCl_3$–methanol–water (7:7:1, by vol.) and (4) 500 ml. of ethanol–$CHCl_3$–water (5:2:2, by vol.). Suitable samples of the washed lipid extract and separated fractions were chromatographed on paper by the method of Marinetti, Erbland & Kochen (1957) and after mild alkaline hydrolysis by the method of Dawson (1954). Smaller samples may be separated on less alumina with proportionately smaller volumes of elution mixtures. Thus a sample of 50–100 mg. of nervous tissue was successfully fractionated on a 20 cm. × 1 cm. alumina column. The column was eluted successively with 8·6 ml. of $CHCl_3$–methanol (98:2, v/v), 93·2 ml. of $CHCl_3$–methanol (1:1, v/v), 109 ml. of $CHCl_3$–methanol–water (7:7:1, by vol.) and 78 ml. of ethanol–$CHCl_3$–water (5:2:2, by vol.).

### *Analytical methods*

The washed $CHCl_3$–methanol extract was analysed directly for serine and phosphorus. When sufficient extract was available ethanolamine, inositol, glycolipid, free and esterified cholesterol determinations were also performed on the unseparated extract. Each separated fraction from the column was then analysed as follows:

*Fraction* 1: *Free and ester cholesterol, triglyceride.* Cholesterol esters are not normally found in detectable amount in adult nervous tissue. However, they may be present in pathological specimens and cholesterol esters are found in nervous tissue during development (Adams & Davison, 1959). Triglycerides are present only in small quantities in normal tissue (Tables 1 and 2). Triglyceride in total lipid extracts of nervous tissue can be quantitatively recovered from the alumina column by elution with $CHCl_3$–methanol (98:2, v/v) (the first solvent mixture). Samples from this fraction were dried and neutral glyceride was determined as described by Adams & Davison (1959). Estimation of carboxylic acid ester by the method of Shapiro (1953) indicated that the neutral glyceride was triglyceride. Results are recorded as triolein.

*Fraction* 2: *Choline phospholipid.* Since alkali-stable phospholipid in total lipid extracts of brain include phospholipids other than sphingomyelin (Dawson, 1960; Ansell & Spanner, 1961), the sphingomyelin content can only be determined by analysis of alkali-stable phospholipid present in the choline-phospholipid fraction. This alkali-stable phospholipid gives one spot on chromatography with an $R_F$ similar to that of authentic sphingomyelin. Total phospholipid less sphingomyelin gives the lecithin present. The amount of phosphatidylcholine present as plasmalogen may be determined by estimation of combined fatty acid aldehyde. No attempt has been made to estimate lysolecithin possibly present in the brain (see Thompson, Niemiro & Webster, 1960).

*Fraction* 3: *Cerebrosides.* This fraction contains almost all the cerebroside present in the original lipid extract, together with traces of an unidentified non-choline phospholipid. Cerebroside can be conveniently determined by analysis for combined galactose.

*Fraction* 4: *Ethanolamine, inositol phosphatides and alkali-stable phospholipid* (*kephalin B*). Analysis for ethanolamine, inositol and plasmalogen on this fraction indicate the concentrations of the respective phosphatides and the amount of phosphatidylethanolamine plasmalogen which is present. This fraction may easily be resolved by paper chromatography and quantitative analysis achieved by phosphorus determination of each spot. Appreciable amounts of lysophosphatidylethanolamine have been detected in brain-lipid extracts, but it is not at present possible to conclude whether or not the lyso compound is formed by decomposition on the column or paper. Alkali-stable non-choline-containing phospholipid (kephalin B) is also present in this fraction. This is determined by hydrolysing the dried lipid sample with 2 ml. of N-KOH for 24 hr. at 37°. After neutralizing, 0·5 ml. of 50% (w/v) trichloroacetic acid is added, and after 2 hr. at 0° the mixture is filtered. The phosphorus content of the precipitate is determined.

*Combined inositol.* Suitable lipid samples were evaporated to dryness and hydrolysed in a sealed tube with 1 ml. of 6N-HCl for 24 hr. Inositol in the neutralized hydrolysate was estimated by a microbiological technique with the yeast *Kloeckera brevis* (Campling & Nixon, 1954).

*Combined aldehyde.* Aldehyde has been estimated by the method of Leupold & Buttner (1953). The mol.wt. of phosphatidylethanolamine plasmalogen has been arbitrarily taken as 800.

*Combined ethanolamine and serine.* Suitable volumes of dried and washed lipid extracts were dried and hydrolysed by refluxing for 3 hr. with 1 ml. of 6N-HCl in methanol. After HCl was removed by drying the sample ethanolamine and serine were determined by the fluorodinitrobenzene method of Axelrod, Reichenthal & Brodie (1953).

*Fraction* 5: *Phosphatidylserine.* Analysis of phosphorus recovered after further elution of the column with ether–ethanol–0·04N-KOH (10:7:5, by vol.; Long, Shapiro & Staples, 1960) does not give a reliable indication of the phosphatidylserine content of the lipid extract. In practice more reproducible results are obtained by serine analysis of the original washed extract. However, a rough assessment of phosphatidylserine content may be obtained by subtracting phospholipid recovered in fractions 2 and 4 from total phospholipid.

Other analytical methods have been described in earlier papers (Davison & Wajda, 1959; Adams & Davison, 1959).

Radioactivity from the brains of white Wistar rats, injected with radioactive phosphorus, was determined as described by Davison & Dobbing (1959).

## RESULTS

Quantitative analysis of tissue lipids separated by chromatography on silicic acid-treated paper has been described by Marinetti and his collaborators (Marinetti *et al.* 1957; Reed, Swisher, Marinetti & Eden, 1960). The method has been applied to separation of certain brain phosphatides labelled with relatively large amounts of $^{32}P$ by Hokin & Hokin (1958). Attempts at a complete separation of whole brain lipids on silicic acid-treated paper in this Laboratory have, however, been unsuccessful. For this reason it was decided to undertake a preliminary separation of cerebral lipids on an alumina column and to analyse each fraction by chemical methods in conjunction with separation on paper chromatography.

*Separation on alumina.* Davison & Wajda (1959) made an examination of a method for the separation of lipids on an alumina column. Recovery of cholesterol, cerebroside and choline-containing phosphatide was satisfactory but some of the non-choline phospholipid was retained on the column. In a preliminary investigation lipids from whole rat brain were separated on an alumina column (20 cm. × 2 cm.) into four fractions. Each fraction was analysed for phosphorus and individual lipids were identified by chromatography on paper treated with silicic acid. In addition, samples of the phosphorus-containing fractions were subjected to mild alkaline hydrolysis (Dawson, 1954), and the water-soluble products were separated and identified on paper chromatograms. In agreement with earlier findings it was shown that fraction 1 contained cholesterol, fraction 2 sphingomyelin and lecithin, and fraction 3 cerebrosides together with traces of an unidentified phospholipid.

Chromatography of a sample of the fourth fraction on silicic acid-impregnated paper indicated that at least three lipids were present: phosphatidylethanolamine, inositol phosphatide and lysophosphatidylethanolamine. However, these chromatograms are run in an acid solvent, and lysophosphatidylethanolamine detected in the original chloroform–methanol extract and in the fourth fraction from the alumina column may well be due to decomposition of some of the acid-labile ethanolamine plasmalogen. Chromatography of the products of mild alkaline hydrolysis of the fourth fraction showed that glycerylphosphorylethanolamine and glycerylphosphorylinositol were present.

Only traces of phosphatidylserine, present in whole lipid extracts of brain, are eluted from the column by the fractionation procedure outlined above. It is therefore probable that retention of this lipid on the alumina column accounts for the incomplete recovery of phospholipid reported earlier. This conclusion was checked by fractionating a mixture of ethanolamine and serine phosphatides on an alumina column. Only 30% of phospholipid equivalent to the phosphatidylethanolamine content was recovered.

These observations confirm those of Long *et al.* (1960), who, using a similar system, demonstrated the retention of serine phospholipids on an alumina column. It therefore appears that the most reliable

Table 1. *Lipid composition of adult human fresh and preserved white matter*

Samples of fresh and preserved cerebral white matter from at least six different adult brains have been analysed for their lipid content. Methods for the analysis of the protein-free chloroform–methanol extract both before and after separation are given in the text. Preserved samples were fixed in formalin–0·9 % sodium chloride solution (1:9, v/v) for at least 1 week. Recorded figures are the mean values ± S.E.M. with the number of samples given in parentheses. Results underlined represent the analytical figures taken to represent the true lipid values as determined by present methods. Correction factors determined for average molecular weights were used as follows: triglyceride is expressed as triolein (glycerol × 9·6), phospholipid as lipid phosphorus × 25, cerebroside as lipid galactose × 4·55, plasmalogen as 4·55 × dodecylaldehyde, phosphatidylinositol as inositol × 5·3, phosphatidylethanolamine as ethanolamine × 13·1 and phosphatidylserine as serine × 7·6.

| | Lipid content (mg./100 g. wet wt. of brain) | | | | |
|---|---|---|---|---|---|
| | | | After formalin fixation | | |
| | Original extract | Lipid recovered from column | Original extract | Lipid recovered from column | Results for fresh brain (previous authors) |
| Total lipid | — | — | — | — | 16000–22000* |
| Cholesterol | 3633 ± 235 (6) | 3850 ± 195 (6) | 3590 ± 234 (3) | 3710 ± 160 (4) | 3600– 5400* |
| Triglyceride | — | 114, 64, 130 | — | — | — |
| Cerebrosides | 3825 ± 358 (4) | 3980 ± 492 (4) | 4312 ± 471 (6) | 4847 ± 585 (6) | 4100– 7400* |
| Total phospholipid | 7533 ± 346 (7) | 6735 (7) | 6824 ± 436 (7) | 5369 (6) | 6200– 9300* |
| Phosphatidylcholine | — | 1832 ± 129 (6) | — | 1639 ± 77 (5) | 900– 1900* |
| Sphingomyelin | — | 1306 ± 115 (6) | — | 1388 ± 177 (5) | 1080 ± 380† |
| Total alkali-stable phospholipid | 1896 ± 137 (6) | 1881 (6) | 2105 ± 185 (4) | 2007 (4) | 1800–4300 * |
| Alkali-stable kephalin (kephalin B) | — | 575 ± 36 (5) | — | 619 ± 78 (4) | 875 ± 350‡ |
| Phosphatidylethanolamine | 3326 ± 280 (3) | 3135 ± 258 (4) | 1244 ± 216 (4) | 466 ± 40 (4) | 1500§ |
| Total plasmalogen | 3387 ± 360 (4) | — | 203 ± 37 (4) | — | 2500–3260‖ |
| Phosphatidylethanolamine plasmalogen | — | 3203 ± 686 (3) | — | 48 ± 10 (4) | — |
| Phosphatidylserine | 1550 ± 76 (3) | 380 ± 112 (3) | 792 ± 92 (4) | — | 1400* |
| Phosphatidylinositol | 233, 213 | 220 | 154, 310 | — | — |
| Water content (%) | 69·6 ± 0·75 (7) | — | 70·34 ± 1·2 (5) | — | 67–74* |

* LeBaron & Folch (1957). † Ansell & Spanner (1961). ‡ Brante (1949). § McIlwain (1959). ‖ Webster (1960).

estimation of phosphatidylserine can be achieved by serine analysis of the original protein-free extract, a procedure generally adopted in this paper. Alternatively, phosphatidylserine may well be separated on a second alumina column as recommended by Long *et al.* (1960).

### *Analysis of human brain lipids and their recovery after chromatography*

Results for the lipid analysis of human cerebral white and grey matter are shown in Tables 1 and 2. Chloroform–methanol (2:1, v/v) extracts of nervous tissue were prepared by using fresh material obtained at autopsy. Samples of the same specimen were placed in neutralized formalin–0·9 % sodium chloride solution and kept for at least a week before the lipid extract was prepared. The first columns of Tables 1 and 2 indicate the result of analysis of the whole washed protein-free chloroform–methanol extract of brain, before separation. In the second columns results obtained after separation on alumina are tabulated. There is reasonable agreement between both sets of figures with the exception of recovery of phosphatidylserine from the column. In order to provide suitable sets of figures for reference purposes, results taken to be the actual lipid content of brain determined by the present method are underlined. These results may be compared with those obtained by other workers for fresh human white and grey matter as shown in Tables 1 and 2. In the same Tables, results for analysis of formalin-fixed brain have been treated in the same way. Figures for the analysis of formalin-fixed brain by previous authors are, however, not shown, for different workers have analysed brains fixed for various periods and there is evidence to

Table 2. *Lipid composition of adult human fresh and preserved grey matter*

Samples of fresh and preserved cerebral grey matter have been tested as described in Table 1.

| | Lipid content (mg./100 g. wet wt. of brain) | | | | |
|---|---|---|---|---|---|
| | | | After formalin fixation | | |
| | Original extract | Lipid recovered from column | Original extract | Recovered from column | Results of previous authors for fresh material (see Table 1) |
| Total lipid | — | — | — | — | 5000–6200* |
| Cholesterol | 916 ± 74 (3) | 803 ± 76 (3) | 896 ± 31 (4) | 901 ± 51 (5) | 600–1400* |
| Triglyceride | — | 23, 41 | — | 82, 81·5 | — |
| Cerebrosides | 313 ± 21 (3) | 320 ± 21 (3) | 590 ± 80 (5) | 514 ± 44 (5) | 300–1900* |
| Total phospholipid | 3347 ± 60 (5) | 3396 (4) | 2905 ± 92 (4)<br>1855¶ (1)* | 2294 (4)<br>1496¶ (1) | 3100–4600* |
| Phosphatidylcholine | — | 1290 ± 32 (3) | — | 1183 ± 33 (4)<br>768¶ (1) | 600–1500* |
| Sphingomyelin | — | 294 ± 31 (3) | — | 326 ± 14 (4)<br>192¶ (1) | 270 ± 173† |
| Total alkali-stable phospholipid | 515 ± 46 (3) | 523 (3) | 780 ± 74 (3)<br>368¶ | 584 (3) | 300–900* |
| Alkali-stable kephalin (kephalin B) | — | 229 ± 50 (3) | — | 181 ± 22 (4)<br>83¶ | 300 ± 160‡ |
| Phosphatidylethanolamine | 1823, 1384 | 778, 984 | 253, 310, 245 | 92, 83, 77 | 1300§ |
| Total plasmalogen | 969, 582 | — | 185, 135 | — | 577–736 |
| Phosphatidylethanolamine plasmalogen | — | 781, 473 | — | 30, 13 | — |
| Phosphatidylserine | 528 ± 97 (5) | — | 234, 155 | — | 600* |
| Phosphatidylinositol | 154 | 160 | 115 | — | — |
| Water content (%) | 84·15 ± 0·14 (4) | — | 84·54 ± 0·72 (5) | — | 81–87* |

¶ Sample kept more than 1 year in formalin–0·9% sodium chloride (1:9).

show that results for brain phospholipid content vary with the period of fixation (Brante, 1949). Since cholesterol ester was not detected in any samples analysed, no results for this lipid have been recorded.

## DISCUSSION

The direct analysis of such complex mixtures of lipids as are present in nervous tissue presents many problems. However, an initial separation of a chloroform–methanol extract of brain into four lipid fractions by chromatography on alumina greatly simplifies the difficulties of analysis. First, it is possible to determine sphingomyelin in the choline-phospholipid fraction by analysis of alkali-stable phospholipid, lecithin content being equal to the alkali-labile phospholipid present. Secondly, the quantity of each phosphatide present as plasmalogen can be estimated by analysis of each fraction for combined aldehyde. It is also possible to determine triglyceride and alkali-stable phospholipid (kephalin B) by simple procedures. Besides these advantages, the use of a preliminary fractionation procedure enables analysis to be performed on smaller quantities of tissue than would be required if each estimation were made on the original extract.

In practice chromatography on alumina is a simple and relatively rapid procedure suitable for use in a laboratory where it is necessary to analyse samples of at least 50 mg. of nervous tissue. Recovery of all lipids is satisfactory (Tables 1 and 2) with the exception of phosphatidylserine, but this lipid may be estimated by analysis of the original dried and washed lipid extract. More detailed analysis of each fraction from the alumina column is possible either by further separation on paper chromatograms or by applying techniques recommended by other workers (e.g. Dawson, 1960).

*Lipids of the normal unfixed human brain.* The results recorded in Tables 1 and 2 are restricted to analysis of the lipids of human cerebral white and

grey matter. In general the present findings fall within the range reported by previous authors. Cholesterol ester has not been detected in the lipid of any samples analysed, confirming a previous observation (Adams & Davison, 1959; Adams, 1960). Figures for the sphingomyelin content of white and grey matter are, however, lower than has hitherto been recorded, since most earlier workers have included alkali-stable phospholipids other than sphingomyelin in their analyses. Similar observations have been recorded recently by Ansell & Spanner (1961) and their results for human grey and white matter sphingomyelin are shown in Tables 1 and 2. Phosphatidylethanolamine of white matter exists largely as its plasmalogen, a finding in conformity with a report by Webster (1960), whereas only small amounts of other phosphatides are found in this form. The present analyses show that little triglyceride is present in normal brain (cf. McIlwain, 1959; 600 mg./100 g. wet wt. of whole brain).

*Lipids of the normal formalin-fixed human brain.* In agreement with earlier investigators it has been shown that after fixation in formalin there is no loss of cholesterol or cerebroside (Brante, 1949; Cumings, 1953, 1955; Rodnight, 1957) from the brain. Even after short periods of fixation, however, there is a decrease in non-choline-containing phospholipid (Brante, 1949). Brante suggested that the initial loss of phospholipid on formalin fixation was due to decomposition of one of the kephalins, possibly phosphatidylserine. This loss has now been shown to be mainly the result of the decomposition of phosphatidylethanolamine plasmalogen and possibly, but to a lesser extent, to the loss of phosphatidylserine. Loss of phospholipid increases with more prolonged formalin fixation.

## SUMMARY

1. A method for the separation of lipids by chromatography on alumina has been investigated and used to simplify the problems of analysis of brain lipids.

2. A procedure suitable for the routine lipid analysis of small samples of brain has been described.

3. The lipid composition of a number of samples of human white and grey matter, both fresh and formalin-fixed, has been determined.

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## REFERENCES

Adams, C. W. M. (1960). *J. Histochem. Cytochem.* **8**, 262.
Adams, C. W. M. & Davison, A. N. (1959). *J. Neurochem.* **4**, 282.
Ansell, G. B. & Spanner, S. (1961). *Biochem. J.* **79**, 176.
Axelrod, J., Reichenthal, J. & Brodie, B. B. (1953). *J. biol. Chem.* **204**, 903.
Brante, G. (1949). *Acta physiol. scand.* **18**, suppl. 63.
Campling, J. D. & Nixon, D. A. (1954). *J. Physiol.* **126**, 71.
Cumings, J. N. (1953). *Brain*, **76**, 551.
Cumings, J. N. (1955). *Brain*, **78**, 554.
Davison, A. N. & Dobbing, J. (1959). *Biochem. J.* **73**, 701.
Davison, A. N. & Wajda, M. (1959). *J. Neurochem.* **4**, 353.
Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
Deuel, H. J. (1951). *The Lipids.* New York: Interscience Publishers Inc.
Folch, J. & Lees, M. (1951). *J. biol. Chem.* **191**, 807.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Hokin, L. E. & Hokin, M. R. (1958). *J. biol. Chem.* **233**, 800.
Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1949). *Biochem. J.* **44**, 494.
LeBaron, F. N. & Folch, J. (1957). *Physiol. Rev.* **37**, 539.
Leupold, F. & Buttner, H. (1953). *Hoppe-Seyl. Z.* **292**, 13.
Long, C., Shapiro, B. & Staples, D. A. (1960). *Biochem. J.* **75**, 17 P.
Long, C. & Staples, D. A. (1959). *Biochem. J.* **73**, 7 P.
Long, C. & Staples, D. A. (1960). *Biochem. J.* **75**, 16 P.
Long, C. & Staples, D. A. (1961). *Biochem. J.* **78**, 179.
McIlwain, H. (1959). *Biochemistry and the Central Nervous System*, 2nd ed. London: J. and A. Churchill Ltd.
Marinetti, G. V., Erbland, J. & Kochen, J. (1957). *Fed. Proc.* **16**, 837.
Reed, C. F., Swisher, S. N., Marinetti, G. V. & Eden, E. G. (1960). *J. Lab. clin. Med.* **56**, 281.
Rhodes, D. N. & Lea, C. H. (1957). *Biochem. J.* **65**, 526.
Rodnight, R. (1957). *J. Neurochem.* **1**, 207.
Shapiro, B. (1953). *Biochem. J.* **53**, 663.
Thompson, R. H. S., Niemiro, R. & Webster, G. R. (1960). *Biochim. biophys. Acta*, **43**, 142.
Webster, G. R. (1960). *Biochim. biophys. Acta*, **44**, 109.