The Metabolism of Phospholipids in Mouse Brain Slices

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1. Slices of mouse brain grey matter were incubated with [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate. Doubly labelled phospholipids were extracted from subcellular fractions prepared from the slices in a mixture of metabolic inhibitors, under conditions where there was negligible change in radioactive labelling during the preparation. Two tissue fractions were studied in detail; one contained a high proportion of mitochondria and the other was mainly microsomal. 2. In all tissue fractions the highest incorporations of both [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate occurred into phosphatidylcholine. 3. After incubation for 1 hr., the $^{32}\text{P}/^{14}\text{C}$ ratios for phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid in the mitochondrial fraction were similar to those in the microsomal fraction. 4. The $^{32}\text{P}/^{14}\text{C}$ ratios were similar in phosphatidylcholine and phosphatidylethanolamine and much lower than those in phosphatidic acid and phosphatidylinositol.

Mitochondrial and microsomal preparations of rat and guinea-pig brain have each been shown to incorporate [^{32}P]phosphate into their constituent phospholipids under appropriate conditions *in vitro* (McMurray, Berry & Rossiter, 1957*a*; McMurray, Strickland, Berry & Rossiter, 1957*b*; Hokin & Hokin, 1958*b*). However, information is required about the metabolism of such subcellular fractions in intact tissue where the spatial and metabolic connexions are preserved. In the present investigation, brain slices were incubated with mixtures of [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate and the extent of the incorporation of each of these isotopes into the phospholipids of different subcellular fractions was determined. A preliminary communication has been given (Clayton & Rowe, 1965).

METHODS

Incubation of tissue and preparation of subcellular fractions. Brain slices were prepared from the grey matter of the brains of male mice (Parke's strain), 4 months old, which were killed by cervical dislocation (McIlwain, 1961). Slices (1.0–1.2 g.) were incubated with shaking at 37° in 10 ml. of a Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) modified so as to contain KH_2PO_4 (2.5 μM), glucose (11.1 mM) and EDTA (disodium salt) (0.36 mM). Carrier-free [^{32}P]orthophosphate (100 μC) and sodium [$1\text{-}^{14}\text{C}$]acetate (50 μC ; specific radioactivity 354 $\mu\text{C}/\text{mg.}$) were added. These labelled compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

For time-course studies slices from several brains were placed in buffer (50 ml. approx.) and mixed. Slices were selected at random, dried with filter paper, weighed and transferred to incubation vessels. A series of independent incubations was started at the same time. The individual incubations were terminated after different time-intervals.

After incubation the slices were homogenized in 10 ml. of a solution containing NaCl (132 mM), NaF (10 mM), NaCN (10 mM), NaN_3 (0.1 mM) and 2,4-dinitrophenol (0.15 mM). A Potter–Elvehjem homogenizer was used and the Teflon plunger was rotated at 1150 rev./min. The outer tube was moved up and down slowly eight times. After dilution (1 ml. of salt solution/200 mg. of tissue), the homogenate was fractionated at 4° in an MSE Superspeed 40 centrifuge. The homogenate was centrifuged at 1000 $g_{av.}$ for 10 min. and the supernatant fluid was removed. The sediment was resuspended in homogenizing fluid, equal in volume to the previous supernatant. The centrifugation was repeated and the sediment collected to give fraction I. The combined supernatant and washings from fraction I were mixed and centrifuged at 12000 $g_{av.}$ for 10 min. to sediment fraction II. The supernatant fluid from fraction II was centrifuged at 105000 $g_{av.}$ for 60 min. to sediment fraction III from the supernatant (fraction IV).

Phase-contrast microscopy of fraction I showed that it contained nuclei and large and small fragments of myelin. The nuclei stained with Mayer's Acid Haemalum (Brontë-Gatenby & Beams, 1950). Electron microscopy indicated that fraction II contained a high proportion of mitochondria but that it was contaminated with synaptosomes and microsomes, and that fraction III consisted almost entirely of microsomes. No significant differences could be detected between fractions isolated from slices that had been incubated for 2 hr. and fractions isolated from fresh tissue.

Extraction of lipids and isolation of total phospholipids. The subcellular fractions were extracted with 20 vol. of chloroform–methanol (2:1, v/v). The extracts were freed from [^{32}P]phosphate and [^{14}C]acetate by a combination of the washing procedures described by Folch, Lees & Sloane-Stanley (1957) and Kennedy (1953) as follows. After one washing with 0.20 vol. of a mixture of 0.1 M-phosphate buffer, pH 7.0, and 0.1 M-sodium acetate, the extract was washed with 'upper phase' (Folch *et al.* 1957) containing chloroform, methanol and a mixture of 0.1 M- NaH_2PO_4 and 0.1 M-sodium acetate (8:4:3, by vol.). The volume of upper

phase was equal to that of the aqueous phase removed in the previous washing. The washed extract was evaporated to dryness and the residue was dissolved in 15 ml. of ethanol-ethyl acetate (1:2, v/v). This solution was washed twice with 3 ml. of a mixture of 0.5 M-NaH₂PO₄ and 2 M-KCl and once with 3 ml. of 0.1 M-KCl.

The washed lipid extract was evaporated to dryness in a rotary evaporator and the residue was extracted twice with 1 ml. portions of diethyl ether. The extract was transferred to a column of Mallinkrodt silica gel (1.5 g.; 3.5 cm. x 1 cm. diam.) that had been prepared as described by Hirsch & Ahrens (1958). Neutral lipids were eluted with diethyl ether (100 ml.). The flask that had contained the lipid was washed twice with 1 ml. of methanol and the washings were transferred to the column. Phospholipids were eluted with methanol (60 ml.). The methanolic eluate was evaporated to dryness and the residue dissolved in 10 ml. of chloroform-methanol (2:1, v/v).

Separation and purification of phospholipid classes. Phospholipids were chromatographed in a series of spots (1 cm. apart), each containing less than 0.15 μ g. atom of P, on silicic acid-impregnated paper with the solvent mixture di-isobutyl ketone-acetic acid-water (9:5:1, by vol.). (Marinetti & Stotz, 1956). Total phospholipid from mouse brain grey matter was used as a marker and the marker strips were developed by immersion in an aqueous solution of Rhodamine 6G (10 μ g./ml.) for 20 min. The separated phospholipids were detected by their fluorescence in ultra-violet radiation. Horizontal strips of the undeveloped chromatogram, corresponding in position to the separated phospholipids, were extracted four times with chloroform-methanol (2:1, v/v) with 1 ml. of solvent per spot. Components extracted were those corresponding to phosphatidylinositol, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and an unseparated mixture of phosphatidic acid and cardiolipin.

Phosphatidylcholine was purified by thin-layer chromatography on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with the solvent mixture chloroform-methanol-acetic acid-water (250:74:19:3, by vol.) (Abramson & Blecher, 1964). Marker strips were developed by spraying with Rhodamine 6G and the corresponding areas of the undeveloped chromatogram were removed with a razor blade and extracted four times with chloroform-methanol-water (49:49:2, by vol.; 2 ml. per spot). Only glycerophosphorylcholine was detected by radioautography of chromatograms of alkaline hydrolysates of this purified material (Dawson, 1960).

Phosphatidylethanolamine was purified by chromatography on alumina (for chromatographic analysis; British Drug Houses Ltd., Poole, Dorset) by the method of Long & Staples (1961). Contaminating phosphatidylcholine was removed with chloroform-methanol (1:1, v/v) and cerebrosides with chloroform-methanol-water (19:19:2, by vol.). Phosphatidylethanolamine was then eluted with chloroform-methanol-water (7:7:2, by vol.).

Phosphatidylinositol was purified on Kieselgel G by using the conditions described above for phosphorylcholine. It was indistinguishable from an authentic specimen (kindly supplied by Dr P. Kemp) in the two chromatographic solvent systems used. Alkaline hydrolysis of the ³²P-labelled lipid isolated by this procedure gave glycerophosphorylinositol with traces of glycerophosphorylcholine and glycerophosphorylethanolamine.

Phosphatidic acid was separated from cardiolipin by chromatography on acid-washed filter paper (Whatman no. 1) in the solvent system butan-1-ol-water-aq. NH₃ (sp. gr. 0.88) (100:15:2, by vol.) (Coulon-Morelec, Faure & Maréchal, 1960). It was extracted from the chromatogram four times with chloroform-methanol (2:1, v/v; 1 ml./0.05 μ g. atom of P). It was indistinguishable from an authentic specimen of phosphatidic acid (kindly supplied by Dr G. Hübscher) in the two chromatographic solvent systems used.

It should be emphasized that the various purified phospholipids may still be mixtures of the diacyl, plasmalogenic and ether forms.

Estimation of radioactivity. ³²P was estimated in solution in a Veal-type Geiger-Müller tube under conditions in which ¹⁴C was not detected. All samples were counted for 10000 counts and background radioactivities were estimated for 1400 counts. The efficiency of counting was 10%. Counts were corrected for radioactive decay from the time of incubation of tissue, and to 100% efficiency.

To estimate ¹⁴C, the phospholipid was hydrolysed in alkali (Lovelock, James & Rowe, 1960), and carrier fatty acid (10 mg.), prepared by the saponification of ox blood, was added. The fatty acids were isolated as described by Lovelock *et al.* (1960). Silica gel (0.01 g.) was added to the solution of the fatty acids in light petroleum (b.p. 40–60°) to adsorb trace components containing ³²P, and then removed and washed four times with 2 ml. of diethyl ether to re-extract any adsorbed fatty acids. Radioactivity was estimated in a Nuclear-Chicago Corp. automatic liquid-scintillation counter. The ¹⁴C-labelled fatty acids from phosphatidylinositol were counted for 1000 counts. All other samples were counted for 10000 counts. Background radioactivities were estimated as for the corresponding samples. The counting efficiency was approx. 80% and all results were corrected to 100% efficiency.

Estimation of phosphorus. Phosphorus was estimated by the method of Berenblum & Chain (1938) with minor modifications (Lyon, Hulse & Rowe, 1965).

RESULTS

Control studies. In experiments to investigate the extent of phospholipid metabolism during the preparation of the subcellular fractions I–IV from brain slices, the results in Tables 1 and 2 were

Table 1. *Lack of incorporation of [³²P]phosphate and [1-¹⁴C]acetate during homogenization*

Brain slices were homogenized in 5 ml. of homogenizing fluid containing 30 μ C of [³²P]phosphate or 10 μ C of [1-¹⁴C]acetate. The homogenate was centrifuged at 105000 *g*_{av} for 1.5 hr. Phospholipids were isolated from the sediment as described in the Methods section.

Radioactive precursor	Wt. of tissue (mg.)	Phospholipid radioactivity (disintegrations/sec./g. of tissue)
[³² P]Phosphate	398	1.1
[³² P]Phosphate	329	0.0
[1- ¹⁴ C]Acetate	371	1.1
[1- ¹⁴ C]Acetate	295	0.0

obtained. There was negligible incorporation of [^{32}P]phosphate and of [^{14}C]acetate into the phospholipids during homogenization (Table 1). There was also negligible loss of radioactivity from pre-labelled phospholipids in brain slices when the slices were homogenized (Table 2).

In further control studies, radioactive fraction II was mixed with a non-radioactive mixture of fractions III and IV and reisolated. Less than 8% of the total phospholipid radioactivity was transferred from fraction II to fraction III (Table 3).

Table 2. *Absence of breakdown of ^{32}P - and ^{14}C -labelled phospholipid during homogenization*

Brain slices (between 290 and 430 mg. of tissue in different experiments) were incubated for 3 hr. in 2 ml. of Krebs-Ringer bicarbonate containing $30\mu\text{C}$ of [^{32}P]phosphate or $10\mu\text{C}$ of [^{14}C]acetate. The slices were either extracted immediately after the incubation and the phospholipids isolated, or the medium was replaced by 5 ml. of homogenizing fluid and the tissue was homogenized. Phospholipids were then isolated after centrifugation as described in Table 1.

Expt. no.	Radioactive precursor	Phospholipid radioactivity (disintegrations/sec./g. of tissue)	
		Immediate extraction	Homogenization and extraction
1	[^{32}P]Phosphate	4160	4080
2	[^{32}P]Phosphate	3340	3260
3	[^{14}C]Acetate	88	90
4	[^{14}C]Acetate	41	41

Table 3. *Transfer of phospholipids between sub-cellular fractions*

Brain slices (350 mg. of tissue) were incubated with $50\mu\text{C}$ of [^{32}P]phosphate for 2 hr. Fraction II was then prepared and separated from the supernatant (fractions III and IV combined) as described in the Methods section. A non-radioactive fraction II and supernatant were also prepared from slices that had been incubated for 2 hr. in the absence of [^{32}P]phosphate. Radioactive fraction II was suspended in the non-radioactive supernatant and reisolated as fraction IIR. From the supernatant, fraction III was prepared. Similarly non-radioactive fraction II was suspended in the radioactive supernatant. From this mixture fraction II was reisolated and fraction IIIR was prepared. Phospholipids were isolated from each fraction.

Expt. no.	Radioactivity in phospholipids (disintegrations/sec.)			
	IIR	IIIR	II	III
1	895	580	72	45
2	1090	728	88	61

Table 4. *Incorporation of [^{32}P]phosphate and [^{14}C]acetate into the phospholipids of subcellular fractions of mouse brain*

Slices (980 mg.) were incubated for 3 hr. with $100\mu\text{C}$ of [^{32}P]phosphate and $100\mu\text{C}$ of [^{14}C]acetate. The phospholipids of cell fractions I, II, III and IV were separated after the incubation as described in the Methods section. In fractions II and IV phosphatidylinositol and sphingomyelin did not separate completely. N.D., Not detected.

Phospholipid	Cell fraction I		Cell fraction II		Cell fraction III		Cell fraction IV	
	Yield (μg . atoms of P)	Radioactivity (disintegrations/sec.)	Yield (μg . atoms of P)	Radioactivity (disintegrations/sec.)	Yield (μg . atoms of P)	Radioactivity (disintegrations/sec.)	Yield (μg . atoms of P)	Radioactivity (disintegrations/sec.)
		^{32}P	^{32}P	^{32}P	^{32}P	^{32}P	^{32}P	^{32}P
		^{14}C	^{14}C	^{14}C	^{14}C	^{14}C	^{14}C	^{14}C
Phosphatidylinositol	2.0	5210	721	95	0.04	138	0.02	24
Sphingomyelin	2.5	1750	0.30	772	0.07	192	—	6
Phosphatidylcholine	15.4	9880	0.99	6670	0.32	873	0.03	70
Phosphatidylserine	6.1	2320	0.26	60	0.04	50	N.D.	36
Phosphatidylethanolamine	7.3	2270	0.47	84	0.15	85	N.D.	—
Phosphatidic acid and cardiolipin	1.7	5110	0.11	23	0.02	39	0.01	5
Total phospholipid before separation	39.5	29500	2.8	3345	1.3	2514	0.20	236
				1146		962		97

Likewise, when radioactive fractions III and IV were mixed with non-radioactive fraction II, reisolation of the fractions revealed that less than 8% of the phospholipid radioactivity had transferred from fraction III to fraction II. The value 8% includes transfer of radioactivity due to incomplete reseparation of the fractions. True exchange of radioactive phospholipid, if it occurred at all, was likely to have been much less.

Incorporation of [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate into the phospholipids of brain slices. When brain slices were incubated for 3 hr. in the presence of [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate, each isotope was incorporated into the various phospholipid classes of the different cell fractions. The detailed findings from one such experiment are shown in Table 4.

The changes with time of the specific activities of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol from fractions II and III with respect to ^{32}P and ^{14}C are shown in Figs. 1 and 2. The $^{32}\text{P}/^{14}\text{C}$ ratios for the isolated phospholipid classes from fractions II and III from the same experiment are shown in Fig. 3. In both tissue fractions, the $^{32}\text{P}/^{14}\text{C}$ ratios of phosphatidic acid and phosphatidylinositol were much higher than those of phosphatidylcholine and phosphatidylethanolamine. The ratios for phosphatidylcholine and phosphatidylethanolamine were similar.

The rates of incorporation of the radioactive precursors varied in different experiments. In all

experiments, however, the relationships, with respect to phospholipid radioactivity, between the tissue fractions and between the phospholipid classes were essentially similar to those that have been described.

DISCUSSION

The aim of the present investigation has been to determine the patterns of incorporation of [^{32}P]phosphate and [$1\text{-}^{14}\text{C}$]acetate into the phospholipids of different subcellular fractions of intact brain slices. For such experiments it is essential that negligible changes in isotope distribution should take place during the isolation of the subcellular fractions. These conditions appear to have been fulfilled in the present study (Tables 1-3).

When brain slices were incubated in a medium containing [^{32}P]phosphate there was considerable incorporation of the ^{32}P into the phosphatidylcholine of the subcellular fractions. Thus, in the experiment shown in Table 4, 44% and 36% of the incorporation of ^{32}P into the total phospholipid of fractions II and III respectively were recovered in phosphatidylcholine. This contrasts with the relatively low incorporation of phosphate into phosphatidylcholine reported for reinforced mitochondrial preparations of both rat brain (McMurray *et al.* 1957*b*) and rat liver (Kennedy, 1953; Marinetti, Erbland, Albrecht & Stotz, 1957), and with the observation by Hokin & Hokin (1958*b*) that with isolated microsomal preparations of guinea-pig brain most of the incorporation of ^{32}P was into

Fig. 1. Incorporation of [^{32}P]phosphate into different phospholipid classes of subcellular fractions II and III from mouse brain slices. (a) Fraction II; (b) fraction III. ●, Phosphatidylcholine; ■, phosphatidylethanolamine; ○, phosphatidic acid; □ phosphatidylinositol.

Fig. 2. Incorporation of [1- 14 C]acetate into the fatty acids of different phospholipid classes of subcellular fractions II and III from mouse brain slices. (a) Fraction II; (b) fraction III. ●, Phosphatidylcholine; ■, phosphatidylethanolamine; ○, phosphatidic acid; □, phosphatidylinositol.

Fig. 3. $^{32}\text{P}/^{14}\text{C}$ ratios of different phospholipid classes from tissue fractions II and III. (a) Phosphatidylcholine; (b) phosphatidylethanolamine; (c) phosphatidic acid; (d) phosphatidylinositol. ○, Fraction II; ●, fraction III.

phosphatidic acid. It appears that isolated tissue fractions lose the ability to synthesize or exchange phospholipids that they possess in tissue slices. Such a loss of ability is also suggested by the reported patterns of incorporation of phosphate into the phospholipid classes of guinea-pig brain slices (Hokin & Hokin, 1958a) and of guinea-pig brain dispersions (Dawson, 1954).

Phosphatidylcholine was also a lipid of major importance in both tissue fractions with respect to incorporation of [^{14}C]acetate. After 3 hr. 67% and 58% of the incorporations of ^{14}C into total phospholipid fatty acid were recovered in the fatty acids of phosphatidylcholine from subcellular fractions II and III respectively. Moreover, phosphatidylcholine was the phospholipid of highest specific

radioactivity (Fig. 2). Incorporation of [^{14}C]acetate could arise from the synthesis of phosphatidylcholine by the Kennedy route (Rossiter & Strickland, 1959; Strickland, Subrahmanyam, Pritchard, Thompson & Rossiter, 1963) or by acylation of lysolecithin (Webster, 1965; Webster & Alpern, 1963).

The isolated phospholipid classes might be expected to include molecules that contained vinyl or saturated ethers. Incorporation of ^{14}C into the fatty acids of these would be measured under the conditions of these experiments but incorporation into the hydrocarbon chains would not.

Although fractions II and III were heterogeneous, they possessed metabolic individuality and the results could not have been due to a single sub-cellular fraction distributed between them. This is indicated by the differences in the specific radioactivity-time-course curves in the two cell fractions, the greatest differences being in the specific radioactivity-time-course curves and $^{32}\text{P}/^{14}\text{C}$ ratios of phosphatidylinositol.

Although the limitations imposed by the inhomogeneity of the tissue fractions and of the phospholipid classes must be borne in mind, the finding that the ratios of the incorporation of [^{32}P]phosphate to that of [^{14}C]acetate into the phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid were very similar in fractions II and III suggests that there may be a close connexion between the metabolism of these phospholipid classes in the fractions. The connexion could be due to a sharing of pools of intermediates, e.g. ATP and acyl-CoA, followed by independent synthesis at different sites, or to independent synthesis followed by intracellular exchange of intact phospholipid.

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