

Distribution of Lipids in Subcellular Particles of Guinea-Pig Brain

By J. EICHBERG, JUN., V. P. WHITTAKER AND R. M. C. DAWSON
*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology,
 Babraham, Cambridge*

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It is becoming increasingly apparent that lipids act as essential structural elements in the multi-enzyme systems associated with the cell organelles. Many of the enzymes of the electron-transport chain in mitochondria can be isolated as discrete lipoproteins (Green, 1959), and the phospholipid contained in these is essential for enzymic activity (Reich & Wainio, 1961). Moreover, lipids play a fundamental role in the microstructure of cell membranes and may even participate metabolically in the transport of cations (Hokin & Hokin, 1964).

Clearly, therefore, the distribution of individual lipids within the cell becomes of fundamental importance. The technique of differential centrifugation has enabled various subcellular fractions to be prepared and numerous lipid analyses of these fractions have been carried out. With liver tissue, consisting predominantly of hepatic cells, the relative cellular homogeneity allows these analyses to apply to defined morphological entities of the cell such as the nuclei or mitochondria (Spiro & McKibbin, 1956; Macfarlane, Gray & Wheeldon, 1960; Getz & Bartley, 1961). However, in tissues like the brain with its complicated cytological differentiation, the application of the same centrifugation technique produces fractions that are grossly heterogeneous. Lipid analyses of such fractions (Peterson & Schou, 1955; Biran & Bartley, 1961) therefore, although of great value, must be regarded as preliminary.

In recent years, the use of density-gradient centrifugation (Hebb & Whittaker, 1958; Whittaker, 1959, 1961; Whittaker, Michaelson & Kirkland, 1963, 1964), coupled with morphological characterization in the electron microscope (Gray & Whittaker, 1960, 1962; Whittaker, 1960; Whittaker *et al.* 1963, 1964), has permitted the isolation of certain morphologically defined structures in relatively pure form. These include pinched-off nerve endings or 'synaptosomes', and synaptic vesicles which are contained within the synaptosomes and can be released from them by suitable disruptive procedures. The present paper describes detailed analyses of the individual lipids present in such subfractions by using, among other techniques, a recently developed method for the determination of all known phospholipids (Dawson, Hemington & Davenport, 1962). It provides new data on the

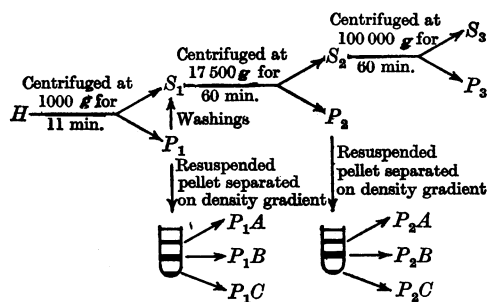
lipid composition of brain mitochondria, nuclei and microsomes, as well as the subcellular particles peculiar to brain: myelin fragments, synaptosomes and synaptic vesicles.

EXPERIMENTAL

Preparation of subcellular fractions

Subcellular fractions were prepared at 0–4° from guinea-pig brain as described by Whittaker (1959), Gray & Whittaker (1962) and Nyman & Whittaker (1963) with some modifications (Scheme 1). The brain tissue rostral to the inferior colliculi (11–12 g. from four animals) was homogenized in ice-cold 0.32M-sucrose to give a tissue concentration of 20% (w/v). After a sample had been withdrawn for analysis the initial homogenate (*H*) was diluted to 10% (w/v) with 0.32M-sucrose before fractionation. Particulate fractions were resuspended in 0.32M-sucrose before analysis.

Nuclear and large-myelin-particle fractions. The first particulate (P_1) fraction, consisting mainly of nuclei, large myelin fragments and tissue debris, was sedimented by centrifuging the homogenate in a Servall type SS-1 centrifuge at 1000g for 11 min. It was washed by suspending in fresh 0.32M-sucrose and resedimenting under the same conditions. In some experiments, this fraction was further fractionated by resuspending the residue in 0.32M-sucrose so that 1 ml. was equivalent to 1 g. of original tissue and centrifuging it (4.5–5.5 ml./tube) into a discontinuous sucrose density gradient formed by pipetting a layer of 0.8M-sucrose (10 ml./tube) on to an equal volume of 1.2M-sucrose contained in Lustroid tubes of the SW25 head of the Spinco model L preparative ultracentrifuge. After centrifuging at 53 000g for 2 hr., three fractions had



Scheme 1. Separation of guinea-pig brain homogenate (*H*) into supernatant (*S*) and particulate (*P*) fractions. Details are given in the text.

separated, a top ivory-coloured band between the 0.32M- and 0.8M-sucrose layers, an intermediate gelatinous pink band between the 0.8M- and 1.2M-sucrose layers, and a small gelatinous pink pellet below 1.2M-sucrose. Light- and electron-microscopic examination with conventional and negative staining (Horne & Whittaker, 1962) showed that the top band consisted almost entirely of large myelin fragments with some smaller vesicular elements, the intermediate band of nuclei together with some mitochondria, myelin fragments, a few synaptosomes and tissue debris, and the pellet mainly of tissue debris and blood cells. The intermediate band was thus less homogeneous than the top band. These two bands were recovered by using a tube slicer, and each was diluted with water to give a final sucrose concentration of approx. 0.32M and sedimented at 100 000g for 1 hr. in the no. 40 head of the Spinco ultracentrifuge. The pellet obtained from the top band was designated fraction P_1A and that from the intermediate band fraction P_1B . The material sedimenting to the bottom of the gradient tube (fraction P_1C) was discarded.

Small-myelin-particle, synaptosome and mitochondrial fractions. The combined supernatants (S_1) from fraction P_1 were centrifuged at 17 500g for 1 hr. in a Servall type SS-34 centrifuge at 0–2° to give fraction P_2 , consisting of small myelin fragments, synaptosomes and mitochondria together with some microsomes. This treatment resulted in microsomal contamination but was essential to sediment the smaller synaptosomes. The fraction was resuspended in 0.32M-sucrose and separated into three subfractions here designated P_2A , P_2B and P_2C exactly as described for the corresponding fractions of P_1 . The compositions of the fractions were examined before sedimentation: fraction P_2A consisted of smaller myelin fragments than did P_1A , contaminated by microsomes and a few synaptosomes; fraction P_2B was an almost pure preparation of synaptosomes—it showed some microsomal contamination but was largely free from myelin fragments and free mitochondria; fraction P_2C consisted of mitochondria with occasional shrunken synaptosomes.

Microsomal and supernatant fractions. The supernatant (S_2) from fraction P_2 was separated in a Spinco ultracentrifuge at 100 000g for 1 hr. into a final supernatant (S_3) and a small gelatinous precipitate consisting mainly of microsomes, i.e. vesicular membrane fragments 0.1–0.2 μ in diameter. Particles less than 0.1 μ in diameter (synaptic vesicles?), small mitochondria, small synaptosomes and non-vesicular membrane fragments were also present as minor constituents.

Synaptic vesicles. These were prepared in two different ways. In method A synaptosomes were ruptured by exposure to water and the vesicles separated on a density gradient as described by Whittaker *et al.* (1963, 1964). In an improved and simplified procedure (method B), the source of synaptosomes was a once-washed P_2 fraction prepared from guinea-pig brain cortex. The cortical tissue (approx. 6 g.) from five animals was homogenized, centrifuged at 1000g for 11 min. to remove nuclei and tissue debris, and then centrifuged at 10 000g for 20 min. to sediment most of the synaptosomes and mitochondria. These were washed once by suspending in 0.32M-sucrose and resedimenting at 10 000g for 30 min. Washing and collection of the synaptosomes at these relatively low speeds greatly diminished microsomal contamination, and the use of cortical tissue largely eliminated myelin. The washed P_2

pellet was suspended in water (approx. 2 ml./g. of original tissue) and transferred (5 ml./tube) to Spinco SW25 Lustroid tubes containing a sucrose density gradient. This consisted of a continuous concentration gradient ranging from 0.4M at the top to 0.6M at the bottom (20 ml./tube) which was layered over 1.6M-sucrose (5 ml./tube). After centrifuging at 53 500g for 2 hr. the region originally containing the suspension of fraction P_2 in water was optically clear; below it, at the top of the continuous gradient, was a hazy band consisting of free synaptic vesicles; and at the boundary between the continuous concentration gradient and the layer of 1.6M-sucrose was a dense layer consisting mainly of partially disrupted synaptosomes and swollen mitochondria. The hazy band of synaptic vesicles and the clearer sucrose below were removed in a tube cutter, diluted with an equal volume of water and centrifuged to give a small gelatinous white pellet; this was resuspended in water and the lipids were extracted as described below. For analysis the extracts of three separate preparations were pooled.

Electron-microscopic examination with negative staining showed that the cortical synaptic-vesicle fraction was remarkably homogeneous, being almost completely free from microsomal contamination. There was slightly more contamination in the preparation from forebrain, but this was still small. The cortical synaptosomes appeared to be more easily ruptured than those from whole brain, the yield, on the basis of acetylcholine determinations (Whittaker *et al.* 1963, 1964), being about 50%.

Extraction of lipids

From cell particles. The lipids of the subcellular fractions were extracted essentially according to the method of Folch, Lees & Sloane-Stanley (1957). The suspended subcellular fractions were mixed with 15 vol. of chloroform-methanol (2:1, v/v) and heated in a water bath at 55° for 5 min. The residue was collected by centrifuging and washed several times with small volumes of chloroform-methanol (2:1, v/v). The combined extracts were filtered by suction through a sintered-glass funnel, porosity 3 (Wherrett & McIlwain, 1962), and the filtrate was made up with chloroform-methanol (2:1, v/v) to a volume 20 times that of the original suspended subcellular fraction. The extract was then shaken with 0.2 vol. of 0.9% NaCl and, after separation, the lower phase was washed twice with half its volume of fresh upper phase made by using 0.9% NaCl (Folch *et al.* 1957). All upper phases were combined and stored at –12° for determination of ganglioside *N*-acetylneuraminic acid.

The washed lower layer was evaporated to dryness *in vacuo* and dissolved in about 20 ml. of chloroform-methanol (2:1, v/v). The extract was then usually again partitioned by the addition of 0.2 vol. of 0.9% NaCl. This step decreased the turbidity due to proteolipids, as well as removing small amounts of residual sucrose. After phase separation, the lower layer was again evaporated to dryness and the residue dissolved in 10–15 ml. of chloroform-methanol (2:1, v/v). The slightly turbid extract was filtered through a sintered-glass funnel, porosity 3, with suction and the filtrate made to volume with chloroform-methanol (2:1, v/v).

From supernatant fluid. Lipids were extracted from the large volume of the sucrose-containing supernatant fraction by the method of Bligh & Dyer (1959) slightly

modified. To the supernatant fluid (82 ml.) were added amounts of chloroform and methanol to produce a monophasic system consisting of chloroform-methanol-water (2:1:0.8, by vol.). The mixture was heated to 60° and then cooled to room temperature, and amounts of chloroform and water were added to produce a diphasic system consisting of chloroform-methanol-water (2:2:1.8, by vol.). The thick emulsion that formed on shaking was broken by centrifuging. The lower layer was recovered and washed twice with an upper phase obtained from chloroform-methanol-water (2:2:1.8, by vol.). The washed lower layer was then treated as described for the other subcellular fractions (see above).

From synaptic vesicles. The synaptic-vesicle preparation was diluted fourfold with methanol and heated for 10 min. at 55°. A few drops of 0.05 M-MgCl₂ were added and the flocculated residue was sedimented by centrifuging. The methanolic supernatant fluid was removed and the residue extracted three times with 5 ml. portions of chloroform-methanol (2:1, v/v). The methanol and chloroform-methanol extracts were combined and chloroform and methanol added to produce a final mixture containing chloroform-methanol (2:1, v/v) with a volume 20 times that of the initial suspension of synaptic vesicles. The lipid extract was then treated as described above for the extracts from particulate fractions.

Examination of extracted tissue residues for residual lipid

The tissue residue remaining after chloroform-methanol extraction was treated with chloroform-methanol (2:1, v/v) acidified with conc. HCl (1 vol. in 400 vol.) to extract protein-bound phosphoinositides (J. Eichberg, jun. & R. M. C. Dawson, unpublished work). The residue was then saponified with 5 ml. of N-NaOH for 18 hr. at 37° and then for a further 2 hr. at 65–70°. The sample was acidified with HCl. An equal volume of ethanol was added and the sample was then extracted three times with twice its volume of diethyl ether. The ether extracts were combined and washed with water until the pH of the water layer was 5–6. The ether layer was then evaporated to dryness under a stream of air and the residue dissolved in 2 ml. of acetone. The fatty acids were titrated with 0.02 N-NaOH from a micro-burette with cresol red as indicator and stirring with a stream of N₂. A blank titration on the reagents was carried out.

Examination of gangliosides from Folch upper phases

The combined upper phases obtained on washing the lipid extracts of the subcellular fractions were evaporated to a small volume on the rotary evaporator at a temperature below 40°. The concentrated solution (10–20 ml.) was dialysed first against running tap water for 48 hr. and then against 2 l. of distilled water for 18 hr. The contents of the dialysis bag were recovered and if necessary evaporated *in vacuo* to a smaller volume. Portions were analysed for N-acetylneuraminic acid.

The remainder of the dialysed solution was evaporated to dryness *in vacuo* below 40° and the residue extracted with a few millilitres of chloroform-methanol (1:1, v/v). The extract was reduced in volume under a stream of air and portions were spotted on plates coated with silica gel G (E. Merck A.-G., Darmstadt, Germany). The solvent system

employed was chloroform-methanol-water-aq. ammonia (sp.gr. 0.88) (60:35:6:2, by vol.) (Wherrett & Cumings, 1963). After development, the plates were air-dried and sprayed with 2% resorcinol in 5N-HCl followed by heating at 100–150° for 15–20 min. to bring up the purple bands.

The separation of the gangliosides was markedly facilitated by developing the chromatogram under conditions where the top of the plate was exposed to the atmosphere. The thin-layer chromatogram (20 cm. long) was run in a glass tank 17 cm. deep. The cover of the tank consisted of two rectangular pieces of glass that could be brought up close to the chromatographic plate and held there by removable stainless-steel clips at either end. The length of the slit between the two pieces of glass was varied by insertion of polythene strips according to the width of the glass plate used. Immediately before the introduction of a plate through the slit the solvent was added and the tank shaken gently. Development was allowed to proceed at room temperature with as little atmospheric disturbance as possible. Evaporation of solvent from the exposed end of the plate increased the effective length of the chromatographic run. The best separation of gangliosides under these conditions was found to take place in 5–6 hr. (Fig. 1); if the run was prolonged further, the bands near the solvent front tended to spread excessively, especially if the chromatogram was somewhat overloaded.

Analytical procedures

Non-diffusible solids. Portions of the subcellular fractions suspended in 0.32 M-sucrose were placed in dialysis bags. The bags were attached to a slowly rotating disk and dialysed against two changes of 18 l. of distilled water for 48 hr. The contents of the bags were then transferred to tared beakers and heated at 95° to constant weight.

Nitrogen. This was estimated by a micro-Kjeldahl procedure.

Distribution of individual phospholipids. This was examined as described by Dawson *et al.* (1962).

Cholesterol. Portions of the lipid solutions were saponified and the non-saponifiable material was extracted according to the method of Koval (1961). Cholesterol was determined by the method of Zlatkis, Zak & Boyle (1953) as modified by Henly (1957). This procedure also gave satisfactory results when employed for the estimation of cholesterol in the acid- and alkaline-stable fraction, containing alkyl ether phospholipid, obtained in the method of Dawson *et al.* (1962). Analysis for cholesterol was carried out on a portion of this fraction when only a limited amount of lipid phosphorus was present in the extracts from subcellular fractions (e.g. synaptic vesicles).

Sulphatide plus cerebroside. This was determined by the procedure of Long & Staples (1961). The lipid galactose values obtained were multiplied by 4.6.

Ganglioside N-acetylneuraminic acid. The thiobarbituric acid method (Warren, 1959) was principally used, after preliminary hydrolysis of the samples in 0.1 N-H₂SO₄ for 1 hr. at 80°. This procedure is the most sensitive available for the detection of N-acetylneuraminic acid but is only applicable to the estimation of the free compound. In combination with the prior acid-hydrolysis step, which releases only a part of the bound ganglioside N-acetylneuraminic acid (Booth, 1962), an estimated 50–60% of the total ganglioside N-acetylneuraminic acid is measured.

Effect of sucrose on lipid extraction and determination

The subcellular fractions were obtained in sucrose solution which could affect the extraction of lipids and interfere with the non-specific orcinol-carbohydrate reaction used for cerebrosides and sulphatides. Total saponification of extracted tissue residues (total homogenate and nuclei) indicated that these contained less than 2% of the total fatty acids removed by the lipid-extraction procedure. Since the nuclei were in the fraction most heavily contaminated with sucrose, this would indicate that the presence of sucrose had not seriously interfered with the extraction of lipids. The addition of [U-¹⁴C]sucrose to some fractions before lipid extraction showed that, in spite of the multiple-washing procedure, the final chloroform layer containing the washed lipids still contained traces of sucrose. Calculation indicated that this residual sucrose was insufficient to affect the determination of lipid galactose (cerebroside plus sulphatide) by more than a few per cent except in those cell fractions where only small amounts were present (mitochondria and synaptosomes). Here the results for lipid galactose represent maximal values, but nevertheless they are useful in indicating the low concentration of cerebroside and sulphatide in such organelles.

RESULTS

Presentation of results. Three bases for expressing the results have been adopted. (1) The amount of lipid contained in the fraction obtained from a unit weight of fresh tissue (Table 1): this is valid for a reasonably quantitative recovery of each cell fraction from the total homogenate; the results in Table 1 indicate that this is true, at least for the primary fractions, and in the secondary fractions the recovery is better than 80%. (2) The amount of lipid present in a defined weight of the non-diffusible solid in the fraction (Table 2). (3) The amount of lipid per unit of total nitrogen in the fraction: this will clearly depend on the composition of the fraction, and one containing relatively more lipid than protein, e.g. myelin, will contain a smaller percentage of nitrogen than will other fractions. Such nitrogen determinations are given in Tables 1 and 2 so that the reader may calculate the results in these terms if desired.

The phospholipid analyses are expressed as percentages of the total lipid phosphorus taken for the analytical procedure (Table 3). Recoveries are also given in Table 3 and were always better than 91%. The results can be calculated in terms of mg./g. of original brain tissue or μg./mg. of non-diffusible solids from the results given in Tables 1, 2 and 3.

To give an indication as to whether a particular cell fraction is enriched in an individual lipid compared with the whole tissue, an enrichment factor for the fractions has been calculated (Table 4). This factor was obtained by comparing the amount (μg.)

Table 1. Analysis of guinea-pig forebrain fractions

Experimental details are given in the text. The results are expressed as mg. of constituent/g. of fresh brain.

Preparation	Non-diffusible solids				Nitrogen			Lipid phosphorus			Cholesterol			Cerebroside + sulphatide		Ganglioside N-acetylneuraminic acid	
	I	II	III	IV	II	III	IV	I	II	IV	I	II	IV	I	II	I	II
Homogenate (H)	155	147	16.2	17.0	6.6	6.6	6.9	1.96	1.94	1.80	16.6	17.5	14.0	9.9	10.6	0.43	0.42
Crude nuclear fraction (P ₁)	73	48	2.1	2.7	2.7	2.7	2.5	0.96	0.87	—	7.8	8.8	—	7.2	7.2	0.13	0.12
Large myelin fragments (P _{1A})	—	21	1.1	3.4	2.1	2.1	4.1	—	0.31	0.44	—	4.4	5.4	—	4.9	—	0.022
Nuclei (P _{1B})	—	26	2.1	3.4	2.1	2.1	4.1	—	0.36	—	—	2.7	—	—	1.3	—	0.087
Cell debris (P _{1C})	—	—	—	0.5	0.7	—	—	—	—	—	—	—	—	—	—	—	—
Crude mitochondrial fraction (P ₂)	52	49	5.0	4.9	3.1	3.1	3.1	0.72	0.66	—	4.4	4.7	—	1.7	1.4	0.18	0.17
Small myelin fragments (P _{2A})	—	12	0.9	1.3	0.8	0.8	0.8	—	0.20	—	—	1.9	—	—	1.5	—	0.05
Synaptosomes (P _{2B})	—	22	2.2	2.2	1.6	1.6	1.6	—	0.33	—	—	1.8	—	—	—	—	0.10
Mitochondria (P _{2C})	—	—	8.8	0.9	0.9	0.9	0.9	—	0.10	—	—	0.2	—	—	0.08	—	0.006
Microsomes (P ₃)	7.2	8.7	1.0	4.4	3.5	3.5	3.9	0.13	0.14	—	1.0	1.0	—	0.27	0.39	0.036	0.040
Supernatant fluid (S ₃)	15	18	—	—	—	—	—	0.034	—	—	0.2	—	—	0.58	—	0.011	—

Table 2. *Content of lipid classes in guinea-pig-forebrain fractions*

Experimental details are given in the text. The results are expressed as $\mu\text{g.}$ of constituent/mg. of non-diffusible solids.

Preparation	Lipid phosphorus		Cholesterol		Cerebroside + sulphatide		Ganglioside <i>N</i> -acetylneuraminic acid		Nitrogen II
	I	II	I	II	I	II	I	II	
Homogenate (<i>H</i>)	12.6	13.3	107	119	64	72	2.5	2.9	111
Crude nuclear fraction (<i>P</i> ₁)	13.2	—	107	—	99	—	1.9	—	—
Large myelin fragments (<i>P</i> _{1<i>A</i>})	—	15.2	—	212	—	233	—	1.1	52
Nuclei (<i>P</i> _{1<i>B</i>})	—	13.7	—	99	—	48	—	3.3	79
Crude mitochondrial fraction (<i>P</i> ₂)	13.9	13.6	86	97	33	29	3.5	3.6	103
Small myelin fragments (<i>P</i> _{2<i>A</i>})	—	16.8	—	165	—	125	—	4.2	79
Synaptosomes (<i>P</i> _{2<i>B</i>})	—	14.7	—	79	—	5.5	—	4.1	98
Mitochondria (<i>P</i> _{2<i>C</i>})	—	10.8	—	19	—	9.1	—	0.8	98
Microsomes (<i>P</i> ₃)	17.7	16.9	104	109	38	45	4.9	4.9	117
Supernatant fluid (<i>S</i> ₃)	2.3	—	14	—	40	—	0.8	—	197

of the component in 1 mg. of the non-diffusible solids in each subcellular fraction with the value for the whole homogenate.

Distribution of gangliosides in subcellular fractions

Our findings on the distribution of gangliosides in the primary fractions of whole forebrain are somewhat at variance with those reported by Wolfe (1961) and Wherrett & McIlwain (1962). The microsomal fractions from grey matter prepared by these authors contained a much larger proportion of the cell gangliosides than that found in the present study (Table 1). On the other hand, our results are in agreement in that they show an enrichment of gangliosides in the microsomal fraction (Table 4).

The ganglioside fractions from the subcellular organelles were examined by thin-layer chromatography to see whether any of the individual gangliosides were specifically localized. The results from a number of such examinations indicated that the ganglioside fractions from the various cell fractions including the synaptic vesicles did not vary greatly in the distribution of individual components (Fig. 1). However, it would appear that the amount of the fastest-running component, probably a monosialoganglioside, is proportionally enriched in the large-myelin-particle fraction, and this was confirmed by scanning the photographs of the chromatograms with a Chromoscan, which gave a semi-quantitative evaluation of the ganglioside distribution. That it was a monosialoganglioside was confirmed by running equivalent chromatograms in propanol-water and showing that it was the only component stable after hydrolysis in 0.01*N*-hydrochloric acid for 15 min. at 100° (Svennerholm, 1963). This difference might account for the finding of Svennerholm (1963) that human-brain grey matter contained more disialoganglioside than did white matter.

DISCUSSION

The present studies represent an advance on previous investigations to determine the lipid composition of defined subcellular organelles of the brain. Of the fractions examined, the mitochondria (fraction *P*_{2*C*}), myelin (fractions *P*_{1*A*} and *P*_{2*A*}), synaptosomes (fraction *P*_{2*B*}) and synaptic vesicles were classed as relatively pure morphological entities on examination under the electron microscope. The microsomes were slightly contaminated with other cell constituents. However, the nuclei (fraction *P*_{1*B*}) were heavily contaminated, mainly with myelin fragments, and further studies will be necessary to obtain the precise lipid composition of nuclei.

In examining the distribution of lipids in the subcellular fractions from tissues, it is necessary to consider the possibility that enzymic degradation of the components may occur during the prolonged centrifuging. The results in Table 1 indicate that more than 80% of the main lipid classes in the whole homogenate is recovered in the primary cell fractions, which suggests that no appreciable autolysis had occurred, especially since manipulative losses may have accounted for at least part of the deficit. The same general picture was obtained after the subfractionation of the crude mitochondria (fraction *P*₂) (Table 1). The results from the equivalent subfractions from crude nuclei (fraction *P*₁) are incomplete because the cell debris was not analysed, but nevertheless at least 75% of the lipid present in fraction *P*₁ was recovered.

The recoveries of the individual phospholipids in the primary fractions were always better than 85% of those in the original homogenate, and the same was true of the recoveries from the subfractions obtained from the crude mitochondria. Again, in the subfractions from the crude nuclei at least 70% of each of the phospholipids was recovered even though the cell debris was discarded.

Table 3. *Distribution of individual phospholipids in subcellular fractions of guinea-pig forebrain*

Experimental details are given in the text. The results are expressed as percentages of total lipid phosphorus.

Fraction ...	Homogenate (H)	Crude nuclear fraction (P ₁)	Large myelin fragments (P _{1A})	Nuclei (P _{1B})	Crude mito-chondrial fraction (P ₂)	Small myelin fragments (P _{2A})	Synapto-somes (P _{2B})	Mito-chondria (P _{2C})	Micro-somes (P ₃)	Synaptic vesicles Prep. A Prep. B
Phosphorus (mg./g. of whole brain) ...	1.96 ± 0.09	0.92	0.38	0.36	0.71	0.20	0.33	0.10	0.14	
Phosphorus (μg./mg. of non-diffusible solids) ...	13.0	13.2	15.2	13.7	13.8	16.8	14.7	10.8	17.3	
No. of analyses ...	4	1	2	1	2	1	1	1	2	1
Phosphatidylcholine	32.6 ± 1.3	27.9	25.6	33.6	36.7	32.1	39.3	40.0	41.4	40.7
Phosphatidylethanolamine	12.8 ± 1.1	11.2	8.9	14.0	15.5	14.1	17.6	23.3	13.0	13.1
Phosphatidylserine	12.3 ± 0.8	12.9	12.8	11.1	12.4	14.1	12.9	5.9	11.9	10.2
Phosphatidylinositol	2.9 ± 0.5	2.7	2.8	2.6	3.5	2.9	4.0	5.5	4.8	5.9
Phosphatidic acid	0.9 ± 0.4	1.0	1.9	0.7	0.4	1.3	0.7	0.6	0	2.3
Cardiolipin	1.4 ± 0.2	1.4	Trace	2.3	3.0	0	1.6	11.1	0.4	0.6
Choline plasmalogen	Trace	0	Trace	0	0	0	0	0	0	0
Ethanolamine plasmalogen	17.9 ± 1.8	19.4	25.5	17.4	14.3	24.4	16.0	9.4	14.8	12.0
Serine plasmalogen	Trace	0	0.3	0	0	0	0	0	0	0
Sphingomyelin	8.3 ± 1.1	10.7	12.4	7.1	5.8	7.3	5.3	3.7	7.5	12.3
Alkyl ether phospholipid	3.2 ± 0.5	3.4	3.8	2.8	2.0	2.3	2.1	2.2	1.9	3.2
Recovery	92.5 ± 4.7	90.6	94.6*	91.6	93.6	98.5	99.5	101.7	95.5	100.3

* Includes 0.6% of a lipid tentatively identified as a higher phosphoinositide.

Table 4. *Relative enrichment of lipid components of guinea-pig forebrain fractions*

Experimental details are given in the text. The results were calculated by dividing the μg. of component/mg. of non-diffusible solids in the fraction by the μg. of component/mg. of non-diffusible solids in the whole-brain homogenate.

Total lipid phosphorus	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Phosphatidylinositol	Phosphatidic acid	Cardiolipin	Ethanolamine plasmalogen	Sphingomyelin	Alkyl ether phospholipid	Cholesterol	Cerebroside + sulphatide	Ganglioside N-acetylneuraminic acid
Homogenate (H)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Crude nuclear fraction (P ₁)	1.06	1.06	1.09	0.84	1.13	1.23	1.15	0.72	0.97	1.00	1.55	0.73
Large myelin fragments (P _{1A})	1.05	0.84	1.14	1.04	2.98	0	1.41	1.32	1.19	1.66	2.96	0.34
Nuclei (P _{1B})	0.95	0.96	0.89	0.81	0.96	1.61	0.94	0.74	1.05	0.83	0.61	1.00
Crude mitochondrial fraction (P ₂)	1.02	1.14	1.23	1.06	1.19	1.80	0.92	0.67	0.91	0.81	0.40	1.24
Small myelin fragments (P _{2A})	1.27	1.25	1.43	1.13	2.60	0	2.56	1.23	1.15	1.39	1.74	1.46
Synaptosomes (P _{2B})	1.15	1.29	1.33	1.32	1.27	1.23	0.97	0.64	0.84	0.66	0.08	1.44
Mitochondria (P _{2C})	0.82	0.88	0.34	1.22	0.70	5.80	0.38	0.31	0.61	0.16	0.13	0.29
Microsomes (P ₃)	1.27	1.53	1.18	1.92	0	0.62	1.02	1.27	0.89	0.92	0.62	1.69
Supernatant fluid (S ₃)	0.18	—	—	—	—	—	—	—	—	0.13	0.63	0.31

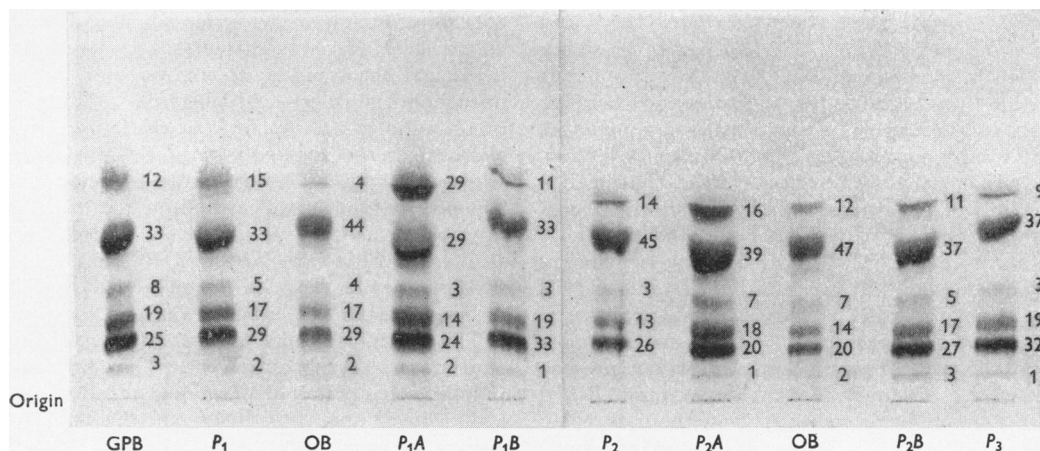


Fig. 1. Thin-layer chromatograms of the gangliosides of brain subcellular fractions. Abbreviations: GPB, guinea-pig-brain homogenate; OB, purified ox-brain ganglioside preparation (kindly supplied by Dr D. Gammack); other abbreviations relate to subcellular fractions from guinea-pig brain as described in the text. Each fraction (about 50 μ g. of *N*-acetylneuraminic acid) was applied to silica-gel-G plates and developed for 5 hr. in chloroform-methanol-water-aq. ammonia (sp.gr. 0.88) (60:35:6:2, by vol.) (see the Experimental section). The number on the right of each band is the intensity (determined by scanning the photograph with a Chromo-scan; Joyce, Loebel and Co. Ltd., Gateshead-on-Tyne), expressed as a percentage of the total of the fraction.

It is possible that some enzymic breakdown of a minor component could have taken place during the short post-mortem period and the initial homogenization.

Lipid composition of myelin. The determination of the lipid composition of myelin by analysing an isolated myelin fraction obtained by centrifuging is likely to give a more accurate picture than that obtained by previous methods. These were based on a comparison of the lipid analyses of white and grey matter (e.g. Branté, 1949) and of brain tissue before, during and after myelination (Johnson, McNabb & Rossiter, 1949; Edgar, 1957; Folch *et al.* 1959), and are liable to error because white and grey matter differ in respects other than their myelin content and because growth of the brain in general will occur during myelination.

In the present studies two myelin fractions were obtained (P_{1A} and P_{2A}) which, on examination by electron microscopy, appeared to consist chiefly of large and small myelin fragments respectively. Some fragments contained cytoplasmic inclusions and may have been fragments of axons. The lipid compositions of these fractions were essentially similar, although the large fragments contained more cerebroside (Table 1) as well as more sphingomyelin and lesser amounts of phosphatidylethanolamine and probably lecithin (Table 3). It is not possible at present to deduce to what extent such differences are due to genuine differences in morphology, to changes occurring during the frac-

tionation procedure or to contamination of the small-myelin-particle fraction by synaptosomes and microsomes.

Myelin appears to have a lipid composition that differs from that of the other cellular particles. Previous studies (Branté, 1949; Edgar, 1957; Folch *et al.* 1959) suggested that the main myelin lipids were cerebroside, cholesterol, sphingomyelin and phosphatidylserine. The present results confirm that these lipids are indeed present in myelin, but indicate also that very appreciable amounts of other lipids occur. None of these four lipids can be considered as characteristic of myelin, and only cerebroside was substantially enriched compared with the whole homogenate (Table 4). It is significant that in the large myelin particles, which formed the predominant fraction, the molar proportions of cholesterol, phospholipid and cerebroside were 2.0:1.94:1. This agrees well with the 2.2:1 molar proportions calculated by Finean & Robertson (1958), and it suggests a well-defined molecular relationship of these lipids in the organized myelin substructure.

One-quarter of the phospholipid consisted of ethanolamine plasmalogen, and its concentration was much greater than that of the phosphatidylethanolamine. This could account for the high content of ethanolamine plasmalogen in human white matter compared with grey matter found by Webster (1961). Little choline or serine plasmalogen was found in myelin or indeed in any of the brain

subcellular fractions, which confirms the observations of both Klenk, Debuch & Daun (1953) and Ansell & Norman (1956). Myelin was appreciably enriched in phosphatidic acid (Table 4), and it is tempting to correlate this with the membranous nature of the myelin sheath. However, as this metabolically active phospholipid occurs in low concentrations, it cannot be assumed that such a situation would pertain *in vivo*. Thus, in the microsome fraction where phosphatidic acid might be expected to occur (Hokin & Hokin, 1964), none, in fact, was detected. Traces of a lipid resembling diphosphoinositide were present in the lipid extract of myelin, but this represented only a small proportion of the total higher phosphoinositides present (J. Eichberg, jun. & R. M. C. Dawson, unpublished work). This emphasizes that such compounds, when combined with protein, are not extracted by neutral chloroform-methanol (Dittmer & Dawson, 1961).

Table 5 compares the lipid analyses of these two myelin fractions with similar but less extensive data on rat myelin that have become available from other Laboratories since the present studies were completed. In general the values do not differ greatly in spite of the differing species examined and variations in the fractionation techniques employed.

Lipid composition of brain mitochondria. Guinea-pig brain-mitochondrial phospholipid contained 11% of cardioliipin P, whereas very little of this constituent was present in the myelin or microsomes. This finding is consistent with the high cardioliipin content of the mitochondria from rat liver (10–12% of lipid phosphorus) (Getz & Bartley, 1959; Strickland & Benson, 1960) and pig heart (13%) (Marinetti, Erbland & Stotz, 1958). Strickland & Benson (1960) and Biran & Bartley (1961) reported that the proportion (2% of lipid phos-

phorus) of this lipid in rat-brain mitochondria was considerably lower than that in the liver mitochondria. However, the fractions analysed in these investigations are likely to compare with our crude mitochondrial fraction which contained only 3% of its phospholipid phosphorus as cardioliipin, and this emphasizes the greater purity of these organelles when obtained by gradient-density centrifuging. The precise function of cardioliipin in mitochondrial physiology has not yet been elucidated, but the studies of Fleischer, Klouwen & Brierley (1961) suggest that it may be an essential part of the cytochrome-oxidase enzyme complex.

The lipids of brain mitochondria were low in cholesterol, cerebroside, ganglioside, sphingomyelin, ethanolamine plasmalogen and phosphatidylserine compared with those of the whole homogenate. Løvtrup & Svennerholm (1963) have reported that rat-brain mitochondria contain 18% of cerebrosides, which is in marked disagreement with our own finding of less than 1% in the guinea pig. It is difficult to explain this discrepancy, for even our crude mitochondrial preparation contained only 7% of cerebroside and the method of isolating mitochondria (Løvtrup & Zelander, 1962) used by Løvtrup & Svennerholm (1963) resulted in limited myelin contamination. In our experience the double washing used by these authors for removing sucrose from chloroform-methanol lipid extracts is inadequate, and a possible explanation might be that a contamination with residual sucrose was adding to the cerebroside values. The species difference between guinea pig and rat is hardly likely to explain such a large discrepancy.

Lipid composition of synaptosomes and synaptic vesicles. Apart from cerebroside the lipid composition of the synaptosomes was rather similar to the

Table 5. *Lipid composition of myelin as reported by various workers*

The values are expressed as μ moles of lipid/mg. of total lipid, where total lipid is the chloroform-soluble lipid after washing by the procedure of Folch *et al.* (1957). In the present study the mg. of total lipid represents the sum of values for total phospholipid (lipid phosphorus $\times 25$), cholesterol and cerebroside + sulphatide.

Species Reference	Guinea pig This paper		Rat Davison (1964)	Rat Nussbaum, Bieth & Mandel (1963)
	Large myelin fragments	Small myelin fragments		
Lipid				
Cholesterol	0.64	0.58	0.548	0.435
Cerebroside + sulphatide	0.32	0.21		
Ganglioside (as <i>N</i> -acetylneuraminic acid)	0.003	0.020		
Total phospholipid	0.62	0.77	0.545	0.534
Phosphatidylcholine	0.159	0.247	0.140	0.135
Phosphatidylethanolamine	0.055	0.109		
Ethanolamine plasmalogen	0.158	0.188	0.236	
Alkyl ether phospholipid	0.029	0.018		0.288
Phosphatidylserine	0.079	0.109		
Phosphatidylinositol	0.018	0.022	0.113	
Phosphatidic acid	0.012	0.010		
Cardioliipin	Trace			
Sphingomyelin	0.077	0.056	0.042	0.035

Table 6. *Content of lipid classes in synaptic-vesicle fraction of guinea-pig forebrain*

Experimental details are given in the text. The synaptic-vesicle fraction prepared by method A had a nitrogen content of 265 $\mu\text{g./g.}$ of fresh brain.

Method of preparation		Total lipid phosphorus	Cholesterol	Ganglioside <i>N</i> -acetylneuraminic acid
Method A	$\mu\text{g. of constituent/g. of fresh brain}$	27.1	136	5.9
	$\mu\text{g. of constituent/mg. of nitrogen in fraction}$	102	514	21.5
Method B	$\mu\text{g. of constituent/g. of fresh cerebral cortex}$	12.9	72.5	3.3

whole homogenate, and there was no evidence of the enrichment of any individual lipid. This result is consistent with the electron-microscopic picture of the fraction: this showed it to contain pinched-off nerve endings composed of a membrane enclosing mitochondria, synaptic vesicles and cell sap. The membrane probably differs from the myelin sheath and microsomes in that the synaptosomes are virtually free of cerebroside. The cardiolipin present may be entirely derived from the mitochondria that are present in the synaptosomes.

Only very limited material was available for lipid analyses of the synaptic vesicles (Table 6). These contain acetylcholine and are believed to be storage sites of this amine within cholinergic nerve endings. In preparations made with either a discontinuous or continuous density gradient the distribution of individual phospholipids was remarkably similar to that of microsomes (Table 3). This is consistent with the electron-microscope appearance of synaptic vesicles in thin sections, where they appear as hollow structures bounded by a 'unit' membrane. Chromatography of the gangliosides of the synaptic vesicles indicated that their distribution closely resembled that of the other cell fractions.

SUMMARY

1. Guinea-pig forebrain was fractionated by differential and gradient-density centrifuging and the distribution of lipids measured in the subcellular fractions.

2. The large myelin particles contained cholesterol, phospholipid and cerebroside in the molar proportions 2:1.94:1. The phospholipids of myelin contained 25% of both lecithin and ethanolamine plasmalogen and also most of the phosphatidic acid of the original homogenate.

3. The mitochondrial phospholipids consisted predominantly of lecithin and ethanolamine-containing phosphoglycerides, and contained 11% of cardiolipin phosphorus. Ganglioside and cerebroside were virtually absent.

4. The microsomes were enriched in phosphatidylinositol and ganglioside.

5. The 'synaptosomes' (nerve-ending particles) contained no cerebroside, but their phospholipids were similar to those of the original homogenate.

6. The phospholipids of synaptic vesicles resembled those of microsomes.

7. The distributions of individual gangliosides in the cell fractions were remarkably similar except that the large myelin particles contained proportionally more of one monosialoganglioside.

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Biochem. J. (1964), **92**, 100

Toxic Fluorine Compounds

20. THE USE OF THE ω -FLUORINE ATOM IN THE STUDY OF THE METABOLISM OF BRANCHED-CHAIN FATTY ACIDS*

BY F. L. M. PATTISON AND R. L. BUCHANAN

Department of Chemistry, University of Western Ontario, London, Ontario, Canada

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Branched-chain fatty acids occur in the lipid fractions of certain bacteria and micro-organisms, and in many types of synthetic fats; hence a study of their mode of breakdown is of some medical importance. In the past, a common approach to this type of problem has been to isolate individual catabolites after administering acid prototypes to animals, and hence to deduce a likely metabolic pathway (see, e.g., Williams, 1959). The present paper describes a 'chemical' approach to this type of biochemical problem, based on the unique toxicological properties of the ω -fluorine atom (Pattison, 1959*b*). The rationale centres around the fact that compounds that ultimately can give rise to ω -fluoroalkanoic acids, $F\cdot[CH_2]_n\cdot CO_2H$, containing an even number of carbon atoms are toxic whereas those that give ω -fluoroalkanoic acids with an odd number of carbon atoms are non-toxic

(Buckle, Pattison & Saunders, 1949; Pattison, Hunt & Stothers, 1956*a*).

For fatty acids with a single branched methyl group, $CH_3\cdot[CH_2]_n\cdot CHMe\cdot[CH_2]_m\cdot CO_2H$, two routes of degradation, depending on the value of m , are possible (Scheme 1). The problem thus reduces to the metabolism of the α - and β -methyl derivatives. Theoretically there are two ways in which each of these can break down. However, work by Pattison & Woolford (1957*b*) indicated that, in the overall β -oxidation process, the α -methyl group is eliminated as part of a C_3 fragment, whereas the β -methyl group is eliminated as part of a C_4 fragment, i.e. route (*a*) in each case. *gem*-Dimethyl groups appeared to inhibit β -oxidation, as expected.

To confirm these conclusions, it was decided to prepare all possible variations of methyl substitution in two adjacent ω -fluoroalkanoic acids, and to determine the toxicities (see Table 1).

* Part 19: Pattison & Dear (1963).