The Incorporation of Radioactive Fatty Acids and of Radioactive Derivatives of Glucose into the Phospholipids of Subsynaptosomal Fractions of Cerebral Cortex

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1. Crude synaptosomal fractions (P_2) from guinea-pig cerebral cortex were incubated in a Krebs–glucose medium containing labelled fatty acids and [^3H]glucose. After the shortest incubation period (7.5 min) a high percentage (50–80%) of the total radioactive fatty acids was found in the P_2 fractions. 2. After the incubation, the synaptosomal fractions were submitted to hypo-osmotic disruption and subsynaptosomal fractionation was carried out by using discontinuous-sucrose-gradient centrifugation. The specific radioactivities of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were determined in fractions D (synaptic vesicles), E (microsomal preparation) and H (disrupted synaptosomes), as were the specific activities of a number of marker enzymes and the distribution of acetylcholine. 3. By using [^{14}C]oleate, [^{14}C]arachidonate, [^3H]palmitate and [^3H]glucose, the order of specific radioactivities in fraction D was found to be: phosphatidylinositol > phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine. 4. The specific radioactivities of phosphatidylcholine and phosphatidylethanolamine were always higher in fraction D than in fraction E . As fraction E had higher specific activities of several membrane marker enzymes, the enhanced labelling found in fraction D was considered to be localized in the synaptic vesicles. In this fraction, phosphatidylinositol made particularly large contributions to the total phospholipid labelling derived from [^{14}C]arachidonate and [^3H]glucose. 5. The similar labelling ratios of fatty acid/glucose in the phospholipids of fractions D and E , and the high specific radioactivities in the total phospholipid of the soluble fraction O , suggested intrasynaptosomal phospholipid transport.

The turnover of phospholipids at the nerve terminal is an interesting field of study and one which has attracted a number of research groups in recent years. By using nerve-ending fractions (synaptosomes) derived from cerebral cortex, acetylcholine has been shown to bring about an increased rate of labelling of phosphatidic acid (Yagihara & Hawthorne, 1972; Yagihara *et al.*, 1973; Abdel Latif *et al.*, 1974) and also possibly of phosphatidylinositol (Lapetina & Michell, 1972; Schacht & Agranoff, 1972; Abdel Latif *et al.*, 1974) in incubations with [^{32}P]P_i. The existence of a cycle involving phosphatidic acid, phosphatidylinositol and *sn*-1,2-diacylglycerol has been postulated for the nerve terminal (Lapetina & Michell, 1972; Bleasdale & Hawthorne, 1975). Yet this metabolic cycle, functioning as such, does not imply a net synthesis of phospholipid *de novo*. Miller & Dawson (1972*a,b*), in analyses of phospholipid synthetic activities and transport phenomena associated with subfractions from brain, presented

evidence for the presence of the terminal enzyme in phosphatidylcholine synthesis in synaptic vesicles. In addition, synaptic vesicles isolated from synaptosomes labelled *in vitro* with CDP-[^{14}C]choline (Miller & Dawson, 1972*a*) and [^{32}P]P_i (Yagihara *et al.*, 1973) showed the highest phospholipid specific radioactivity of any of the membranous subfractions and for ^{14}C -labelled synaptosomes was second only to phospholipid derived from the soluble subsynaptosomal fraction O . During the course of experiments designed to study the turnover of phospholipids during neurotransmitter release (Baker *et al.*, 1975), several noteworthy observations were made with respect to the uptake and metabolism of radioactive fatty acids and of the radioactive derivatives of glucose in synaptosomes and their subfractions. This motivated more thorough studies of the nature of the phospholipid labelling in several of the fractions, particularly with regard to the distribution of membrane marker enzymes and acetylcholine. We present evidence of the labelling of synaptic-vesicle phospholipids which supports the proposed existence of intrasynaptosomal phospholipid synthesis (Miller &

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Dawson, 1972a; Abdel Latif *et al.*, 1973) and the characteristics of the labelling of several subfractions which are consistent with intrasynaptosomal phospholipid transport (Miller & Dawson, 1972b).

Experimental

Materials

The Triton X-100 used in the scintillation mixture was supplied by ICN Chemicals, Cleveland, Ohio, U.S.A. The radiochemicals used in the incubations were supplied by Amersham-Bücher G.m.b.H., Wenden über Braunschweig, F.R. Germany, and had the following specific radioactivities: [1-¹⁴C]arachidonate, 60 mCi/mmol; D-[1-³H]glucose, 11 mCi/mmol (adjusted); [1(3)-³H]glycerol, 50 mCi/mmol (adjusted); [1-¹⁴C]oleate, 58 mCi/mmol; [9,10-³H]-palmitate, 500 mCi/mmol. Guinea pigs were provided by Volksschule, Moringen, F.R. Germany.

Lipid standards were purchased from Supelco, Bellefonte, Pa., U.S.A. and from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tyramine and bovine serum albumin were provided by Serva G.m.b.H., Heidelberg, F.R. Germany, and butylated hydroxytoluene by Fluka A.G., Neu-Ulm, F.R. Germany. Boehringer Mannheim G.m.b.H., Mannheim, F.R. Germany, supplied NADPH, adenosine 5'-monophosphate and cytochrome *c*. 5,5'-Dithiobis-(2-nitrobenzoic acid), NADH and acetylthiocholine iodide were purchased from Sigma (London) Chemical Co. Thin-layer plates (H) were provided by Supelco and by Schleicher und Schüll, G.m.b.H., Dassel, F.R. Germany. [1-¹⁴C]Tyramine (10 mCi/mmol) was supplied by New England Nuclear Chemicals G.m.b.H., Dreieichenhain, F.R. Germany.

Methods

Treatment of radioactive precursors. The radioactive fatty acids, supplied in organic solvents, were evaporated to dryness under a stream of N₂ and approximately neutralized in a small portion of 20 mM-NaOH. Tris/HCl buffer, pH 7.4, containing bovine serum albumin was then added, giving a buffer concentration of 5 mM and a molar ratio of fatty acid/albumin of 5:1. This mixture was then solubilized with mixing at 40°C for a few minutes. In certain cases portions of aqueous solutions of radioactive glucose or glycerol were evaporated to dryness before the addition of the fatty acid/albumin mixture or were added directly to it. Small volumes (50–60 µl) of such a mixture were added to each incubation to give a final concentration of fatty acid or of glycerol of approx. 5 µCi/ml ([³H]-glucose at 112 µCi/ml).

Incubation conditions. Crude synaptosomal fractions (P₂), prepared by the method of Whittaker

et al. (1964) from scraped guinea-pig cerebral cortex, were suspended in Krebs incubation medium, containing 10 mM-glucose and 2.8 mM-CaCl₂ as described by Bradford (1969), at 3 ml/g wet wt. of original cortex. The suspensions were incubated at 37°C in an atmosphere of O₂+CO₂ (95:5) with shaking and preincubated for 15 min. Then a small volume of radioactive mixture was added. After a further 7.5 or 15 min the incubations were stopped by the addition of 4 vol. of ice-cold 0.32 M-sucrose. They were then sedimented at $2.0 \times 10^4 g_{\max}$ for 20 min, washed again and resedimented at $2.5 \times 10^4 g_{\max}$ for 40 min before hypo-osmotic disruption. Fractions were subsequently separated by discontinuous-sucrose-gradient centrifugation of a supernatant fraction W_s, as described by Whittaker *et al.* (1964).

O₂ uptake studies. In respiration studies P₂ fractions from cerebral cortex were suspended in the Krebs medium containing 10 mM-glucose and the O₂ uptake was measured with a Clarke oxygen electrode at 37°C.

Acetylcholine, protein and enzyme assays. Fractions were analysed for acetylcholine after heating samples at 100°C and pH 4 for 10 min. The leech dorsal-muscle strip was used for the bioassay (Whittaker & Barker, 1972).

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Total lactate dehydrogenase (EC 1.1.1.27) and cholinesterase (without inhibitor) activities were assayed as described by Whittaker & Barker (1972). NADPH-cytochrome *c* reductase (EC 1.6.2.4) and cytochrome *c* oxidase (EC 1.9.3.1) were measured by the method of Sottocasa *et al.* (1967). Monoamine oxidase (EC 1.4.3.4) (McCaman *et al.*, 1965) and 5'-nucleotidase (EC 3.1.3.5) (Emmelot *et al.*, 1964) were also assayed.

Lipid extraction, chromatography and analysis. Lipid extracts of subcellular fractions were prepared and purified by the method of Folch *et al.* (1957). In certain cases neutral lipids and phospholipids in the chloroform phase were separated by using short silicic acid columns with chloroform and methanol elutions. Phospholipids were separated by two-dimensional t.l.c. (Turner & Rouser, 1970) or by one-dimensional t.l.c. with the solvent system chloroform/methanol/acetic acid/water (65:43:1:3, by vol.). Butylated hydroxytoluene (5 mg/100 ml) was added to the developing solvent mixtures. The phospholipids were detected with I₂ vapour. The gel areas were collected by aspiration into short Pasteur pipettes plugged with glass wool and eluted with methanol. Dried portions of the eluates or of the extracts were submitted to acid hydrolysis and analysed for phosphate (Bartlett, 1959).

Aqueous radioactive samples, up to 1 ml in volume, were dissolved in 10 ml of a scintillation solution

[toluene/Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (3.3 g/litre) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.13 g/litre)]. Samples of radioactive lipid in organic solvent were evaporated to dryness under a stream of N₂ and dissolved in a similar scintillation solution which did not contain Triton X-100. ³H and ¹⁴C were determined simultaneously in a Packard model 3390 liquid-scintillation spectrometer, with an external standard for calculating the efficiencies (efficiencies of counting: ³H, 37–46%; ¹⁴C, 56–66%). The specific radioactivities of labelled phospholipids were determined by taking portions for radioactivity and for phosphate assays.

The distribution of radioactivity in isolated phospholipids was determined in two ways. Samples of phospholipid were evaporated to dryness and trans-methylated as described by Parkes & Thompson (1970). The fatty acid methyl esters were extracted into *n*-hexane and the radioactivity was determined or the species were separated on the basis of unsaturation by argention t.l.c. (Baker & Thompson, 1972). In a second method, radioactive phosphatidylcholine was hydrolysed by using *Crotalus adamanteus* venom (Keenan & Hokin, 1964). The fatty acids and the lysophosphatidylcholine were separated by one-dimensional t.l.c. as described above.

Results

Radioactive labelling of P₂ fractions

In the presence of 10 mM-glucose, the P₂ fractions suspended in the Krebs incubation medium were found to respire at a rate of 39.8 μmol of O₂/h per 100 mg of protein. This is a value which is comparable with the rate of respiration of more highly purified synaptosomal preparations (Bradford, 1969).

Table 1 presents values for the radioactive labelling of the crude synaptosomal fraction (P₂) after 7.5 and 15 min at 37°C *in vitro* and the corresponding rate of incorporation of radioactivity into the phospholipids of the D fraction after 15 min, based on the specific radioactivities of the labelled compounds used. There was a significant uptake of radioactive glucose into the P₂ fractions, about 1% of that available in the medium after 15 min incubation. A small fraction (0.1%) of that taken up in 15 min was incorporated into the phospholipids of the synaptic-vesicle fraction D, presumably via dihydroxyacetone phosphate formed during the course of glycolysis. More than 90% of the radioactivity derived from [³H]glucose or [³H]glycerol was associated with the glycerol backbone after fatty acids had been removed by trans-methylation. [³H]Glycerol, at lower concentrations than glucose, showed a higher percentage uptake by the P₂ fraction and a higher percentage of that taken up was incorporated into phospholipid. [³H]Glycerol labelled at the 1(3) position can be used to assess both the glycerol 3-phosphate and acyldihydroxyacetone phosphate pathways of phospholipid biosynthesis (Manning & Brindley, 1972).

The rate of labelling of the P₂ fractions by radioactive fatty acids was substantial, as 50–80% was found in the P₂ fraction after 15 min *in vitro*. Similar values were obtained after the shorter incubation period (7.5 min). This was in contrast with the rate of uptake of glucose, which showed a more linear progression from 7.5 to 15 min. The highest percentage labelling was shown for [³H]palmitate, followed by [¹⁴C]-oleate and [¹⁴C]arachidonate. Of the radioactivity taken up, 0.2–0.6% was found in the D fraction phospholipid after 15 min incubation. A rapid rate of uptake and of esterification of radioactive palmitate has been shown in incubations using isolated liver

Table 1. Rate of labelling of crude synaptosomal fractions by radioactive fatty acids, glucose and glycerol and the rate of their incorporation into phospholipid of synaptic vesicles

Crude synaptosomal fractions (P₂) were incubated in the presence of radioactive compounds for 7.5 or 15 min at 37°C. The concentrations of the radioactive compounds in the incubation medium were: glucose, 10 mM; glycerol, 0.1 mM; palmitate, 0.01 mM; oleate, 0.09 mM; arachidonate, 0.09 mM. The P₂ fractions were washed and the uptake was calculated on the basis of the specific radioactivities of the compounds used. The rates of incorporation were calculated in a similar manner based on the labelling of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol in the derived synaptic-vesicle fraction D. The results are means ± S.D. (glucose, oleate, arachidonate) or averages ± range (glycerol, palmitate). The uptake of radioactivity after 15 min, expressed as a percentage of that available in the medium, is given in parentheses. N.D., Not determined.

No. of independent experiments	Radioactive compound	Labelling of fraction P ₂ (nmol/g wet wt. of cortex)		Incorporation into vesicle phospholipid (nmol/g wet wt. of cortex) 15 min
		7.5 min	15 min	
3	[³ H]Glucose	146	255 ± 40 (1.0%)	0.186 ± 0.04
2	[³ H]Glycerol	N.D.	4.8 ± 0.7 (1.7%)	0.041 ± 0.01
2	[³ H]Palmitate	21.0	20.0 ± 1.7 (81%)	0.103 ± 0.01
6	[¹⁴ C]Oleate	132	137 ± 10 (60%)	0.800 ± 0.13
3	[¹⁴ C]Arachidonate	111	114 ± 14 (50%)	0.193 ± 0.02

cells (Ontko, 1972). Argentation t.l.c. of the derived fatty acid methyl esters indicated less than a 5% change in the degree of unsaturation of the radioactive fatty acids used in the incubations.

With each fatty acid used the labelling ratios of the disrupted P_2 fractions, W_s (vesicle-rich supernatant): W_p (mitochondria-rich pellet), were similar and fell within a range of 0.25–0.30. Thus the values for the rate of labelling of P_2 fractions by radioactive fatty acids were representative of the labelling of W_s and the derived subsynaptosomal fractions. Further, 60% of the total glucose taken up was found in the W_s fraction after both incubation times.

Table 2 gives the percentage distribution of the radioactive compounds among the subsynaptosomal fractions. Radioactive glucose was found predominantly in the soluble fraction O (similar findings were made for radioactive glycerol), whereas the radioactive fatty acids were localized mainly in fractions D , E and $F+G$. Fraction D (synaptic vesicles) made the highest percentage contribution with radioactive oleate and arachidonate. Less than 9% of the fatty acid radioactivity was associated with the soluble fraction O .

Phospholipid labelling in subcellular fractions

Fig. 1 shows the specific radioactivities of phospholipids of subsynaptosomal fractions D (synaptic vesicles), E (microsomal fractions) and H (disrupted synaptosomes) derived from P_2 fractions after 7.5 or 15 min incubations in the presence of radioactively labelled fatty acids or glucose. There are a number of features of interest. With each radioactive compound used, the specific radioactivities of phosphatidylcholine and phosphatidylethanolamine in the synaptic-vesicle fraction D were approximately twice the corresponding values for the E fraction. However,

in comparing the specific radioactivities of phosphatidylserine plus phosphatidylinositol, the values for fractions D and E were quite similar for labelling derived from [^3H]glucose and [^{14}C]arachidonate, whereas the D values were actually lower (approx. 60% of the E values) for ^3H , 16:0 and ^{14}C , 18:1 labelling. The specific radioactivity of phosphatidylinositol of fraction D was found to be several if not many times the value for phosphatidylserine. The results of some preliminary experiments suggested that this was true for the phosphatidylinositol of fraction E as well. The specific radioactivities of the phospholipids of fraction H were generally 50% or less of the corresponding values for the D and E fractions. Work with synaptosomes *in vitro* using [^{32}P]P_i (Yagihara *et al.*, 1973) or CDP-[^{14}C]choline (Miller & Dawson, 1972a) has shown that the D fraction phospholipids had higher specific radioactivities than those of the E fraction.

With each radioactive precursor used, the order of specific radioactivities in fraction D was found to be phosphatidylinositol > phosphatidylcholine > phosphatidylserine > phosphatidylethanolamine. This was also found to be the case in some preliminary studies with [^3H]glycerol. The largest difference in labelling between phosphatidylinositol and phosphatidylcholine of fraction D was found with [^{14}C]arachidonate, whereas the smallest was observed for [^3H]palmitate. Phosphatidylcholine and phosphatidylserine of fraction D had specific radioactivities that were quite close, except for [^{14}C]arachidonate where phosphatidylcholine had values twice as high. Studies *in vivo* (Miller & Dawson, 1972a) and *in vitro* (Yagihara *et al.*, 1973) using [^{32}P]P_i and [^{14}C]glycerol (Lunt & Lapetina, 1970) with synaptosomal preparations indicated a similar order of labelling of these phosphoglycerides. However, work with [^{14}C]glucose and brain slices (Lapetina & Michell, 1974)

Table 2. Distribution of radioactive glucose and fatty acids among subsynaptosomal fractions

Crude synaptosomal fractions (P_2) were incubated in the presence of radioactive compounds for 15 min at 37°C. After the incubation the P_2 fractions were washed, hypo-osmotically disrupted and a derived supernatant fraction (W_s) was fractionated by discontinuous-sucrose-gradient centrifugation. The distribution of the radioactivity among the subfractions is expressed as a percentage of the total recovered, and individual values are means $\pm$ S.D. or averages (palmitate, arachidonate). The number of independent determinations is given in parentheses. The concentrations of the radioactive compounds are given in Table 1.

Subfraction	Percentage distribution			
	[^{14}C]Oleate (4)	[^3H]Palmitate (2)	[^{14}C]Arachidonate (2)	[^3H]Glucose (3)
<i>O</i>	7.3 $\pm$ 1.9	2.4	8.2	86.6 $\pm$ 1.2
<i>D</i>	29.2 $\pm$ 1.0	23.5	32.6	10.3 $\pm$ 1.0
<i>E</i>	23.8 $\pm$ 2.6	15.3	17.4	2.9 $\pm$ 0.7
<i>F+G</i>	25.7 $\pm$ 5.0	36.5	21.9	—
<i>H</i>	11.0 $\pm$ 2.6	15.2	13.1	—
<i>I</i>	3.1 $\pm$ 1.4	5.9	6.9	—

Fig. 1. Histogram of specific radioactivities of phospholipids of fractions D, E and H derived from crude synaptosomal fractions of guinea-pig cerebral cortex

The specific radioactivities of the major phosphoglycerides expressed per μmol of phospholipid phosphate are presented as means $\pm$ s.d. of three independent determinations (for [^3H]palmitate, averages $\pm$ ranges). The shaded blocks for the fatty acid labelling represent the specific radioactivities determined after 7.5 min incubation (averages or means of two or three independent determinations) and the upper limiting bars represent the value after 15 min incubation. Radioactivity was added after a 15 min preincubation period and incubations were carried out at 37°C under an $\text{O}_2 + \text{CO}_2$ (95:5) atmosphere. The amounts of radioactivity in the incubation medium were $5 \mu\text{Ci/ml}$ for fatty acid and $112 \mu\text{Ci/ml}$ for [^3H]glucose. After incubation, P_2 fractions were washed, hypo-osmotically disrupted and submitted to discontinuous-sucrose-density-gradient centrifugation to give the subsynaptosomal fractions that were subsequently analysed for phospholipid. PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. The radioactive precursors used were (a) [^3H]glucose, (b) [^3H]palmitate, (c) [^{14}C]oleate and (d) [^{14}C]arachidonate.

Table 3. *Marker enzyme, protein and acetylcholine concentrations in cerebral-cortex subfractions*

The methods of enzyme, protein and acetylcholine assay are given in the text. P_2 is the crude synaptosomal fraction and W_s and W_p are the supernatant and pellet fractions respectively derived after osmotic shock. The fractions $O-I$ (Whittaker *et al.*, 1964) are the subfractions derived from W_s ; D and E denoted synaptic vesicles and microsomal fraction respectively. The spectrophotometric assays used cuvettes with a 1 cm light-path. The results are means $\pm$ S.D. of three independent experiments. The units used are: total lactate dehydrogenase, $\Delta E_{340}/\text{min}$; cholinesterase, $\Delta E_{412}/\text{min}$; NADPH-cytochrome *c* reductase, $10 \times \Delta E_{550}/\text{min}$; cytochrome *c* oxidase, $\Delta E_{550}/\text{min}$; monoamine oxidase, $\mu\text{mol}/30\text{min}$; 5'-nucleotidase, $\mu\text{mol}/20\text{min}$. N.D., Not determined.

Fraction	Acetylcholine (nmol/g wet wt.)	Protein (mg/g wet wt.)	Activity (units/g wet wt. of cortex)					
			Lactate dehydrogenase	Cholinesterase	NADPH-cytochrome <i>c</i> reductase	Cytochrome <i>c</i> oxidase	Monoamine oxidase	5'-Nucleotidase
P_2	3.65 ± 0.62	26.3 ± 0.3	52.2 ± 6.0	7.1 ± 1.6	4.1 ± 1.1	28.8 ± 4.0	0.12 ± 0.04	17.0 ± 0.3
(% distribution of activities based on fraction P_2)								
W_p	31.5	60.1	29.6	47.7	37.3	76.7	82.6	64.6
W_s	67.5	31.2	60.3	38.7	50.0	23.3	17.4	35.4
Wash	N.D.	8.7	10.1	13.5	12.7	—	N.D.	N.D.
	Acetylcholine (% of that in fraction W_s)	Protein (% of that in fraction W_p)	Activity (units/mg of protein)					
			Lactate dehydrogenase	Cholinesterase	NADPH-cytochrome <i>c</i> reductase	Cytochrome <i>c</i> oxidase	Monoamine oxidase	5'-Nucleotidase
O	9.0	38.1	4.8	0.13	0.63	—	—	0.23
D	52.0	11.1	2.8	0.41	0.42	—	0.21	0.73
E	10.4	7.0	1.3	1.01	1.01	—	2.35	1.41
$F+G$	10.0	18.7	1.1	1.01	0.46	—	1.75	1.02
H	15.0	15.6	1.3	0.38	0.25	0.83	4.90	1.34
I	3.6	9.4	0.4	0.17	0.08	4.38	9.39	0.63
Recovery	110%	105%	103%	104%	120%	90%	106%	102%

has shown that phosphatidylcholine had a higher specific radioactivity than phosphatidylinositol.

In rat brain *in vivo*, phosphatidylinositol has been shown to have the highest specific radioactivity on a molar phosphate basis, after the intracerebral injection of [^{14}C]glycerol, [^3H]arachidonate or [^3H]stearate (Baker & Thompson, 1972). An analogous situation was demonstrated in a synaptosome-rich fraction of mouse brain after the intracerebral injection of radioactive oleate (Sun, 1973), but not with palmitate (Sun & Horrocks, 1973).

Distribution of marker enzymes and acetylcholine

Table 3 shows the characterization of the subsynaptosomal fractions with respect to the distribution of marker enzyme activities and acetylcholine for a sample after 15 min incubation (i.e. 30 min *in toto*). The D fraction was the richest in acetylcholine whereas the H fraction showed a decreased percent-

age concentration of acetylcholine when compared with values obtained with unincubated P_2 fractions (Whittaker *et al.*, 1964). The distribution of lactate dehydrogenase activity between the W_s and W_p fractions was noteworthy, as it reflected the distribution of [^3H]glucose in these two fractions. On the basis of the specific activities of the marker enzymes, the E fraction had a higher specific content of microsomal material (NADPH-cytochrome *c* reductase) (Miller & Dawson, 1972a), mitochondrial outer membrane (monoamine oxidase) and plasma membrane (5'-nucleotidase) than did fraction D . Cholinesterase also had a higher specific activity in the E fraction. These relations were still valid after corrections were made for the contribution of soluble protein to fractions D and E . The enzyme and acetylcholine distributions were similar in fractions derived from P_2 samples incubated for the shorter incubation time (7.5 min) after the preincubation period. The distributions of NADPH-cytochrome *c* reductase, lactate dehydrogenase and cholinesterase among the synaptosomal

Table 4. Ratios of labelling of incorporated fatty acid/radioactivity derived from glucose in phospholipids of fractions D and E

Synaptosomal fractions (P_2) were incubated with radioactive fatty acids and glucose for 15 min after a 15 min preincubation period at 37°C. The fractions were washed, hypo-osmotically disrupted and the derived subfractions were analysed for phospholipid. Results are means (three independent experiments) or averages (two independent experiments); the numbers of determinations are given in parentheses.

Fraction	Phospholipid	Labelling ratio (fatty acid/glucose)		
		[^3H]Palmitate (2)	[^{14}C]Oleate (3)	[^{14}C]Arachidonate (3)
D	Phosphatidylcholine	27.1	26.6	4.9
	Phosphatidylethanolamine	47.7	33.9	5.6
	Phosphatidylserine plus phosphatidylinositol	14.8	15.2	6.1
E	Phosphatidylcholine	27.4	19.7	4.7
	Phosphatidylethanolamine	70.3	43.4	8.3
	Phosphatidylserine plus phosphatidylinositol	20.3	20.0	5.4

Table 5. Comparison of specific radioactivities of phospholipid in fractions O and D

The phospholipid of fraction O was separated from neutral lipid by silicic acid chromatography. The specific radioactivity of the total phospholipid extract was expressed as a relative specific radioactivity of the combined phospholipids of fraction D. The results are means $\pm$ s.d. (three independent experiments) for a 15 min incubation. The values given in parentheses are $10^{-3} \times$ radioactivity (d.p.m./g wet wt. of cortex) for total phospholipid.

	Relative specific radioactivity in phospholipid	
	O	D
[^3H]Palmitate	8.9 $\pm$ 1.9 (4.17)	1.0 (11.4)
[^{14}C]Oleate	3.4 $\pm$ 0.8 (13.5)	1.0 (104)
[^{14}C]Arachidonate	48 $\pm$ 24 (48.8)	1.0 (24.9)
Phospholipid (μmol of phosphate/g wet wt. of cortex)	0.041 $\pm$ 0.014	0.61 $\pm$ 0.15

fraction E and a similar situation existed for phosphatidylserine plus phosphatidylinositol with [^3H]palmitate and [^{14}C]oleate labelling. Phosphatidylserine plus phosphatidylinositol had comparable ratios in fractions D and E labelled with [^{14}C]arachidonate.

Table 5 presents the specific radioactivities of the total phospholipid in the soluble fraction O as ratios of the corresponding values for fraction D phospholipid. Also shown is the total radioactive phospholipid labelling in fractions O and D. The O fraction had very low concentrations of phospholipid phosphate and it was not possible to calculate specific radioactivities for the individual phospholipids of this fraction. Phosphatidic acid was isolated from the D fraction in several experiments. An approximation of the contribution of this phospholipid to the specific radioactivity of the D fraction did not greatly decrease the relative specific radioactivity ratios in the O fraction, which were found to be quite high. The difference between the O and D fractions was most pronounced for [^{14}C]arachidonate labelling. To the total radioactive phospholipid labelling a higher contribution was made by fraction D with ^3H 16:0 and ^{14}C 18:1 and a contribution of 50% of the fraction O value with ^{14}C 20:4. Miller & Dawson (1972a) have reported a high specific radioactivity of the phospholipid derived from the O fraction of intact synaptosomes labelled *in vitro* with CDP-[^{14}C]choline. Also of interest was a decrease in this value of almost 90% if the isolated O fraction was incubated with this radioactive compound (Miller & Dawson, 1972a).

Table 6 gives the percentage of the incorporated radioactivity in fraction D contributed by each of the four phosphoglycerides under study. Phosphatidylcholine accounted for the largest portion of the radioactivity incorporated into phospholipid except for [^{14}C]arachidonate, where phosphatidylinositol made

subfractions were similar to values presented earlier (Whittaker *et al.*, 1964; Miller & Dawson, 1972a).

Phospholipid labelling characteristics

Table 4 presents the ratios of the specific radioactivities of incorporated fatty acids to those derived from [^3H]glucose in the phospholipids of fractions D and E after 15 min incubation. [^{14}C]Oleate was usually incubated in the presence of [^3H]glucose, and [^{14}C]arachidonate in the presence of [^3H]glucose or of [^3H]palmitate. Thus the ratios for palmitate are derived values. The ratios for phosphatidylcholine in fractions D and E labelled with [^3H]palmitate or with [^{14}C]arachidonate were quite similar. The ratios for phosphatidylethanolamine were always higher in

Table 6. Rate of incorporation of radioactivity into phospholipids of fraction D

By using the specific radioactivities and relative abundancies of phospholipids of fraction D (Baker *et al.*, 1975), values for the rates of incorporation are expressed as percentages of the total incorporation into the combined phospholipids of fraction D derived from 1 g of cortex. The results are taken from 15 min incubations and are means $\pm$ s.d. of three independent determinations, except for [^3H]palmitate where values are averages $\pm$ ranges of two determinations.

Phospholipid	Incorporation into combined phospholipid/g wet wt. of cortex (%)			
	[^3H]Glucose	[^3H]Palmitate	[^{14}C]Oleate	[^{14}C]Arachidonate
Phosphatidylcholine	42.2 $\pm$ 3.9	45.6 $\pm$ 6.8	49.3 $\pm$ 6.8	37.5 $\pm$ 3.7
Phosphatidylethanolamine	15.5 $\pm$ 3.1	29.1 $\pm$ 2.2	22.8 $\pm$ 4.2	15.6 $\pm$ 2.4
Phosphatidylserine	12.3 $\pm$ 3.0	12.6 $\pm$ 2.3	12.6 $\pm$ 2.3	6.8 $\pm$ 0.8
Phosphatidylinositol	30.0 $\pm$ 6.1	12.6 $\pm$ 1.3	15.3 $\pm$ 2.2	40.1 $\pm$ 2.8

essentially an equal contribution. There was also a substantial contribution made by phosphatidylinositol with label derived from [^3H]glucose. Phosphatidylethanolamine made its largest contributions with [^3H]palmitate and with [^{14}C]oleate labelling, and phosphatidylserine generally made the smallest contribution of the four phosphoglycerides.

The positional incorporation of the radioactive fatty acids has been analysed for phosphatidylcholine from fraction D. The distribution of [^{14}C]oleate was essentially random between positions 1 and 2, but [^3H]palmitate showed a somewhat higher distribution at position 1 (60–70%) and [^{14}C]arachidonate was predominantly localized at position 2 (>80%). Somewhat similar distributions of fatty acids have been reported for the phosphatidylcholine of rat brain synaptic vesicles (Kishimoto *et al.*, 1969), particularly with regard to the polarized distribution of arachidonate. An essentially random incorporation of radioactive oleate and of palmitate has been noted for phosphatidylcholine of rat brain slices *in vitro* (Webster, 1967).

A number of the differences in phospholipid labelling and enzyme marker distributions between fractions D and E have been statistically analysed for their significance, e.g. the higher specific radioactivities of the phosphatidylcholine and phosphatidylethanolamine of fraction D (Fig. 1), the higher specific radioactivities of phosphatidylserine plus phosphatidylinositol labelled with [^3H]palmitate and [^{14}C]oleate in fraction E (Fig. 1) and the higher specific activities of membrane marker enzymes in fraction E (Table 3). By using Student's *t* test, the differences were significant at $P < 0.05$.

Discussion

The Krebs–glucose medium described by Bradford (1969) has been used in these incubations as this supported a linear respiration of the P_2 fractions *in vitro*. Such media, devoid of other substrates or cofactors, have also been shown to support the synthesis of phospholipid in synaptosomes *in vitro* (Lunt & Lapeina, 1970). The concentration of labelled oleate and

of arachidonate in the incubation medium was close to 0.1 mM, a value which approximates to the physiological concentrations of unesterified fatty acid in mammalian brain (Bazan & Joel, 1970). Palmitate was used at a much lower concentration to gain an optimum solubilization of this saturated fatty acid.

The rapid labelling of the P_2 fraction by radioactive fatty acids was quite an interesting finding. A rapid turnover of radioactive fatty acids (5 min half-life) has been shown in experiments with mouse brain *in vivo* (Sun, 1973; Sun & Horrocks, 1973; Yau & Sun, 1974); certainly our data indicated a maximum rate of radioactive labelling of the P_2 fraction within 7.5 min *in vitro*. The P_2 fraction, as judged by its pellet volume before and after incubation, made only a small contribution to the volume of the incubation mixture. Yet 50–80% of the radioactive fatty acid was associated with the P_2 fraction. Further, radioactive palmitate, a saturated fatty acid, showed the highest percentage of radioactive labelling even though its concentration was the lowest of the three fatty acids used. The similar distribution of labelled glucose, of lactate dehydrogenase and of acetylcholine between fractions W_s and W_p , suggested primarily a synaptosomal uptake of glucose. There is a high-affinity transport of glucose into synaptosomes (Heaton & Bachelard, 1973) and the rate of uptake of radioactivity derived from glucose after 7.5 min was close to the corresponding values for labelling with radioactive oleate and arachidonate (although some radioactivity derived from glucose would be lost during oxidative phosphorylation). This finding was even more significant as the molar concentrations of fatty acid used were 1% (oleate, arachidonate) and 0.1% (palmitate) of that of glucose in the incubation medium. This would suggest that more than just a passive diffusion of fatty acids into the P_2 fraction occurs. The ATP- and CoA-dependent formation of palmitoylcarnitine from palmitate by preparations of erythrocyte 'ghosts' has been taken as evidence for the involvement of carnitine in the uptake of unesterified fatty acids by erythrocytes (Wittels & Hochstein, 1967). A similar hypothesis has been

made on the basis of the formation of palmitylcarnitine in synaptosomal preparations (Abdel Latif *et al.*, 1973). As subsynaptosomal localization of the radioactively labelled palmitoylcarnitine or of palmitoyl-CoA synthetase activities was not carried out, an unequivocal statement on the role of carnitine in synaptosomes cannot be made. Nonetheless, the relatively high percentage of radioactive labelling by palmitate (as compared with other fatty acids) in our experiments is noteworthy with regard to this theory. The ratios of fatty acid labelling in the W_s and W_p fractions suggested the existence, in part, of a non-synaptosomal fatty acid uptake. The mitochondria of the P_2 fraction are possible candidates. However, the W_s labelling, on subfractionation, was found to be mainly associated with fractions D , E and $F+G$, which have low amounts of mitochondrial contamination as judged by marker enzyme analyses. The substantial contribution of the D fraction to the total labelling by each of the radioactive fatty acids (Table 2) indicated that the radioactive labelling of the P_2 fractions was, in part, a reflexion of synaptosomal fatty acid uptake. This proposal was strengthened as the amount of fatty acid radioactivity in fraction D could not be solely ascribed to contamination by other membranes on the basis of distribution of membrane marker enzymes (Table 3). The cross-contamination is considered in more detail below.

Our interests in the phospholipid labelling of these fractions centred mainly on the subsynaptosomal fractions D , which was shown to be richest in bound acetylcholine and H , which has been shown by Barker *et al.* (1972) to have a compartment of acetylcholine with a particularly high turnover. Fraction E was studied to assess the labelling contributed primarily by microsomal membranes in comparison with the amounts of phospholipid labelling in fractions D and H .

The specific radioactivities of labelled phospholipids isolated from the subsynaptosomal fractions D , E and H indicated that the values for phosphatidylcholine and phosphatidylethanolamine were highest in fraction D . This situation was not only true for the labelled fatty acids used, which can enter into phospholipids independently of synthesis *de novo* in brain (Webster & Alpern, 1964; Baker & Thompson, 1973). It was also true for the radioactivity derived from [3H]glucose and [3H]glycerol, which, being localized in the glycerol backbone of the phospholipid, were better indications of synthesis *de novo*.

The significant labelling of the phospholipids of fraction D , which is rich in synaptic vesicles, prompted a more thorough characterization of the synaptosomal subfractions with respect to marker enzyme activities. Fraction E had the highest specific activity of NADPH-cytochrome *c* reductase, which is a marker in brain for the microsomal fraction (Miller & Dawson, 1972a). This was also true for cholinesterase,

which is a marker for plasma membranes and for microsomal fractions derived from cholinergic cells in mammalian brain. It is also true for 5'-nucleotidase, which is a marker for plasma membranes. Monoamine oxidase, a marker for the mitochondrial outer membrane, had a tenfold higher specific activity in fraction E than in fraction D . These findings with respect to fractions D and E held even when corrections were made for soluble-protein contamination. As in earlier analyses of the labelling of phospholipids of subsynaptosomal fractions (Dawson *et al.*, 1972; Miller & Dawson, 1972a; Yagihara *et al.*, 1973) the lower specific activities of these marker enzymes in fraction D , compared with fraction E , suggested that the labelling of fraction D phospholipids was not due to contamination by these non-vesicular membranes.

As the O -fraction phospholipid had high specific radioactivities (Table 5), it was considered that soluble contamination might account for the radioactive phospholipid labelling of fraction D . Although it was not feasible to determine specific radioactivities of individual phospholipids of fraction O , a comparison of the total phospholipid radioactivities (per g wet wt. of cortex) of fractions O and D was made. Fraction D had a higher total radioactive phospholipid labelling than fraction O with 3H 16:0 and ^{14}C 18:1 and a value which was 50% of that of fraction O for ^{14}C 20:4. Assays of lactate dehydrogenase (Table 3) showed a 70% and 11% distribution of enzyme activity in fractions O and D respectively. Thus the distribution of radioactivity did not follow that of the soluble protein marker. It was therefore considered unlikely, particularly for radioactive palmitate and oleate, that the D fraction phospholipid labelling could be accounted for by soluble contamination.

On the basis of these considerations, the phospholipid labelling in fraction D was probably associated with synaptic vesicles. Similar conclusions have been forwarded for synaptic vesicles of guinea-pig cerebral cortex, labelled with [^{32}P]P_i (Yagihara *et al.*, 1973; Bleasdale & Hawthorne, 1975) and CDP-[^{14}C]choline (Miller & Dawson, 1972a).

Fraction H , which is derived from unincubated P_2 fractions, has been shown to have a high acetylcholine turnover and it is suggested that it has vesicles that are more involved during neurotransmitter release than synaptic vesicles of fraction D (Barker *et al.*, 1972). We found only relatively low phospholipid labelling associated with the former fraction. This is not inconsistent with the earlier acetylcholine turnover studies (Barker *et al.*, 1972), since the amount of vesicular phospholipid in fraction H probably only constitutes a small fraction of the total phospholipid. We have also shown that stimulation *in vitro* of neurotransmitter release from the P_2 fractions does not significantly increase phospholipid labelling in fractions D , E or H (Baker *et al.*, 1975).

The lower specific radioactivities of phosphatidylcholine and phosphatidylethanolamine in fraction *E* compared with fraction *D* suggested a lack of significant extrasynaptosomal phospholipid synthesis. This was a reasonable finding, as extrasynaptosomal microsomal and mitochondrial fractions were deprived of substrates and of cofactors which are essential for phospholipid turnover, by the rudimentary nature of the Krebs glucose incubation medium used. In the absence of suitable substrates for mitochondrial respiration and because of the concentration of Na^+ in the medium (Bradford, 1969), no significant ATP generation outside the synaptosomes would also be expected.

A clear distinction should be made between labelled phospholipids occurring in membranes and the potential of membranes to metabolize phospholipids. By using liver (Wirtz & Zilversmit, 1968; McMurray & Dawson, 1969) and brain (Miller & Dawson, 1972*b*) subfractions, a transfer of phospholipids between microsomal and mitochondrial fractions and also between a brain supernatant fraction and subsynaptosomal fractions (Miller & Dawson, 1972*b*) has been demonstrated *in vitro*. Miller & Dawson (1972*a*) also demonstrated the ability of synaptic vesicles to synthesize phosphatidylcholine from CDP-choline and *sn*-1,2-diacylglycerol. These authors, as well as Abdel Latif *et al.* (1973), proposed the existence of a limited amount of endoplasmic reticulum within synaptosomes. Our results, particularly the labelling of the *D* fraction phospholipids, support the proposal for intrasynaptosomal phospholipid synthesis.

The similarity of the labelling ratios of fatty acid to radioactivity derived from [^3H]glucose in corresponding phospholipids of fractions *D* and *E* was noteworthy. The double-label ratios permit an assessment of the radioactive labelling of different portions of the phosphoglyceride molecule, namely the esterified fatty acids and the glycerol backbone. This technique is useful, as fatty acids may be introduced by acyl-exchange mechanisms at the level of the phosphoglyceride end product (e.g. phosphatidylcholine, phosphatidylinositol; Webster & Alpern, 1964; Baker & Thompson, 1973), as well as by synthesis *de novo*, which is more accurately reflected by the labelling of the glycerol backbone by derivatives of [^3H]glucose. Thus if there were, for example, two separate and isolated sites of phosphatidylcholine synthesis, the two product phosphatidylcholines could differ in their labelling ratios because of differing relative rates of labelling by fatty acid and derivatives of glucose, the existence of different acyl-transferase activities or the possibility of precursor pools with different specific radioactivities. As phospholipid double-labelling ratios were obtained which had similar values in fractions *D* and *E*, a close metabolic relationship between the membranes of the two fractions was suggested. For example, there was the

possibility of phospholipid exchange between the membranes.

The high phospholipid specific radioactivities in fraction *O* indicated the possibility of a soluble phospholipid transport agent within the synaptosome. It would seem unlikely that all the labelled phospholipids attributable to the synaptic vesicles were synthesized by the vesicles. Thus there may be more than one site of synaptosomal phospholipid synthesis, as suggested by Cotman *et al.* (1971). On the basis of these considerations, an exchange of products, e.g. between vesicles and putative intrasynaptosomal endoplasmic reticulum, may be a more likely estimation of the situation *in vivo*.

A comparison of the absolute rates of incorporation of the different fatty acids into the phospholipids of fraction *D* is complicated by the probable release of unesterified fatty acids (by the action of lipases possibly acting on neutral glycerides) during the incubations and by the subsequent dilution of radioactive precursor. However, on the basis of data for the release of unesterified fatty acids in rat brain *post mortem* (Bazan, 1970) and from incubated cerebral-cortex synaptosomal fractions (Price & Rowe, 1972), the higher rate of incorporation of oleate than of arachidonate into the *D* fraction phospholipid (Table 1) was likely to be valid. Comparisons of the percentage contribution of each phospholipid to the total, with each precursor used, were of interest. The results indicated quite a substantial contribution of phosphatidylinositol with [^{14}C]arachidonate and radioactivity derived from radioactive glucose. The former observation may reflect the high concentration of arachidonic acid in the inositides (Holub *et al.*, 1970; Baker & Thompson, 1972; Breckenridge *et al.*, 1973). However, the relatively high rate of incorporation of radioactivity derived from [^3H]glucose (localized in the phospholipid glycerol backbone) was in contrast with results of studies using brain slices and [^{14}C]glucose, in which phosphatidylcholine was the major contributor (Lapetina & Michell, 1974). This would emphasize the relatively high rate of synthesis *de novo* of phosphatidylinositol at the nerve terminal as compared with whole brain and underline the possible functional importance of this phosphoglyceride at the synapse.

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