Biochem. J. (1961) **79**, 159

Distribution of Fatty Acids in Lipids of Rat Brain, Brain Mitochondria and Microsomes

By L. A. BIRAN
Department of Biochemistry, University of Oxford

and W. BARTLEY
Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(*Received* 24 *August* 1960)

The importance of lipids as structural elements of particulate enzyme systems is being increasingly realized (e.g. Green, 1959; Hokin & Hokin, 1959). The extent to which the lipids themselves participate in the metabolic function of the structures in which they are found and the specificity of the lipids for particular structures and functions are uncertain. The present study represents part of a search for lipids of specific structure in subcellular fractions of different organs. The underlying assumption is that the occurrence of a lipid with a particular structure in the corresponding cell organelle (or fraction thereof) of organs with different total lipid composition may give an indication of the metabolic function of the lipid.

Experimental data on the detailed composition of lipids in different tissues are scarce, and on the fatty acid distribution within lipids almost non-existent. In view of the variety of tissues used for research on subcellular organelles, and of the probable metabolic importance of lipids, a more detailed knowledge of tissue lipids is desirable.

The present study provides some new data for rat brain, brain mitochondria and microsomes. The study is based on partition of total lipid extracts by silicic acid chromatography and gas–liquid-chromatographic analysis of the fatty acid content of separated phospholipids.

EXPERIMENTAL

Materials

Rats. These were of the Wistar strain, approx. 300 g. in weight, fed on a standard diet of rat cubes supplied by Oxo Ltd. (London).

Chemicals. All solvents were of analytical grade, except ethanol, which was 'azeotropic'. Palmital dimethylacetal was kindly supplied by Dr Marjorie G. Macfarlane and Dr G. M. Gray.

Diazomethane was prepared from *N*-methyl-*N*-nitroso-toluene-*p*-sulphonamide (British Drug Houses Ltd., Poole, England). Choline chloride was recrystallized 'Laboratory reagent' (British Drug Houses Ltd.).

Analytical methods

Dry weights. Dry weights of tissue fractions were determined as 'acid-insoluble dry matter' (Werkheiser & Bartley, 1957). Dry weights of lipid extracts were determined after evaporation of the extracting solvent *in vacuo* at room temperature.

Phosphorus. This was determined by the method of Berenblum & Chain (1938), as modified by Bartley (1953). Wet ashing was by the method of Hanes & Isherwood (1949) as modified by Werkheiser & Bartley (1957).

Determination of acyl ester. The method used was a modification of that of Rapport & Alonzo (1955), giving linear standard curves with up to 4 μmoles of ester, but with variable colour yields.

The concentrations of NaOH and hydroxylamine were increased to 5%, w/v (2 g. of reagent dissolved in 2 ml. of water and diluted with 38 ml. of ethanol). Esters were dissolved in 3 ml. of diethyl ether and treated with 0·1 ml. of the alkaline hydroxylamine. After addition of ether and alkaline hydroxylamine all the samples and standards of a batch were evaporated to dryness together in a water bath at 65°. They were left in the water bath for 5–7 min. and then cooled. Butanolic ferric perchlorate was added from a burette and the colours were read after 30–40 min.

Determination of nitrogen. Amino nitrogen was determined by the method of Cocking & Yemm (1954). Total nitrogen was determined by direct nesslerization after Kjeldahl digestion.

Determination of choline. Samples were hydrolysed under the conditions of Wheeldon & Collins (1958). The acid hydrolysate was extracted with light petroleum (b.p. 40–60°) to remove fatty acids and evaporated to dryness at 90° under a stream of N_2 to remove HCl. The residue was resuspended in 1–2 ml. of water and centrifuged. A portion of the clear supernatant, containing 0·02–0·6 μmole of choline, was used in the estimation by the following modification of the method of Appleton, La Du, Levy, Steele & Brodie (1953).

The aqueous choline solution (0·5 ml.) in a thick-walled glass centrifuge tube (5 cm. long, 5 ml. volume) was treated at 0° with one drop of a 5% Teepol solution and 0·5 ml. of the KI_3 reagent. After being stirred with a glass fibre the solution was left in ice for 30 min.

The tubes were capped and centrifuged at 0° (25 000 *g*, 45 min.). The supernatant was decanted and the tubes were inserted upside down into close-fitting celluloid

centrifuge tubes, the upper parts of which had been cut off. They were centrifuged in the upside-down position at 0° (200 *g*, 20 min.). The open ends of the tubes were wiped with filter paper to remove the last traces of KI_3 and the precipitates of choline iodide (choline I_9) were dissolved in redistilled ethylene dichloride. Extinctions were measured against ethylene dichloride at 365 mμ after about ½ hr. at room temperature in glass cells with a 2 mm. light-path. Choline chloride was used as standard.

The standard curve was linear in the region tested (0·02–0·8 μmole of choline chloride). It passed through the origin, indicating a complete removal of KI_3 from the precipitate. The colour densities of the ethylene dichloride solutions of choline I_9 were somewhat variable from one estimation to another. A set of standards was therefore included in each batch of samples.

Aldehydes and cholesterol. Aldehydes were measured by the method of Gray & Macfarlane (1958) and cholesterol (total) was determined by the method of Zlatkis, Zak & Boyle (1953).

Treatment of tissue

Rats (300 g. wt., Wistar strain) were killed by decapitation. The brains were immediately removed and minced in a stainless-steel Fisher mincer (Jouan, Paris). Approximately 1 g. of the mince was taken for direct saponification of the tissue lipids with ethanolic KOH (see below: 'Preparation of methyl esters').

The mince (12 g.) was homogenized in 4 vol. of sucrose (0·25 M) in a stainless-steel Potter–Elvehjem homogenizer (vol. 40 ml.) with the pestle revolving at 2000 rev./min. The homogenizer was moved up and down four times.

A volume of the homogenate containing 5 g. (wet wt.) of brain was removed for extraction of lipids. A further 2 vol. of sucrose (0·25 M) was added to the remaining homogenate. This was fractionated according to the scheme in Fig. 1, suggested by Dr D. E. Bellamy and similar to his published procedure (Bellamy, 1959).

Extraction of lipids

The mitochondrial and microsomal pellets and whole homogenate were separately homogenized in a MSE homogenizer with 10 vol. of $CHCl_3$–methanol (2:1, v/v). After 2 hr. at room temperature the material was filtered through a fat-free filter paper.

The filter paper containing the residue was re-homogenized with a further 10 vol. of $CHCl_3$–methanol (2:1, v/v), left for 2 hr. and the solid material filtered off and discarded. To the combined extracts was added one-fifth of their volume of NaCl (0·29 M). After shaking, the emulsion was left overnight at room temperature to separate the two phases. The upper (aqueous) phase was removed and the walls of the vessel and surface of the lower phase were washed twice with 'upper-phase liquid' [aq. 0·58% NaCl–methanol–$CHCl_3$ (47:48:3, by vol.); cf. Folch, Lees & Sloane-Stanley, 1957].

Insoluble 'lipoprotein' material at the interface (Folch, Ascoli, Lees, Meath & Le Baron, 1951) was removed and treated separately (see 'Treatment of lipoprotein').

Methanol was added to the washed lower phase until one phase resulted and the solution was evaporated to dryness at 45° in a stream of N_2. The residue was extracted with 2% methanol in $CHCl_3$ and the extract containing 'total lipids' was filtered and stored at −15°.

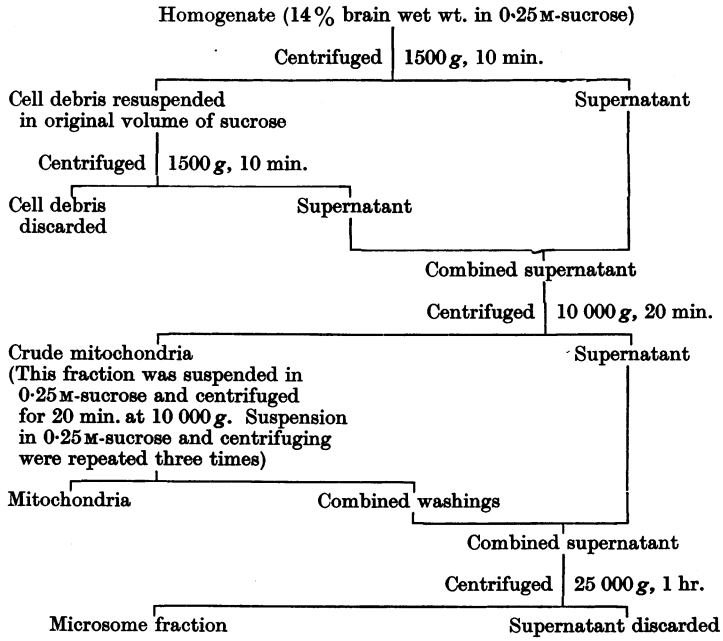

Fig. 1. Procedure for isolation of rat-brain mitochondrial and microsomal fractions. All operations were carried out at 0–2° immediately after the death of the animal.

Table 1. *Eluting system employed for silicic acid-column chromatography of rat-brain lipids* (100–200 *mg.*)

Fraction no.	Composition of eluent	Vol. of eluent (ml.)	Expected composition of fraction
1	1% of diethyl ether in light petroleum	350	Steroid esters
2	10% of diethyl ether in light petroleum	500	Triglycerides, free cholesterol
3	Diethyl ether	300	Monoglycerides, diglycerides
4	Chloroform–methanol (7:1, v/v)	100	Polyglycerophosphatide etc. (unknown)
5	Chloroform–methanol (4:1, v/v)	200	Ethanolamine phosphatides, serine phosphatides
6	Chloroform–methanol (3:2, v/v)	100	Phosphoinositide
7	Chloroform–methanol (3:2, v/v)	400	Lecithin
8	Chloroform–methanol (1:4, v/v)	200	Sphingolipids
9	Methanol	400	Unknown lipids

Treatment of lipoprotein

The residues in the flask and on the filter from 'total lipid' extraction were combined and extracted with $CHCl_3$–methanol (1:1, v/v) (Folch *et al.* 1951). To this extract was added 'lipoprotein' from the interface of total lipid washing (above); the suspension was evaporated to dryness under N_2 and the residue was saponified.

Analysis of total lipid extracts

Extracts of mitochondria, microsomes and homogenate were analysed for lipid dry weight, total phosphorus and total acyl glyceryl ester of the original lipid. Extracts of mitochondria and microsomes were also analysed for total nitrogen, amino nitrogen, choline, aldehyde and total cholesterol. A portion of mitochondrial and microsomal extracts was taken for direct saponification and fatty acid analysis.

Separation of total lipids into lipid classes

For more detailed analysis total lipids were chromatographed on a silicic acid (100 mesh, Mallinckrodt Chemical Works, N.Y., U.S.A.) column prepared as described by Hirsch & Ahrens (1958). A system of stepwise elution based on systems of Hirsch & Ahrens (1958) and Hanahan, Dittmer & Warashina (1957) was used.

Lipids (100–200 mg.) were introduced on the column in light petroleum (b.p. 60–80°). The eluting solvents and expected composition of eluates are shown in Table 1. Eluted fractions were evaporated to dryness under a stream of N_2 and redissolved in $CHCl_3$.

Analysis of fractions from silicic acid columns

Paper chromatography. Phospholipid fractions were chromatographed at 0° on silicic acid-impregnated paper with di*iso*butyl ketone–acetic acid–water (40:20:3, by vol.) (Marinetti, Erbland & Kochen, 1957). Another solvent described by Marinetti *et al.* (1957), di*iso*butyl ketone–*n*-butyl ether–acetic acid–water (20:20:20:3, by vol.) gave poor separation.

Non-phosphatides were chromatographed at 23° on silicic acid-impregnated paper with *n*-heptane–di*iso*butyl ketone (96:6, v/v) (Marinetti, Erbland & Stotz, 1958).

The following reagents were used on a single chromatogram in the order shown: Rhodamine-6 G (fluorescent lipid spots, Marinetti *et al.* 1957); dipicrylamine for choline (Beiss & Armbuster, 1958); acid ammonium molybdate for phosphorus (Hanes & Isherwood, 1949). Colour was developed by heating and u.v.-irradiation.

A duplicate chromatogram was sprayed with ninhydrin to detect lipids containing amino groups.

R_F values of identified lipids were in the order given by Marinetti *et al.* (1958).

Chemical estimations. All fractions were analysed for acyl glyceryl ester, total phosphorus and amino nitrogen. Phospholipid fractions and mitochondrial Fraction 2 (Table 1) were analysed for aldehyde. Choline, cholesterol and total nitrogen were determined in those fractions where their presence was indicated by paper chromatography.

Preparation of methyl esters for gas–liquid chromatography

The fatty acids of all lipid fractions were analysed by gas–liquid chromatography of their methyl esters, which were prepared by one of two methods.

Diazomethane method. Lipids were saponified for 3 days at 30° with 2N-KOH in aq. 50% (v/v) ethanol containing 0·1% (w/v) of quinol as antioxidant. The unsaponifiable lipids were removed by shaking with light petroleum (b.p. 40–60°) and fatty acids were extracted into light petroleum after acidification of the mixture. Fatty acid extracts were washed with water, dried over Na_2SO_4 and esterified with diazomethane. This procedure was applied to the lipids of whole-brain mince, total lipid extracts of microsomes and mitochondria and proteolipids from whole-homogenate mitochondria and microsomes. Silicic acid fractions of homogenate were treated similarly, but extraction of unsaponifiable material was omitted, since the methyl esters were purified by sublimation (see below).

Interesterification with methanolic hydrochloric acid. This procedure, described by Stoffel, Chu & Ahrens (1959), was applied to silicic acid fractions of mitochondria and microsomes. All methyl esters were further purified by sublimation (Stoffel *et al.* 1959).

Gas–liquid chromatography

Treatment of sample. The instruments used were a chromatograph fitted with an ionization detector (Lovelock, 1958), built by Dr G. S. Getz and Mr A. Renshaw in

this laboratory, and an Argon Chromatograph (Pye Co. Ltd., Cambridge, England). Columns were 4 ft. long and 4 mm. in diameter. The solid supports used were Celite 545 (mesh 100–120) or Embacel (May and Baker Ltd., Dagenham, Essex). Apiezon L high-vacuum grease (20%) was used as non-polar stationary phase and polyethylene glycol adipate or polyethylene glycol adipate cross-linked with pentaerythritol (Cambridge Industries Ltd., Cambridge, Mass., U.S.A.; stationary phase LAC-2-R-446; 10 or 20%) were used as polar stationary phases. Column temperatures were between 175° and 195° and did not vary significantly during analysis.

Samples (0·05–0·1 μl.) of dry methyl esters were introduced by means of a Pye micropipette. Smaller samples were introduced with the ground end of a glass rod. For introducing the sample the flow of argon through the column was stopped and the pressure at the top of the column was allowed to drop to atmospheric. The pipette was introduced through a stopcock at the top of the column. Immediately after discharge of the sample on the stationary phase the pipette was withdrawn, the stopcock closed and gas flow resumed.

Calculation of proportions of methyl esters. Areas of peaks, on a recording of detector potential against time, were approximately proportional to mass of component eluted in the peak. The areas were measured by drawing the base line, and the tangents to the peak at the point of inflexion (Fig. 2, AB, BC), and measuring the area of triangle ABC (Keulemans, 1957).

Distances from the intersection of tangents B to peak *X* (due to resumption of gas flow through detector) were measured.

Retention volumes were expressed relative to stearate.

Identification of peaks. Fatty esters were identified by the following methods. (1) Comparison of their relative retention volumes with those of authentic samples of easily available esters and with data of James & Martin (1956) and Insull & Ahrens (1959) for stationary phases of similar polarity. (2) Hydrogenation and bromination (James & Martin, 1956) of unsaturated esters and gas chromatography of the products.

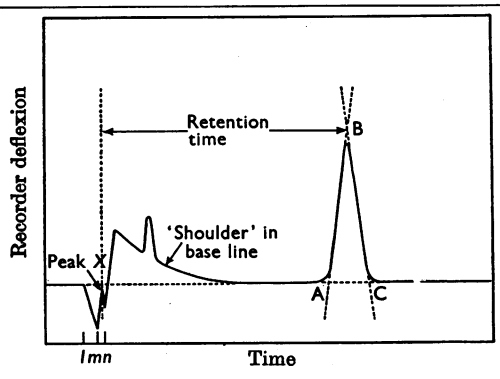

Fig. 2. Quantitative interpretation of gas chromatograms. Area ABC is proportional to mass of methyl ester eluted in the peak. During introduction of sample flow of argon is discontinued at *l* and resumed at *m*. Peak *X*, from which retention times were measured, corresponds to the beginning of negative deflexion *n* due to passage through the detector of air introduced together with the sample.

EVALUATION OF METHODS

Precautions against oxidation of lipids. Exposure of dry lipid material to atmospheric O_2 was kept to a minimum. Lipid was never left for long periods without organic solvent. It was stored at −15° dissolved in solvent. Solvent was removed by evaporation at 45–50° under a stream of dry O_2-free N_2. The experiment described below indicates the adequacy of these precautions.

An extract of sheep-heart lipids containing large proportions of polyunsaturated fatty acids was evaporated to dryness under O_2. It was saponified with 2N-KOH in aq. 50% ethanol without antioxidant in an atmosphere of O_2. The fatty acids were isolated and methylated with diazomethane. The solution was evaporated to dryness and the methyl esters were heated for 30 min. at 70° in a stream of O_2. Another portion of the same extract was saponified and methylated with diazomethane as described above ('Preparation of methyl esters'). Chromatograms of fatty acid esters obtained by both methods showed no appreciable differences.

The experiment indicates that fatty acids detected by gas chromatography under the conditions described in the Experimental section are not significantly oxidized during isolation and methylation.

Lipid-extraction procedure. Comparison of recoveries of methyl esters after extraction of brain mince with $CHCl_3$–methanol and after direct saponification indicated complete extraction of (fatty) acyl glyceryl esters. The methods of analysis used were unsuitable for the detection of cerebrosides or small amounts of sphingomyelin.

Silicic acid fractionation. Recoveries of various functional groups after fractionation are shown in Table 2. The figures may be in error by ±10% since minimum

Table 2. *Recovery of cholesterol, total phosphorus, total nitrogen and functional groups from silicic acid fractionation*

$$\text{Percentage recovery} = \frac{100 \times \Sigma \text{ moles of component in fractions analysed}}{\text{moles of component in total-lipid extract of same tissue fraction}}.$$

Tissue fraction	Percentage recovery: Total phosphorus	Acyl ester	Total nitrogen	Amino nitrogen	Choline	Aldehyde	Cholesterol
Homogenate	103	108	*	*	*	*	*
Mitochondria	96	123	86	111	81	93	17
Microsomes	93	93	93	105	97	63†	14

* No analyses were made for these on total-homogenate extract.

† The apparent low recovery is probably an artifact of aldehyde estimation (see Evaluation of Methods section).

Table 3. *Elution pattern of brain lipids*

Mitochondrial Fraction 3 was lost. Fraction 9 (100% methanol) was collected only from homogenate lipids. Serine–ethanolamine–inositol phosphatide fraction of homogenate also contained 'polyglycerophosphatide'. For composition of eluents used for separating the Fractions and their expected lipid contents see Table 1.

Lipid class	Silicic acid fractions in which lipid classes were isolated: Homogenate	Mitochondria	Microsomes
Non-phospholipids (mainly 'neutral' lipids, fatty acids)	1	1	1
	2	2	2
	3	.	3
Polyglycerophosphatide	Not isolated	4	4
Serine phosphatides	4	5	5
Ethanolamine phosphatides	5	6	.
Inositol phosphatides	6	.	.
Lecithin	.	.	6
	7	7	7
Polar lipids (unidentified)	8	8	8
	9	.	.

Table 4. *Percentage recovery of ester groups after methylation and sublimation*

$$\text{Percentage recovery} = \frac{100 \times [\text{methyl ester in sublimate (moles)}]}{[\text{acyl glyceryl ester in original lipids (moles)}]}$$

For expected composition of silicic acid fractions see Table 1. For composition of fractions as isolated see Table 2.

Tissue fraction	Silicic acid fraction no.	Recovery (%)
Homogenate	Total extract	Not esterified
	1	590
	2	219
	3	114
	4	102
	5	108
	6	102
	7	80
	8	124
	9	580
Mitochondria	Total extract	103
	1	89
	2	78
	3	Not esterified
	4	55
	5	92
	6	97
	7	147
	8	120
Microsomes	Total extract	87
	1	37
	2	121
	3	74
	4	60
	5	71
	6	97
	7	96
	8	170

amounts of material were used for analysis. The recoveries appear sufficient for the fractionated lipids to be considered representative of original lipids.

The recovery of cholesterol was low (15%). Cholesterol esters, however, constitute only 2–3% of non-phospholipids by weight (Williams *et al.* 1945), so that the poor recovery is not likely to affect fatty acid distribution significantly. Silicic acid fractionation did not separate all lipid classes completely. Sphingomyelin, if present, was eluted with lecithin. In calculating the results analyses of eluate fractions were combined in groups to give analytical data for the major lipid class present in those fractions (see Table 3). The minor components were treated as contaminants. The differences in elution pattern between tissue fractions (Table 3) are probably caused by the small differences in fatty acid composition found.

Preparation of methyl esters for gas chromatography. When esterification was carried out with diazomethane, peak E (Table 5) appeared in trace quantities on chromatograms on polar stationary phase. The peak was not observed in esters prepared by prolonged methanolysis (Stoffel *et al.* 1959). It is probably the product of a side reaction of diazomethane.

Table 4 shows the recoveries of ester groups after methylation and sublimation. The too-high recoveries in homogenate neutral lipids (Fractions 1–3) and in polar lipids (Fractions 8 and 9) will be referred to in the discussion of results for these lipids. Polyglycerophosphatide fractions and microsomal kephalin fraction gave very low recoveries of methyl esters (60 and 70% respectively). Other fractions gave yields approaching 100%.

The methods of preparation and purification of methyl esters used do not separate them from aldehydes resulting from plasmalogen hydrolysis. As a result aldehyde peaks appear on gas chromatograms.

Identification of peaks in gas–liquid chromatography. Identification of peaks and their retention volumes relative to stearate are shown in Table 5. For minor unsaturated or branched esters assignment of structure is based upon the comparison of their retention volumes with other peaks.

Table 5. *Identification and retention volumes of compounds appearing on gas chromatograms*

*, Indicates tentative identification; un., unsaturated; br., branched. Chain $n-x$ contains n C atoms and x double bonds [notation after Ahrens *et al.* (1959)].

$n-x$	Description or trivial name	Retention volume relative to methyl stearate: Apiezon L	Retention volume relative to methyl stearate: Polyethylene glycol adipate
	Esters		
8–0*	.	0·015	.
10–0*	.	0·035	.
12–0	Lauric	0·076	0·103
12– or 13–un.*	.	.	0·135
13–0	.	0·128	0·150
13–un.*	.	0·081	0·196
14–0	Myristic	0·186	0·230
14–un.*	.	0·170	0·235
14–1*	.	0·160	0·263
15–0	.	0·307	0·339
15–1*	.	0·251	0·394
16–0	Palmitic	0·435	0·501
16–1	Palmitoleic	0·380	0·560
17–br.*	.	0·499	0·695
18–0	Stearic	1·00	1·00
18–1	Oleic	0·862	1·11
18–2	Linoleic	0·801	1·34
19–0*	.	1·64	.
20–0*	Arachidic	2·32	2·02
20–1	.	1·95	2·17
20–4	Arachidonic	1·49	3·25
22–5*	.	3·25	6·61
22–6*	.	2·99	8·73
C	Unidentified highly	0·186	1·62
D	polar components	0·435	2·83
E	Unidentified	.	6·05
F	Unidentified	1·10	.
G	Unidentified	1·34	.
	Aldehydes		
H	Unidentified	0·289	0·409
A	Unidentified	0·545, 0·562, 0·602	0·790
B	Unidentified	0·657, 0·688, 0·717, 0·747	0·867

Whereas the use of two stationary phases of widely different polarities is an aid to identification, the structures given for these peaks (marked with an asterisk in Table 5) are only tentative. This also applies to peaks 8–0, 10–0 and 19–0, which were detected only on one stationary phase, and to peak 20–0, which was a trace component of one of the fractions.

Unidentified components in gas–liquid chromatography. Peaks A, B and H are due, at least partly, to aldehydes. The nature of the aldehydes has not yet been elucidated. It is possible that some of these peaks also contain a methyl ester.

On non-polar stationary phase the relative retention volumes of peaks A and B varied from sample to sample more than those of other peaks. A possible explanation for this variability is a partial reaction of diazomethane with the aldehydes during methylation of esters in the sample. These may run with retention volumes different from original aldehydes.

Peaks C and D, due to highly polar compounds, are discussed under Results ('Neutral lipids').

Peak E is a trace component, probably produced by interaction of diazomethane with a double bond (see above, 'Preparation of methyl esters').

Peaks F and G are unidentified trace components of some fractions.

Quantitative estimation of esters by gas–liquid chromatography. Fatty acid-distribution figures are calculated as:

$$\text{fatty acid (\%)} = \frac{100 \times (\text{peak area of component})}{\Sigma\,(\text{peak area of all measured components})}.$$

Thus components detected but not measurable because of small or overlapping peaks were neglected.

In most samples the sum of measurable peaks accounted for over 95% of esters present. In those fractions where only very small samples were available for gas chromatography (polar lipids, some neutral-lipid fractions), measured peaks may represent a much smaller fraction of total esters. This source of error does not affect the ratios between compounds with measurable peaks on any single chromatogram.

The accuracy and reproducibility of gas chromatography were not critically examined, but some general observations are given. Short-chain acids (C_8–C_{14}), which gave very narrow peaks lying in some chromatograms on a shoulder (see Fig. 2), and long-chain acids (C_{22}) with long retention times, which gave low broad peaks, are estimated less accurately than acids in the C_{16}–C_{20} range. Small amounts of C_{23} and C_{24} acids would probably not have been detected at the temperature and flow rates used.

For well-resolved esters containing 20–30% of total ester groups the error in the percentage figure due to the above causes may be up to 12% of that figure and for esters containing 2% of total ester groups up to 100% of the figure (i.e. from trace quantity to 4%). More exact figures are given, since peaks of similar magnitude, especially within the C_{16}–C_{20} chain-length range, can be compared with a much higher degree of accuracy.

Small amounts of acids with retention volumes similar to those of major components were difficult to estimate accurately (palmitoleate, linoleate, 22–5). Inadequate resolution may also interfere with estimation of major peaks. Thus oleate was overestimated by about 10% of its content on the polyethylene glycol adipate column, on which it was inadequately resolved from preceding stearate.

Chemical estimations. (*a*) Choline estimation. In the silicic acid fractions containing only lecithin and/or kephalin the ratios choline/phosphorus or (choline + amino nitrogen)/phosphorus exceeded 1. On this basis it was assumed that choline was over-estimated by 10–15%.

The overestimation is attributed to coprecipitation of water-soluble organic material with the choline I_3. The contaminating material can trap KI_3 during centrifuging and give additional colour in ethylene dichloride solution.

(*b*) Aldehyde estimation in microsomal Fraction 5. About 50% of the aldehyde–rosaniline complex was not extracted into octan-2-ol from the aqueous layer. The effect was not observed with total microsomal lipids, where all the rosaniline complex was extracted into the octan-2-ol phase.

It is assumed that all microsomal aldehyde was in fact recovered in the kephalin fraction, as was the case in mitochondrial lipids.

RESULTS

Whole-brain analysis

Tissue fractionation. The yield of mitochondria was 20·2 mg. dry wt./100 mg. dry wt. of homogenate and the yield of microsomes 20·5 mg. The procedure used aimed at purity of subcellular organelle fractions rather than maximum yields. In particular a part of the mitochondrial fraction was discarded with the cell debris. Of the mitochondrial pellet 15% (by vol.) consisted of nuclei and cell debris. Of the microsomal pellet 10% (by vol.) consisted of mitochondria and unidentified small vesicles. (These data were kindly supplied by Dr D. E. Bellamy.)

Lipid extraction. Table 6 shows the content of some constituents and functional groups in the total lipids. On the basis of these data approximate contents of different lipids by weight are given in Table 7 together with the total lipid dry weight. Mitochondria have the highest content of phospholipids and neutral lipids.

The recoveries of total lipids as well as of individual lipid classes are substantially lower than those obtained by Williams *et al.* (1945) from brains of 70-day-old rats (Table 7). Differences in animals and technique make the significance of these lower yields difficult to assess. Low yield of phospholipids may be partly due to lack of recovery of sphingomyelin.

Table 6. *Content of total phosphorus, cholesterol and neutral lipids in total-brain lipids*

	Concentration (μmoles/100 mg. dry wt. of tissue)		
	Total phosphorus	Cholesterol	Neutral lipids (esters of fatty acids after methylation)
Homogenate	30·6	Not analysed	2·79
Mitochondria	34·6	14·8	12·0
Microsomes	32·2	17·1	3·51

Table 7. *Recoveries of lipids calculated on basis of analyses in Table* 6

For comparison, recoveries obtained by Williams *et al.* (1945) for brain lipids of 70-day-old rats are given. Total lipid dry weights are maximal since the lipid extracts on which they were determined may have contained sucrose from the homogenizing medium, which was not removed by washing the lipid extracts.

Phospholipid dry weight was calculated from the phosphorus content by using an arbitrary molecular weight of 750 (mean of distearyl phosphatidylethanolamine and distearyl lecithin).

Neutral fat was calculated as grycerol tristearate on the basis of sublimed methyl esters (to include free fatty acid content).

	Concentration (mg./100 mg. dry wt. of tissue)							
	Total lipids (dry wt.)		Phospholipids		Cholesterol		Neutral fat	
	Present study	Williams *et al.*	Present study	Williams *et al.*	Present study	Williams *et al.*	Present study	Williams *et al.*
Homogenate	39·5	44·63	22·9	27·19	Not determined	7·05	0·8	1·95
Mitochondria	51·7	.	25·8	.	6·0	.	3·6	.
Microsomes	34·2	.	22·2	.	6·6	.	1·1	.

The relatively high proportion of neutral lipids in mitochondria (3·6% of the dry weight) is a striking feature of the total lipid distribution. The major part of this lipid consists of the polar compounds 'C' and 'D' (see above: 'Identification of peaks in gas–liquid chromatography').

Composition of rat-brain phospholipids

General assumptions and interpretation of data. Amino-nitrogen content of a fraction is considered equivalent to the content in that fraction of serine and ethanolamine phosphatides (kephalins). Where the identity of the other component of a fraction is known, but its content has not been directly measured, its proportion is expressed as its phosphorus content; thus 'moles of inositide P in kephalin fraction = moles of total P in fraction − moles of amino N'.

Lysophosphatides are calculated from the following equation:

moles/100 moles of total lipid P contained in lysophosphatides
= 2[moles of total P − (moles of acyl glyceryl ester + moles of aldehyde)].

This is a very rough approximation, since the figure obtained is a small difference of two large numbers. The lysophosphatide content may be in error by 50% for some fractions.

Plasmalogen is calculated on the basis of one ether linkage per molecule.

Content of individual lipid classes. Table 8 shows the composition of total phospholipids based upon analyses of total extracts and of tissue fractions. The proportion of lecithin found is similar to the figure given by Williams *et al.* (1945) for a 70-day-old brain. Brain appears to contain less lecithin in phospholipids than do other rat organs (Williams *et al.* 1945), ox heart (Gray & Macfarlane, 1958) or pig heart (Marinetti *et al.* 1958). The differences are of the order of 10–15% of total phospholipid phosphorus.

Polyglycerophosphatide and inositol phosphatide were not measured directly. The figures given are approximations. The proportion of polyglycerophosphatide in brain mitochondria is considerably smaller than in other organs [cf. ox heart (Gray & Macfarlane, 1958), 9% of phospholipid phosphorus; rat-liver mitochondria (Getz & Bartley, 1959), 10%; pig-heart mitochondria (Marinetti *et al.* 1958), 8%]. The polyglycerophosphatide also differs in its fatty acid structure.

The content of mitochondrial inositol phosphatide was similar to that of pig-heart mitochondria [Marinetti *et al.* (1958), 14%]. In microsomes it was much higher than in pig-heart microsomes (2%). Gray & Macfarlane (1958) give a somewhat lower figure for ox-heart mitochondria (6%) and higher for ox-heart microsomes (10%) than Marinetti *et al.* (1958).

Lipid distribution in mitochondria and homogenate is very similar. Microsomes have a higher proportion of lecithin with respect to kephalin. The composition of brain phospholipids obtained in the present study differs considerably from that given by Petersen & Schou (1955).

An unusual feature is the large excess of total nitrogen over the sum of nitrogeneous bases (choline + serine + ethanolamine) in all fractions. The nature of compounds containing this nitrogen is unknown. Since proteolipids were not rigorously

Table 8. *Composition of brain phospholipids calculated on a molar basis from analyses of total extracts and silicic acid-chromatography fractions*

'Serine and ethanolamine phosphatides' ≡ total amino-N content. 'Lecithin' ≡ phosphorus − amino N in lecithin fraction. Polyglycerophosphatide ≡ phosphorus − amino N in polyglycerophosphatide fraction. Inositol phosphatide ≡ phosphorus − amino N in 'kephalin' fraction. (Polyglycerophosphatide + inositol phosphatide) ≡ (phosphorus − amino N in 'kephalin' fraction) [in homogenate only]. Plasmalogen = aldehyde content (after hydrolysis). Lysophosphatides ≡ 2[(total phosphorus) − (acyl glyceryl ester + aldehyde)].

	Composition (moles/100 moles of phospholipid P)		
	Homogenate	Mitochondria	Microsomes
Acyl glyceryl ester (in lipids before methylation)	175	181	170
Serine and ethanolamine phosphatides	48·0	50·0	41·4
Lecithin	31·4	32·1	38·9
Polyglycerophosphatide	.	1·6	2·2
Inositol phosphatide	.	13·4	12·2
Inositol + polyglycerophosphatide	17·8	.	.
Polar lipids fraction	4·0	0·5	1·4
Plasmalogen	14·4	16·8	16·1
'Lysophosphatides'	Not determined	15·2	13·2
Total N − (amino N + choline)	75	50	68

Table 9. *Fatty acid distribution in total-brain lipids*

Chain $n-x$ contains n C atoms and x double bonds. br., Branched.

		Methyl ester in measurable peaks (%)		
		Saponified whole brain	Saponified extracts	
$n-x$	Trivial name		Mitochondria	Microsomes
	Esters			
13–0	.	–	1·8	2·5
14–0	Myristic	–	0·5	1·5
14–1	.	–	0·6	1·2
16–0	Palmitic	23·9	28·4	30·7
16–1	Palmitoleic	1·3	1·0	0·5
17–br.	.	–	–	1·7
18–0	Stearic	22·8	22·7	24·5
18–1	Oleic	30·0	31·9	24·0
18–2	Linoleic	1·4	–	–
20–1	.	3·7	1·6	1·4
20–4	Arachidonic	9·5	7·1	6·8
22–6	.	7·6	4·3	6·8
	Trace components (esters)			
10–0	.	+	+	–
12–0	Lauric	+	+	+
	Summation of esters			
$n-0$	.	46·7	50·5	59·3
$n-1$	.	34·5	34·5	25·9
$n-2$	.	1·4	–	–
$n-x>2$	.	17·0	11·4	13·5
	Aldehydes			
H	.	2·2	1·0	1·6
A (See Table 5)	.	3·3	2·2	1·1
B	.	3·1	1·9	3·1

Table 10. *Distribution of fatty acids in proteolipids*

Chain $n-x$ contains n C atoms and x double bonds. Proteolipids in column II were extracted by the procedure given in the Experimental section from a different batch of rat brains without homogenization with sucrose before extraction.

		Methyl ester in measurable peaks (%)			
$n-x$	Description or trivial name	Homogenate extract (I)	Whole-brain extract (II)	Mitochondria extract	Microsomes extract
	Esters				
14–0	Myristic	+	+	0·3	+
16–0	Palmitic	25·9	26·6	26·3	29·8
16–1	Palmitoleic	1·1	1·3	0·6	0·5
18–0	Stearic	23·8	25·3	20·9	22·4
18–1	Oleic	31·7	32·0	29·5	24·0
18–2	Linoleic	+	+	3·9	3·6
20–1	.	3·8	–	3·1	1·8
20–4	Arachidonic	7·6	6·9	7·4	7·7
22–6	.	6·1	7·8	8·0	10·0
	Trace components (esters)				
12–0	Lauric	+	+	+	+
15–0	.	–	+	+	–
G	.	–	–	+	–
	Summation of esters				
$n-0$	.	49·7	51·9	47·5	52·2
$n-1$	.	36·6	33·3	33·2	26·3
$n-2$	.	+	+	3·9	3·6
$n-x>2$	.	13·7	14·7	15·4	17·7
	Aldehydes				
H	.	2·0	2·9	1·7	1·9
A	.	5·0	0·9	2·5	1·6
B	.	5·0	4·5	–	–

removed from the extracts the nitrogen may derive from contaminating protein.

Fatty acid composition of total lipids of whole mince and subcellular fraction extracts is shown in Table 9. The general pattern of fatty acid distribution is similar in all three extracts. Mitochondria and microsomes contain slightly higher proportions of saturated fatty acids and less 'essential' acids (linoleic acid and polyenoic acids) than does the whole mince.

Brain lipids are remarkable for their high content of saturated acids and very low content of 'essential' fatty acids relative to other organs. Essential fatty acids were present in the diet (Dr G. S. Getz, unpublished work).

Comparison of particular brain lipids with lipids found in other organs will be made in the discussion of lipid fractions.

Fatty acid composition of 'proteolipids' is shown in Table 10. The distribution of fatty acids in all the proteolipids closely resembles distribution in the corresponding total lipids. In view of the isolation by Folch *et al.* (1951) of proteolipids in crystalline form with specific proportions of lipid and protein a greater specificity of fatty acid distribution might be expected. It is possible, however, that the proteolipid fraction isolated in the present experiment contains a large proportion of 'total' lipids occluded on protein during extraction.

In a separate experiment the influence of age on total brain fatty acids was studied by saponification of whole mince or whole-lipid extracts from brains of animals at different ages (Table 11).

The brain fatty acids of 5-day-old rats contained as much as 48% of palmitic acid, and a total of 60% of saturated acids. The proportion of palmitic acid decreases with age to 21% in the adult animal (cf. also 'whole brain' in Table 9) whereas the proportions of stearic acid and oleic acid increase.

Since lecithin is the component lipid richest in palmitic acid (cf. Table 14), it would be interesting to find out whether the high proportion of palmitic acid in young brain correlates mainly with an increased lecithin content (as found by Williams *et al.* 1945) or whether it reflects a general change in fatty acid composition.

Distribution of fatty acids within individual lipid classes. (*a*) Kephalin fraction (kephalin comprises serine and ethanolamine phosphatides). Paper chromatography indicated the presence in this fraction of amino-nitrogen-containing lipids with R_F values corresponding to phosphatidylserine and phosphatidylethanolamine, lysophosphatides, and of non-nitrogenous phosphatides with R_F values similar to phosphoinositide (Marinetti *et al.* 1958).

Table 11. *Influence of age on brain lipids*

Chain $n-x$ contains n C atoms and x double bonds.

		Methyl ester in measurable peaks (%)		
$n-x$	Trivial name	Saponified whole brain (adult)	Lipid extract of whole brain (22 days old)	Saponified whole brain (5 days old)
	Esters			
14–0	Myristic	1·0	0·5	–
14–1	.	–	–	2·9
16–0	Palmitic	23·4	32·6	48·2
16–1	Palmitoleic	1·2	2·4	6·3
18–0	Stearic	24·9	27·5	16·6
18–1	Oleic	30·9	21·8	15·1
18–2	Linoleic	–	–	1·3
20–1	.	4·6	–	–
20–4	Arachidonic	8·4	11·2	9·7
22–6	.	5·6	3·8	–
	Trace components (esters)			
18–0	.	–	+	–
22–5	.	–	+	–
	Summation of esters			
$n-0$	.	49·4	60·8	64·7
$n-1$	.	37·9	24·2	21·4
$n-2$	.	–	–	1·3
$n-x>2$	.	14·0	15·0	13·5
	Aldehydes			
H	.	2·7	3·8	2·8
A (See Table 5)	.	3·1	0·9	–
B	.	4·2	3·9	0·9

The recovery of amino nitrogen and the components present in the fractions are shown in Table 12.

The recovery of amino nitrogen (85–100%) is considered sufficient to give a representative fatty acid distribution.

Kephalin fraction contains a large amount of phosphoinositide. Assuming equal distribution of lyso compounds between nitrogenous phospholipids and phosphoinositide, the latter accounts for approx. 30% of fatty acids in this fraction in whole homogenate, 20% in mitochondria and 25% in

Table 12. *Composition of kephalin fractions on a molar basis*

Lecithin ≙ choline in fraction. (Inositol + polyglycerophosphatide) ≡ Total P − (amino N + choline). Plasmalogen ≡ aldehyde. Lysophosphatide ≡ 2[(total P) − (glyceryl acyl ester + aldehyde)].

	Homogenate	Mitochondria	Microsomes
Recovery (% of total amino N recovered in fraction)	95·2	98·8	83·3
	Composition (moles/100 moles of amino N)		
Total P	146	128	135
Glyceryl acyl ester	218	200	209
Lecithin	2·8	2·2	0
Inositol and polyglycerophosphatide	42·7	26·2	35·2
Plasmalogen	36·7	32·2	45·2 (28·1)*
Lysophosphatides	36	25	10
Total N − (amino N + choline)	126	81·2	79·2

* Figure in parentheses represents aldehyde actually measured. Other figure gives the plasmalogen content on the basis of estimation on total-microsome lipid extract.

Table 13. *Fatty acid distribution in kephalin fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%) Homogenate	Mitochondria	Microsomes
	Esters			
13–0	.	+	0·3	0·9
14–1	.	+	+	0·4
16–0	Palmitic	7·1	8·7	9·3
16–1	Palmitoleic	1·7	1·0	–
18–0	Stearic	34·3	32·4	41·8
18–1	Oleic	35·2	30·3	25·8
18–2	Linoleic	+	1·0	+
20–1	.	3·8	3·2	1·6
20–4	Arachidonic	9·9	10·9	8·7
22–5	.	+	3·1	2·4
22–6	.	7·6	9·0	9·1
	Trace components (esters)			
8–0	.	+	–	–
10–0	.	+	+	–
12–0	Lauric	–	–	–
13–un.		–	–	+
14–0	Myristic	–	+	+
15–1	.	–	+	–
17–br.	.	+	+	–
19–0	.	+	–	–
C	Methylation (diazomethane) artifact	–	–	+
E, F, G (See Table 5)	Unidentified	+	–	+
	Unidentified	+	–	+
	Unidentified	+	–	+
	Summation of esters			
$n-0$	.	41·8	41·4	52·0
$n-1$	.	40·7	34·5	27·4
$n-2$	.	–	1·0	–
$n-x>2$	.	17·6	23·0	11·1
	Aldehydes			
H, A, B (See Table 5)	.	2·0	2·0	3·1
	.	3·9	0·6	0·9
	.	3·6	0·3	6·3

microsomes. As a result of this contamination, the fatty acid distribution in Table 13 is only a rough approximation to the distribution in brain kephalins.

All the aldehyde of total-lipid extracts was recovered in the kephalin fractions on silicic acid chromatography.

Anomalies in the aldehyde estimation in the microsomal fraction are discussed in the 'Evaluation of Methods' section. When allowance is made for relatively low recovery of amino nitrogen in the microsomal kephalin, plasmalogen content approaches that of the whole homogenate.

In contrast to the proportions of plasmalogen found for brain lipids, Gray & Macfarlane (1958) found in ox heart nearly 50% of kephalin and 60% of lecithin in the plasmalogen form. Even allowing for inaccuracies in the computation of lysophosphatides the figure obtained both in kephalin and lecithin fractions (see below) is surprisingly high. No comparable amount of fatty acids was detected (free fatty acids would cause yields of esters after methylation of over 100%). Incomplete recovery of methyl esters may, however, have obscured their presence.

Even if the lysophosphatide content was entirely due to breakdown of the lipids on the silicic acid column or on storage, only 18% of the fatty acids was lost in the homogenate, 13% in mitochondria and 5% in microsomes. Thus possible breakdown does not invalidate the results of gas–liquid chromatography, unless the breakdown is confined to one constituent lipid class or a narrow group of fatty acids.

Although the proportion of saturated fatty acids is only slightly lower than in total lipids there is a remarkably low proportion of palmitate relative to stearate in all three fractions. This has no parallel in the fatty acids of kephalin of rat-liver mitochondrial, microsomal (Macfarlane, Gray & Wheeldon, 1960) or whole-liver kephalin fatty acids (Dittmer & Hanahan, 1959).

The proportion of 'essential' polyene fatty acids is low in comparison with heart and liver, with only about 1% of linoleic acid, but the sum of the polyenes in mitochondrial and microsomal kephalin is about twice that in the whole lipids of the corresponding tissue fractions.

(*b*) Lecithin fraction. Paper chromatography of the lecithin fractions showed one strong, choline-positive spot and two other weak lipid spots, one with a higher R_F corresponding to phosphatidylserine or phosphatidylethanolamine and one with a lower R_F corresponding possibly to sphingomyelin. The weak spots were ninhydrin- and dipicrylamine-negative, but may have contained insufficient material for detection. Table 14 shows the composition of this fraction for the three tissue fractions.

The recovery of choline (85–100%) was sufficient to yield representative fatty acid samples. The composition is expressed as a percentage of moles of lecithin phosphorus (calculated as moles of total P − moles of amino N), since no significant amounts of other components were present on the basis of paper chromatography.

The contamination with kephalin is highest in homogenate lecithin, where the kephalin accounts for 20% of total fatty acids. Contamination in the other tissue fractions is much smaller. Only traces of aldehyde were detected. This is in sharp contrast with the results of Gray & Macfarlane (1958) for ox heart, where 60% of the lecithin contained plasmalogen.

It is possible that unsaturated ether linkages were hydrolysed on the silicic acid column in the presence of the polar solvents used to elute the kephalin fractions. Such hydrolysis would, however, be expected to occur earlier in the elution (cf. Gray & Macfarlane, 1958), giving rise to significant aldehyde recoveries from 'neutral fat' and polyglycerophosphatide fractions. In view of the absence of reports of lecithin plasmalogen in brain, this lipid class is not likely to be present in brain in large amounts.

The negative lysophosphatide content of the homogenate is a calculation artifact. The figures for the other two fractions are surprisingly high. They

Table 14. *Composition of lecithin fractions*

Lecithin P ≡ total P − amino N. Kephalin ≡ amino N. Plasmalogen ≡ aldehyde. Lysophosphatide ≡ 2[(total P) − (acyl glyceryl ester + aldehyde)].

	Homogenate	Mitochondria	Microsomes
Recovery (% of total choline recovered in fraction)	95·8	87·2	98·3
	Composition (moles/100 moles of lecithin P)		
Total P	128	107	116
Acyl glyceryl ester	282	184	210
Choline	111	116	114
Kephalin	27·7	6·7	15·8
Plasmalogen	0	0	1·0
Lysophosphatides	−26	30	21
Total N − total P	67	32	−5

Table 15. *Fatty acid distribution in lecithin fractions*

Chain $n-x$ contains n C atoms, x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%) Homogenate	Mitochondria	Microsomes
	Esters			
12–0	Lauric	+	–	0·2
13–0	.	–	+	0·1
13–un.	.	–	+	0·2
14–0	Myristic	0·4	0·2	0·5
15–0	.	–	+	0·2
16–0	Palmitic	49·7	38·9	44·7
16–1	Palmitoleic	0·8	0·8	1·8
18–0	Stearic	12·8	17·1	15·9
18–1	Oleic	30·2	32·2	26·6
18–2	Linoleic	0·9	0·8	1·1
20–0	Arachidic	–	+	0·3
20–1	.	2·1	1·3	1·2
20–4	Arachidonic	3·3	4·7	4·1
22–6	.	+	3·6	2·6
	Trace components (esters)			
14–1	.	–	+	+
15–1	.	–	+	+
17–br.	.	–	+	+
22–5	.	–	+	+
	Summation of esters			
$n-0$	.	62·9	56·2	61·4
$n-1$	.	33·1	33·3	29·6
$n-2$	.	0·9	0·8	1·1
$n-x>2$	.	3·3	8·3	6·7
	Aldehydes			
H	.	–	+	0·3
A (See Table 5)	.	–	+	+
B	.	+	0·2	0·2

are unlikely to represent lysolecithin content *in vivo*.

If the lysolecithin content given by the computation is correct and is entirely due to breakdown, the proportions of fatty acids lost amount to 15% in mitochondria and 10% in microsomes. The possible breakdown is not likely to affect significantly the fatty acid-distribution figures.

Fatty acid composition of brain lecithin is shown in Table 15. The distribution patterns in homogenate, mitochondria and microsomes are similar.

Lecithin shows the highest proportion of saturated acids of all lipid fractions. Some di-saturated lecithin is probably present, since saturated acids comprise over 50% of the total. Palmitic acid is present in high concentration (cf. kephalins, Table 13). In contrast with kephalin, in lecithin the proportion of 'essential' fatty acids in all tissue fractions is lower than in whole lipids.

The fatty acid distribution found in lecithin differs widely from that of whole-liver lecithin (Dittmer & Hanahan, 1959) or the lecithin of liver mitochondria and microsomes (Macfarlane *et al.* 1960). Both these groups found a much smaller proportion of saturated fatty acids (35% in liver mitochondria and microsomes) and a much higher proportion of linoleic acid and polyenoic acids (45% in liver mitochondria and microsomes).

Table 16. *Composition of polyglycerophosphatide fractions on a molar basis*

Polyglycerophosphatide P ≡ (total phosphorus) – (amino N). Polyglycerophosphatide ester (minimum) ≡ (acyl glyceryl ester) – 2(amino N). Kephalin ≡ amino N.

	Composition (moles/100 moles of polyglycerophosphatide P) Mitochondria	Microsomes
Acyl glyceryl ester	450	260
Kephalin	43	27
Polyglycerophosphatide ester / Polyglycerophosphatide P	3·6:1	2·0:1
Total N – amino N	213	No analysis

The proportion of oleic acid (25–30%) is remarkably constant in the major phospholipids of each tissue fraction (cf. Tables 9, 11, 13 and 15).

(*c*) 'Polyglycerophosphatide' fractions. Chromatography of these fractions on silicic acid-impregnated paper showed a fast-moving, ninhydrin-negative spot followed by a ninhydrin-positive spot with R_F corresponding to phosphatidylethanol-

Table 17. *Fatty acid distribution in polyglycerophosphatide fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)	
		Mitochondria	Microsomes
	Esters		
13–0	.	2·1	3·2
14–0	Myristic	0·8	+
14–un.	.	1·2	1·7
15–0	.	–	2·2
16–0	Palmitic	12·5	12·1
16–1	Palmitoleic	5·0	2·3
17–br.	.	1·0	+
18–0	Stearic	21·3	24·5
18–1	Oleic	18·6	21·6
18–2	Linoleic	10·3	2·3
19–0	.	+	0·8
20–0	Arachidic	1·8	1·3
20–1	.	1·3	1·6
20–4	Arachidonic	7·5	6·6
22–6	.	7·6	8·4
C, D	Unidentified, highly polar component	1·8; 6·9	1·8; 9·3
	Trace components (esters)		
8–0	.	+	–
12–0	Lauric	+	+
13–un.	.	–	+
14–un.	.	+	+
15–1	.	+	+
F	Unidentified	+	+
	Aldehydes		
H, A, B	See Table 5	+; +; +	1·4; 0·7; +

Table 18. *Composition of polar lipid fractions*

	Composition (moles/100 moles of total P)		
	Homogenate	Mitochondria	Microsomes
Acyl glyceryl ester	50	200	220
Amino N	16	Not analysed	57

amine. The fast-moving spot was not detected in other fractions. Since these fractions, however, had a much higher lipid content a substantial portion of the fast-moving spot may have been present in them and escaped detection. The R_F of the fast spot is consistent with it being polyglycerophosphatide.

The composition of the fraction (Table 16) is given as a percentage of polyglycerophosphatide phosphorus, calculated as total P – amino N. Owing to the high ester/P ratio of polyglycerophosphatide only 20% of acyl glyceryl esters in the fraction are derived from contaminating kephalins. Hence the fatty acid distribution in the fraction is unlikely to be considerably influenced by kephalin contamination.

Polyglycerophosphatide fractions from both mitochondria and microsomes gave very low recoveries of methyl esters after sublimation (55–60%, Table 4, Fraction 4). Distribution of fatty acids in polyglycerophosphatide fractions is shown in Table 17. The most interesting feature is the relatively high content of 'essential' fatty acids in mitochondria (25%) and microsomes (17%).

The polyglycerophosphatide fraction may be compared with the 'cardiolipin' fraction of Gray & Macfarlane (1958) from ox heart and of Getz & Bartley (1959) from rat liver with a linoleic acid content of 80%. Although the polyglycerophosphatide fraction is the most unsaturated brain lipid it does not correspond in its fatty acid composition to the cardiolipin fraction. Even if all the fatty acid 'missing' as a result of low yield from sublimation were linoleic acid (which is unlikely), the fraction would contain a smaller proportion of linoleic acid and larger proportion of saturated acids than cardiolipin.

(*d*) Polar lipid fraction. Paper chromatography showed a weak lipid spot, whose R_F corresponded roughly to that of sphingomyelin. It was choline-negative but the amount of choline may have been

below limits of detection. A ninhydrin-positive spot remained at the origin.

The composition of polar fractions is given in Table 18. The fraction gave a very high yield of methyl esters after sublimation (cf. Table 4: homogenate Fractions 8 and 9, mitochondria Fraction 8, microsomes Fraction 8).

The lipids present in this minor fraction have not been characterized. Their fatty acid distribution is shown in Table 19. The detection of only a small number of fatty acids in polar lipids of mitochondria and microsomes is probably due to the insufficient amount of material available for gas chromatography.

(*e*) Neutral lipids. Paper chromatography showed spots corresponding to mono-, di- and triglycerides, cholesterol and cholesterol esters, possibly free fatty acids and a lipid spot in Fraction 1 (Table 1) moving with the solvent front.

The phosphorus content shows that phospholipid contamination is small (Table 20). The analyses (except of cholesterol) were done at the lower limits of their sensitivity and are only approximate. The low recovery of cholesterol is discussed in the 'Evaluation of Methods' section (Lipid fractionation).

Table 21 shows the fatty acid distribution in neutral-lipid fractions. Neutral-lipid fractions are remarkable for the large variety of fatty acids present in them. They also contain high proportions of compounds C and D.

As may be seen from Table 5, on the non-polar stationary phase compound C co-chromatographed with myristate and compound D with palmitate. The compounds ran much slower on polar stationary phases: C in front of 20–1 and D in front of arachidonate. The change in retention volumes between the two types of stationary phase is larger than would be expected to result from unsaturation of the fatty acid chain and suggests the presence of a more polar group.

The compounds constitute 70 % of mitochondrial and 30 % of microsomal neutral lipids. Their proportion in homogenate neutral lipids is uncertain, since these lipids were chromatographed only on the non-polar stationary phase.

Trace amounts of compound C were also detected in some kephalin and polar lipid fractions.

Table 19. *Fatty acid distribution in polar lipid fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)		
		Homogenate	Mitochondria	Microsomes
	Esters			
14–0	Myristic	1·9	–	4·4
14–1	.	+	–	1·9
15–0	.	–	–	2·7
15–1	.	+	–	1·6
16–0	Palmitic	34·5	32·2	25·8
16–1	Palmitoleic	3·1	–	12·7
17–br.	.	–	–	1·4
18–0	Stearic	25·0	40·2	24·1
18–1	Oleic	30·3	23·3	24·5
18–2	Linoleic	+	+	1·0
20–4	Arachidonic	4·5	–	–
22–5	.	–	4·3	–
	Trace components (esters)			
8–0	.	–	–	+
10–0	.	–	–	+
12–0	Lauric	–	–	+
13–0	.	–	–	+
13–un.	.	–	–	+
20–0	Arachidic	–	–	+
20–1	.	+	–	+
C	Unidentified highly polar component	–	–	+
	Summation of esters			
$n-0$	.	61·4	72·4	57·0
$n-1$	.	33·4	23·3	38·8
$n-2$	.	+	+	1·0
$n-x>2$	.	4·5	4·3	–
	Aldehyde			
H, A, B	See Table 5	0·8	–	2·7
		–	–	1·6
		–	–	2·1

Table 20. *Composition of neutral-lipid fractions*

	Composition (moles/100 moles of methyl ester)		
	Homogenate	Mitochondria	Microsomes
Total P (≡ phospholipid contamination)	0·47	4·6	12
Amino N	6·0	0·3	1·8
Total N	.	20	.
Cholesterol recovered in fraction	148	33	95

Table 21. *Fatty acid distribution in neutral-lipid fractions*

Chain $n-x$ contains n C atoms, x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)		
		Homogenate	Mitochondria	Microsomes
	Esters			
8–0	.	–	–	2·6
12–0	Lauric	–	0·7	+
12- or 13–un.	.	–	–	2·3
13–0	.	0·7	+	+
14–0	Myristic	0·9*	0·3	+
14–1	.	+	0·3	+
15–0	.	0·2	0·3	0·4
15–1	.	0·4	+	0·4
16–0	Palmitic	29·0*	6·7	14·5
16–1	Palmitoleic	4·5	1·0	1·2
17–br.	.	0·3	+	+
18–0	Stearic	26·6	2·5	22·2
18–1	Oleic	23·9	2·6	11·9
18–2	Linoleic	1·3	1·3	–
20–1	.	+	1·8	0·7
20–4	Arachidonic	9·1	–	8·0
22–5	.	–	5·2	–
22–6	.	1·4	–	2·0
C, D	Unidentified polar component	*, *	17·8, 59·6	7·2, 26·6
	Trace components (esters)			
10–0	.	–	–	+
13–un.	.	–	–	+
14–1	.	–	–	+
20–0	.	–	–	+
F	Unidentified	–	–	+
K	Unidentified	–	+	–
	See Table 5			
	Aldehydes			
H	.	0·9	0·3	4·0
A	.	0·8	1·1	–
B	.	+	0·2	2·1

* Figures for homogenate acids 14–0 and 16–0 may include C and D. No separation was possible as samples were chromatographed only on non-polar stationary phase (see Results section: 'Neutral lipids').

DISCUSSION

Similarity of mitochondrial and microsomal lipids

Mitochondrial, microsomal and total-brain phospholipids contain similar proportions of the two major components, lecithin and kephalin (phosphatidylserine and phosphatidylethanolamine and corresponding plasmalogens). The fatty acid composition of either of these lipid classes shows only minor variations from one centrifugal fraction to another. These findings are similar to those of Macfarlane *et al.* (1960) for rat liver and Marinetti *et al.* (1958) for pig heart.

The similarity of mitochondrial and microsomal phospholipids despite the diverse functions of these organelles suggests that a large proportion of the phospholipid in cell organelles has no direct metabolic function, but serves as a structural component common to mitochondria and microsomes. The differences in the distribution of fatty acids in the same phospholipid class in mitochondria and microsomes may represent the content of a small proportion of the lipid with a distinctive fatty acid composition, derived from specific structures more directly involved in metabolism than the bulk of the lipid. Similar con-

siderations may apply to minor phospholipid components (polyglycerophosphatides, phosphoinositides, sphingomyelin) on which the present study throws little light.

Two lines of approach may lead to the identification of lipids with specific metabolic functions. One is the study of lipids in subfractions of mitochondria and microsomes (Green, 1959; Siekievitz, 1959). Some of these particles may contain a metabolically important lipid in higher concentration than total cell subfractions (cf. Holman & Widmer, 1959). The other, parallel, approach is more exact characterization of the tissue lipids by improvements in methods of fractionation and analysis.

Specific composition of brain-lipid classes

The fatty acid composition of each brain phospholipid is specific both in proportions of fatty acids with different numbers of double bonds and in relative proportions of fatty acids with the same degree of saturation (cf. differences in palmitate/stearate ratio between lecithin and kephalin). The differences in fatty acid distribution between brain lecithin and kephalin bear no close relationship to the differences between corresponding lipid fractions in rat liver.

This specificity indicates that the fatty acid structure of a particular phospholipid is not simply a function of total fatty acid distribution in the organ. It is possible that a mechanism of diglyceride synthesis exists which is specific for each lipid in the selection of fatty acids esterified as well as (at least for lecithin) in the position of the saturated fatty acids on glycerol (cf. Tattrie,1960; Hanahan, Brockerhoff & Barron, 1960).

Fatty acid distribution in total-brain phospholipids

All classes of brain phospholipids analysed contained considerably more saturated fatty acids (40–60%) and much less polyethenoid fatty acids (10–25%) than has been reported for rat liver (Dittmer & Hanahan, 1959; Macfarlane *et al.* 1960) or ox-heart mitochondria (Holman & Widmer, 1959). A polyglycerophosphatide fraction rich in linoleic acid which has been isolated from rat-liver mitochondria (Getz & Bartley, 1959) and ox heart (Macfarlane *et al.* 1960) is either absent from brain or present to the extent of below 2% of the lipid phosphorus in mitochondria and microsomes.

In view of these findings it seems that in brain mitochondria a high total content of polyethenoid fatty acids in the constituent phospholipids is not essential for the functioning of respiratory enzyme systems.

SUMMARY

1. Lipids from rat-brain homogenate, mitochondrial and microsomal fractions were analysed for phosphorus, total nitrogen, amino nitrogen, choline, cholesterol, acyl ester and aldehyde content. They were separated into lipid classes by silicic acid-column chromatography. The distribution of fatty acids in whole lipids and the lipid classes was obtained by gas-chromatographic analysis of methyl esters of the fatty acids.

2. Lipids from all three tissue fractions were similar in their content of different lipid classes and in the fatty acid distribution both in total-lipid extracts and in individual lipid classes. Proteolipids resembled total-lipid extracts in their fatty acid distribution.

3. In comparison with other lipids, brain phospholipids are remarkable for their high content of saturated fatty acids (50%) and low proportion of essential (polyenoic) fatty acids (16%). Linoleic acid accounts for only 1% of total fatty acids.

4. Mitochondria contained 26% of their tissue dry weight as phospholipid, 6% as cholesterol and 4% as neutral fat.

5. The composition of mitochondrial phospholipid was 32% of lecithin, 50% of phosphatidylserine and phosphatidylethanolamine, 13% of phosphoinositide, 2% of polyglycerophosphatide, 17% of plasmalogens (serine and ethanolamine). No lecithin plasmalogens were found.

6. Fatty acids from total mitochondrial extract contained 28% of palmitic acid, 23% of stearic acid, 32% of oleic acid, 11% of essential acids (linoleic acid and polyenoic acids) and traces of short-chain acids ($C_{n<16}$).

7. Mitochondrial serine–ethanolamine phosphatide fraction contained 9% of palmitic acid, 32% of stearic acid, 30% of oleic acid and 23% of essential fatty acids. Mitochondrial lecithin fatty acids consisted of 39% of palmitic acid, 17% of stearic acid, 32% of oleic acid and 8% of essential fatty acids. Polyglycerophosphatide fraction contained 13% of palmitic acid, 21% of stearic acid, 19% of oleic acid, 25% of essential acids and 4% of short-chain acids.

8. No close resemblance was found between the composition of any brain-lipid class and similar lipids from other organs, but some general trends were apparent. The findings are discussed in connexion with possible metabolic functions of lipids.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and advice, Dr G. S. Getz for helpful discussion, Dr M. G. Macfarlane and Dr G. M. Gray for a gift of palmital dimethylacetal and Mr A. Renshaw for technical assistance. The work was aided by grants from the Rockefeller Foundation and the United States Department of Public Health. One of us (L.A.B.) wishes to thank the Colonial Office for a Medical Research Studentship.

REFERENCES

Ahrens, E. H., Insull, W., Hirsch, J., Stoffel, W., Petersen, M. L., Farquhar, J. W., Miller, T. & Thomasson, H. J. (1959). *Lancet*, i, 115.
Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953). *J. biol. Chem.* **205**, 803.
Bartley, W. (1953). *Biochem. J.* **54**, 677.
Beiss, von U. & Armbuster, O. (1958). *Z. Naturf.* **13B**, 79.
Bellamy, D. (1959). *Biochem J.* **72**, 165.
Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.
Dittmer, J. C. & Hanahan, D. J. (1959). *J. biol. Chem.* **234**, 1976.
Folch, J., Ascoli, I., Lees, M., Meath, J. A. & Le Baron, F. N. (1951). *J. biol. Chem.* **191**, 833.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Getz, G. S. & Bartley, W. (1959). *Nature, Lond.*, **184**, 1229.
Gray, G. M. & Macfarlane, M. G. (1958). *Biochem. J.* **70**, 409.
Green, D. E. (1959). *Advanc. Enzymol.* **21**, 118.
Hanahan, D. J., Brockerhoff, H. & Barron, E. J. (1960). *J. biol. Chem.* **235**, 1917.
Hanahan, D. J., Dittmer, J. C. & Warashina, E. (1957). *J. biol. Chem.* **228**, 685.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hirsch, J. & Ahrens, E. H. (1958). *J. biol. Chem.* **233**, 311.
Hokin, M. R. & Hokin, L. E. (1959). *J. biol. Chem.* **234**, 1387.
Holman, R. T. & Widmer, C. (1959). *J. biol. Chem.* **234**, 2269.
Insull, W. & Ahrens, E. H. (1959). *Biochem. J.* **72**, 27.
James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
Keulemans, A. I. M. (1957). *Gas Chromatography*, 2nd printing, p. 32. New York: Reinhold Publishing Corp.
Lovelock, J. E. (1958). *J. Chromat.* **1**, 34.
Macfarlane, G. M., Gray, M. G. & Wheeldon, L. W. (1960). *Biochem. J.* **74**, 43P.
Marinetti, G. V., Erbland, J. & Kochen, J. (1957). *Fed. Proc.* **16**, 837.
Marinetti, G. V., Erbland, J. & Stotz, E. (1958). *J. biol. Chem.* **233**, 562.
Petersen, V. P. & Schou, M. (1955). *Acta physiol. scand.* **33**, 309.
Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 193.
Siekievitz, P. (1959). *Ciba Foundation Symp., Regulation of Cell Metabolism*, p. 17.
Stoffel, W., Chu, F. & Ahrens, E. H. (1959). *Analyt. Chem.* **31**, 307.
Tattrie, N. H. (1960). *J. Lipid Res.* **1**, 60.
Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.
Wheeldon, L. W. & Collins, F. D. (1958). *Biochem. J.* **70**, 43.
Williams, H. H., Galbraith, H., Kaucher, M., Moyer, E. Z., Richards, A. J. & Macy, I. G. (1945). *J. biol. Chem.* **161**, 475.
Zlatkis, A., Zak, B. & Boyle, G. J. (1953). *J. Lab. clin. Med.* **41**, 486.

Biochem. J. (1961) **79**, 176

Studies on Cerebral Sphingomyelin

BY G. B. ANSELL AND SHEILA SPANNER
Department of Experimental Psychiatry, The Medical School, Birmingham 15

(*Received* 21 *September* 1960)

Sphingomyelin was first isolated from brain by Thudichum (1884) but little was known of its metabolism until recently. Brady & Koval (1958) and Brady, Formica & Koval (1958) have now carried out extensive investigations on sphingosine biosynthesis, and more recently Sribney & Kennedy (1958) have shown that the biosynthesis of sphingomyelin in liver proceeds by the reaction of a ceramide (*N*-acylsphingosine) with cytidine diphosphate choline. It is quite likely that such a reaction proceeds in nervous tissue (Rossiter, Thompson & Strickland, 1960).

In tissues other than brain the function of sphingomyelin is unknown. In nervous tissue the lipid probably does not make its appearance until the onset of myelination when it rapidly increases in concentration (Folch-Pi, 1955). Earlier investigations (Branté, 1949; Johnson, McNabb & Rossiter, 1949) have shown that the sphingomyelin is preferentially located in the white matter (myelinated fibres), and Finean and his colleagues have suggested that the lipid could fit well into the structure of the repeating units of myelin (Finean & Robertson, 1958). In this sense therefore, white-matter sphingomyelin would appear to have a structural role in the nervous system, although this may not be so for sphingomyelin occurring outside the myelin sheath, e.g. in cerebral cortex. From experiments with [3-^{14}C]serine, Davison, Morgan Wajda & Payling-Wright (1959) have concluded that the sphingomyelin in myelin has a very slow rate of replacement and may even be permanent