The Incorporation of Long-Chain Fatty Acids into Phospholipids of Respiring Slices of Rat Cerebrum

By G. R. WEBSTER

Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E. 1

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1. Respiring slices of adult rat cerebrum have been shown to incorporate long-chain ^{14}C -labelled fatty acids into phospholipid. 2. Labelling was almost entirely confined to lecithin and ethanolamine phospholipid, only traces being present in serine phospholipid. 3. Palmitic acid, oleic acid and linoleic acid were incorporated more actively into lecithin than into ethanolamine phospholipid, but the converse was found with stearic acid. 4. All four acids labelled the 1- and 2-positions of both lipids; palmitic acid, oleic acid and linoleic acid were approximately evenly distributed, but stearic acid was incorporated predominantly at the 1-position. 5. It is considered that incorporation is most likely brought about through acylation of endogenously derived lysophosphatides. 6. The possible implications of this pathway of lipid metabolism in nervous tissue are discussed.

It was reported by Webster & Thompson (1964) that when slices of rat cerebrum were incubated in a physiological medium at pH 7.4 lysolecithin was formed. The amounts accumulating during 4 hr. incubation at 37° were considerably greater in anaerobic than in aerobic conditions, and this difference was considered to be due to re-esterification of long-chain fatty acids to lysolecithin in the presence of oxygen, when ATP and acyl-CoA formation could take place by the sequence of reactions described by Kornberg & Pricer (1953).

The results of these experiments with brain slices were consistent with previous studies which demonstrated that phospholipase A-like activity (phosphatide acyl-hydrolase), capable of forming lyso compounds from diacylglycerophosphatides, was present in aqueous-glycerol extracts of human and rat brains (Gallai-Hatchard, Magee, Thompson & Webster, 1962), and that lysolecithin is a normal minor constituent of brain tissue (Webster & Thompson, 1962). In addition, other studies (Webster, 1962, 1965; Webster & Alpern, 1964) showed that brain homogenates and subcellular particles, in a system containing ATP, CoA, Mg^{2+} ions and ^{14}C -labelled fatty acids, could bring about acylation of added lysophosphatides by an enzymic mechanism similar to that described originally in liver by Lands (1960).

It is known from the work of Korey & Orchen (1959) and of Pritchard (1963) that ^{14}C -labelled fatty acids can be incorporated into brain lipids *in vivo*, after intraperitoneal administration to young rats. Moreover, variation of the fatty acid composition of the diet of weanling rats (Witting, Harvey, Century & Horwitt, 1961; Mohrhauer &

Holman, 1963) and of more mature (8–12 weeks-old) rats (Rathbone, 1965) results in changes in the fatty acid composition of lipids in the brain. It seemed possible that incorporation of exogenous fatty acids into glycerophosphatides in brain could be brought about by acylation of endogenously derived lysophosphatides, and thus provide a mechanism for the exchange of long-chain fatty acids between brain and body fluids. Since the acylating enzyme systems of adult rat brain have appreciable activity (Webster & Alpern, 1964) such a process could be of importance in mature animals. To investigate this possibility it was decided to study first the incorporation of various ^{14}C -labelled acids into the lipids of respiring slices of rat cerebrum. Initial experiments with [^{14}C]oleic acid showed that appreciable incorporation occurred only into lecithin and ethanolamine phospholipid, and studies were also made on the distribution of the incorporated acids between the 1- and 2-positions of these lipids. A preliminary account of some of this work has already been reported (Webster, 1966).

MATERIALS AND METHODS

Fatty acids (99% pure) and butylated hydroxytoluene were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks. The 1- ^{14}C -labelled fatty acids and [^{14}C]hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks. AMP (sodium salt) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and crystalline bovine plasma albumin from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. Silicic acid (A.R. 100-mesh) was from Mallinckrodt Chemical Works, New York, N.Y., U.S.A., and silica gel (Kieselgel G and H) from E. Merck A.-G., Darmstadt, Germany. 2,5-Diphenyloxazole and

1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh, and from the Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A. Xylene (sulphur-free) was from May and Baker Ltd., Dagenham, Essex. Other chemicals, except for naphthalene (G.P.R.), were of A.R. grade; CHCl_3 and methanol were redistilled before use, CHCl_3 being immediately restabilized with 2% (by vol.) of methanol.

Freeze-dried *Agkistrodon piscivorus piscivorus* venom was obtained from Ross Allen's Reptile Institute, Silver Springs, Fl., U.S.A.

Solutions. Stock solutions of unlabelled fatty acids were made in CHCl_3 and of radioactive fatty acids in benzene; these solutions were stored in a deep-freeze. [^{14}C]Hexadecane for use as a radioactive reference standard was dissolved in xylene to give solutions with about 2000 disintegrations/min./100 μl . Krebs-Ringer phosphate (Krebs, 1933), pH 7.4, with the CaCl_2 omitted, and solutions of AMP (15 mM) and of glucose (165 mM) in this medium were freshly prepared for each experiment.

Solutions of fatty acid-albumin complexes were also freshly prepared; portions of stock fatty acid solutions containing 10 μC of ^{14}C -labelled acid and 10 μmoles of unlabelled acid were pipetted into a glass-stoppered tube and the solvents evaporated; 2 ml. of 0.9% NaCl and 0.1 ml. of 0.1 N-NaOH were then added and the tube was heated at 100° for 5 min.; after cooling to room temperature 125 mg. of bovine plasma albumin was added, and the tube was shaken mechanically for 5 min.

Incubation procedure. Adult rats (about 200 g. body wt.) were killed by decapitation and the cerebral hemispheres immediately removed and blotted free of obvious blood on a filter paper. Coronal slices were cut free-hand with a safety-razor blade moistened in buffer. After draining off excess of buffer, slices totalling 200–300 mg. were placed in tared Warburg flasks containing 2.4 ml. of buffer and the flasks reweighed; 0.2 ml. each of glucose and AMP solutions were added to the main compartments of the flasks, to give final concentrations of 11 mM and 1.0 mM respectively, and 0.2 ml. of fatty acid-albumin solution to the side arms. Four drops of 20% KOH solution and a small roll of filter paper were placed in the centre well of each flask and the flasks flushed out with O_2 for 3 min. After temperature equilibration in the bath for 10 min. the fatty acid solution was tipped in and the side arms and manometer taps were closed. Incubation at 37° with shaking was carried out for 60 min., O_2 consumption being measured during the first 30 min. usually, but in some experiments for the second 30 min. also.

Lipid extraction. The contents of the Warburg flasks were transferred to a small Potter-Elvehjem-type of all-glass homogenizer, together with two 1 ml. lots of buffer used to wash out the flasks, and the slices were homogenized in the suspending medium. The homogenates were transferred to glass-stoppered tubes and shaken for 5 min. with 20 ml. (4 vol.) of CHCl_3 -methanol mixture (2:1, v/v) containing 0.1% butylated hydroxytoluene and 10 μmoles of carrier fatty acid. The tubes were centrifuged and the lower CHCl_3 -rich phase removed quantitatively to another tube. Samples (0.2 ml.) of the fatty acid-albumin solution, diluted with 0.8 ml. of buffer, were similarly extracted. Solvents were removed from these total lipid extracts by warming in a stream of N_2 immediately before chromatography.

Chromatography. Separation of lipid classes was carried

out on columns of silicic acid by one of two methods. (1) Columns 1 cm. in diameter containing 1 g. of silicic acid were used. Non-phospholipids were eluted with 10 ml. of ether and total phospholipids with 10 ml. of methanol-water (24:1, v/v). (2) Lecithin and ethanolamine phospholipid fractions were eluted from columns containing 6 g. of silicic acid (Webster & Thompson, 1962). Free fatty acids were first removed with 50 ml. of CHCl_3 (fraction a). Ethanolamine phospholipid, together with some serine phospholipid, was then eluted as a peak (fraction b) with 100 ml. of CHCl_3 -methanol mixture (4:1, v/v). A small mixed fraction (c) including some serine phospholipid, inositol phospholipid and the tail of the ethanolamine phospholipid peak was next eluted with 35 ml. of CHCl_3 -methanol mixture (4:1, v/v) containing 1.25% (v/v) of water. A further 100 ml. of this latter solvent eluted the lecithin peak (d). Sphingomyelin- and lysolecithin-containing fractions were subsequently eluted as described by Webster & Thompson (1962).

Thin-layer chromatography was carried out on glass plates coated with silica gel (0.25 mm. thick); Kieselgel G was used for earlier experiments, but when isolation of lyso compounds was required Kieselgel H was found to give better separation. Samples in 100 μl . of CHCl_3 -methanol mixture (2:1, v/v) containing 0.1% butylated hydroxytoluene were applied to the plates with a constriction pipette. Chromatograms of phospholipids were developed in CHCl_3 -methanol-water (14:6:1, by vol.) (Müldner, Wherrett & Cumings, 1962). Chromatograms of non-phospholipid fractions, to which cholesterol oleate was added as a carrier, were developed in *n*-hexane-diethyl ether-acetic acid (75:23:2, by vol.). Lipids were located by exposing the chromatograms to iodine vapour for 1 min. and after marking the stained spots the plates were left in air for 60 min. before scraping off the lipid-containing regions for assay of radioactivity.

Hydrolysis of phospholipid with snake venom. Samples of labelled lecithin and ethanolamine phospholipid fractions (about 1 mg.) were dissolved in 1 ml. of diethyl ether and treated with a solution of venom in 0.005 M- CaCl_2 (Long & Penny, 1957). After 18 hr. at room temperature the ether was evaporated and the residue was dissolved in a small volume of solvent for thin-layer chromatography.

Measurement of radioactivity. Samples of lipid extracts from the brain slices and from the fatty acid-albumin solutions, samples of each fraction from the silicic acid columns and of lecithin and ethanolamine phospholipid after treatment with venom were evaporated to dryness and dissolved in 10 ml. of scintillation fluid (0.3% 2,5-diphenyloxazole in xylene). Lipid-containing regions on thin-layer chromatograms were scraped off the plates into counting cells containing 10 ml. of the scintillation fluid described by Snyder (1964). All counts were recorded for 100 sec. on a Nuclear Enterprises scintillation counter; samples of total phospholipid, lecithin and ethanolamine phospholipid giving at least 2000 counts were generally used. The counting efficiency in either scintillator was about 60%; [^{14}C]hexadecane was used as an internal standard.

RESULTS

In the first series of experiments oxygen uptake by rat cerebrum slices was found to be linear throughout the whole 60 min. period of incubation,

and in later experiments measurements were made usually during the first 30 min. only. The average oxygen uptake from all experiments was 2600 μ l. of oxygen/g. of fresh tissue/hr., with a range 2204–2864 μ l. of oxygen/g./hr. Uptake was unaffected by the type of fatty acid added to the incubation medium.

In two experiments slices were incubated with [14 C]oleic acid in oxygen and in nitrogen. The lipids were then extracted and in each case separated into non-phospholipid and total phospholipid fractions by method 1 (see above). The ratio of phospholipid radioactivity to that of the 1 μ mole of 14 C-labelled fatty acid added originally to the flasks was calculated and the results expressed in $m\mu$ moles of labelled acid incorporated/g. of tissue/hr. As shown in Table 1 average rates of incorporation of 146.8 and 21.9 $m\mu$ moles/g./hr. were found in oxygen and nitrogen respectively. The phospholipid fractions in these experiments were examined by thin-layer chromatography (Kieselgel G) to determine the distribution of incorporated fatty acid between individual lipid classes. Of the total phospholipid radioactivity 89% was recovered from the chromatograms; 68% was present in lecithin and 19% in ethanolamine phospholipid. The region corresponding to serine phospholipid, which may also have included some inositol phospholipid, contained only 2% and was not further investigated. There was, however, some evidence of streaking of ethanolamine phospholipid on the Kieselgel G plates used for these experiments and the rather low recovery of total radioactivity (89%) from the plates probably reflects some preferential loss of this lipid fraction; the value in Table 1 thus may slightly underestimate incorporation of [14 C]oleic acid into ethanolamine phospholipid. Thin-layer chromatography of the non-phospholipid fractions showed no evidence of esterification of cholesterol with [14 C]oleic acid. In the experiments reported in Table 1 duplicate flasks were incubated in oxygen and nitrogen and the lipids from each flask processed separately

throughout; excellent agreement was found between duplicates in both experiments.

The incorporation of fatty acids into ethanolamine phospholipid and lecithin was further studied in subsequent experiments with column fractions *b* and *d* obtained by method 2 described above. The total recovery of radioactivity in the first four fractions (*a–d*) from these columns was essentially complete, averaging 98.1% (95.1–101.0%). The ethanolamine phospholipid and lecithin fractions, *b* and *d*, together accounted for 92% of the labelled phospholipid, the balance being present in the small intermediate fraction *c*. In a preliminary experiment only traces of radioactivity, amounting to 0.16 and 0.08% of that applied to the column, were found in the two fractions eluted after the lecithin peak, thus indicating little if any labelling of sphingomyelin. It is likely that part of the radioactivity in fraction *c* was present in ethanolamine phospholipid, but this fraction was not investigated further. Results shown in Tables 2 and 3, expressed as $m\mu$ moles of fatty acid incorporated/g. of tissue/hr., are therefore minimal values, but any underestimate, particularly of ethanolamine phospholipid labelling, should not exceed 8%. Examination of fractions *b* and *d*, referred to below as ethanolamine phospholipid and lecithin respectively, confirmed the earlier findings (Table 1) as regards incorporation of [14 C]oleic acid into lecithin, but the value for ethanolamine phospholipid was higher (see Table 3).

Experiments were next carried out with different 14 C-labelled fatty acids to determine their relative degrees of incorporation into glycerophosphatides of rat cerebrum slices during incubation for 1 hr. in oxygen; in these experiments lipid extracts from duplicate flasks were pooled before chromatography, or only single flasks were incubated with each acid. Table 2 shows the total incorporation into lecithin and ethanolamine phospholipid fractions, and Table 3 the incorporation into each of these fractions considered separately. Four 14 C-labelled acids were studied, namely palmitic acid,

Table 1. *Incorporation of [14 C]oleic acid into total phospholipid of respiring slices of rat cerebrum*

Slices were incubated with oleic acid in O_2 and N_2 and phospholipids were extracted by method 1, as described in the text. Lipid classes were separated on thin-layer chromatograms. Mean results of two experiments are given and incorporation is expressed as $m\mu$ moles of acid/g. of tissue/hr.

Gas phase	Incorporation	Distribution of [14 C]oleic acid in glycerophosphatides (%)		
		Serine phospholipid	Ethanolamine phospholipid	Lecithin
N_2	21.9	—	—	—
O_2	146.8	2.2	18.6	68.0
Increase in O_2	124.9	—	—	—

stearic acid, oleic acid and linoleic acid, in the seven experiments reported. In Table 2 results of the individual experiments are given, together with the mean values for each acid. There was some variation in the results for each acid and, although the mean value of $196 \mu\text{moles/g./hr.}$ for palmitic acid is considerably higher than those for the other acids tested, the ranges of the individual results overlapped, and no clear differences between the extents of labelling by the four acids were discernible. However, when the incorporation of palmitic acid and of other acids was studied in the same experiments (Expts. B, C and D, Table 2) it is evident that palmitic acid was consistently slightly more active than stearic acid, oleic acid or linoleic acid, all of which showed similar values.

Mean results and the ranges of values for the labelling of lecithin and ethanolamine phospholipid fractions, considered separately (Table 3), are expressed both as $\mu\text{moles/g./hr.}$ and as percentages of total labelling in the two fractions.

Although some variation between experiments was again apparent, certain differences in the patterns of labelling by the four acids can be seen. Palmitic acid, oleic acid and linoleic acid consistently labelled lecithin more actively than they did ethanolamine phospholipid, but with $[^{14}\text{C}]$ stearic acid there was a more even distribution of the incorporated acid between the two lipids. The former three acids were all incorporated into lecithin at a mean rate of about $100 \mu\text{moles/g./hr.}$, but the rates for stearic acid were markedly less and averaged $66 \mu\text{moles/g./hr.}$ Ethanolamine phospholipid fractions, on the other hand, were more actively labelled by the two saturated acids, at rates of $70\text{--}80 \mu\text{moles/g./hr.}$, than by oleic acid or linoleic acid, for which values of about $50 \mu\text{moles/g./hr.}$ were found.

In some experiments described above the distribution of incorporated ^{14}C -labelled acids between the 1- and 2-positions of the glycerol moiety of lecithin and ethanolamine phospholipid was investi-

Table 2. *Incorporation of ^{14}C -labelled fatty acids into the glycerophosphatides of respiring slices of rat cerebrum*

Slices were incubated with labelled acids in O_2 . Lipids were extracted and their radioactivity was determined as described in the text. The values given are the total μmoles of labelled acid incorporated/g. of tissue/hr. into lecithin and ethanolamine phospholipid.

Expt.	^{14}C -labelled acid			
	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid
A	—	—	136.0	—
B	233.9	—	212.2	—
C	155.6	122.1	136.0	120.6
D	198.0	191.8	167.9	168.5
E	—	137.4	—	145.0
F	—	118.7	—	—
G	—	133.5	—	—
Means	195.8	140.7	163.0	144.7

Table 3. *Incorporation of ^{14}C -labelled fatty acids into lecithin and ethanolamine phospholipid fractions of rat cerebrum*

Slices were incubated with labelled acids in O_2 . Lecithin and ethanolamine phospholipid fractions were isolated, and their radioactivity was determined, as described in the text. Mean results and the ranges of values are given, expressed both as μmoles of labelled acid incorporated/g. of tissue/hr., and as percentage of total incorporation into the two fractions. Numbers of experiments with each acid are shown in parentheses.

Acid	Lecithin		Ethanolamine phospholipid	
	($\mu\text{moles/g./hr.}$)	(%)	($\mu\text{moles/g./hr.}$)	(%)
Palmitic acid (3)	115.9	59	80.0	41
	84.0–134.8	54–68	63.2–105.1	32–46
Stearic acid (5)	65.9	46	74.8	54
	48.0–101.5	36–53	57.0–90.3	47–64
Oleic acid (4)	107.5	67	55.5	33
	89.8–120.8	54–77	31.2–97.6	23–46
Linoleic acid (3)	96.5	67	48.2	33
	85.6–111.2	64–71	35.0–57.3	29–36

gated. Portions of each lipid fraction were treated with snake venom to liberate the 2-linked acid and yield the corresponding 1-acyl lyso derivative. Equivalent samples of lipid, before and after hydrolysis by venom, were applied in adjacent lanes on thin-layer plates (Kieselgel H). After development of the chromatograms and location of the separated lipids with iodine vapour the radioactivity in regions corresponding to free fatty acids, lysophosphatides and diacylglycerophosphatides in each lane was determined. The distribution of ^{14}C -labelled acids incorporated at the 1- and 2-positions of the two lipid fractions was calculated from the relative amounts of radioactivity appearing in the lyso derivatives and free fatty acids respectively, after hydrolysis by venom; the extent of hydrolysis by the venom was calculated from the difference in radioactivity in the diacylglycerophosphatide regions of the chromatograms of treated and untreated samples.

The results obtained for lecithin fractions are set out in Table 4; the individual values for each experiment are shown, as well as the mean values for each acid, to indicate the pattern of results more clearly. Hydrolysis by venom was essentially complete in all experiments and recovery of radioactive products from chromatograms averaged

91.3%. With palmitic acid, oleic acid or linoleic acid as the labelled substrate a fairly even distribution between the two positions was found, although in all experiments except one (Expt. D with palmitic acid and oleic acid) all values showed a slightly greater incorporation at the 1-position, the mean values being 56, 54 and 56% for palmitic acid, oleic acid and linoleic acid respectively. A markedly uneven distribution was found with stearic acid, however, in four experiments. Only 24% of the labelling by this acid was at the 2-position and an average of 76% in the 1-position, and this value is significantly different ($P < 0.01$) from the combined values for 1-labelling by the other three acids.

The results of similar experiments carried out with ethanolamine phospholipid fractions are shown in Table 5. In these the degree of hydrolysis by venom was less complete, especially in experiments with linoleic acid, than was found for lecithin. Nevertheless it was evident that, with this fraction also, labelling of both positions had occurred with all four acids studied. Palmitic acid, oleic acid and linoleic acid were again fairly evenly distributed, but there was a marked and very consistent preponderance (84%) of [^{14}C]stearic acid incorporated at the 1-position of the ethanolamine phospholipid

Table 4. *Distribution of ^{14}C -labelled fatty acids incorporated into phospholipids in respiring slices of rat cerebrum, between the 1- and 2-position of lecithin*

Rat cerebrum slices were incubated with ^{14}C -labelled fatty acids in O_2 and the radioactive lecithin formed was extracted and hydrolysed with snake venom as described in the text. The percentage distribution of labelled acid between the 1- and 2-positions of lecithin was calculated from the proportions of counts found in lysolecithin and free fatty acid respectively, after this treatment; results of individual experiments and the mean values obtained with each acid are shown. The percentage hydrolysis of lecithin brought about by the venom is also given.

Acid	Expt.	Hydrolysis (%)	Distribution (%)	
			Position 1	Position 2
Palmitic acid	B	100	68	32
	C	100	56	44
	D	95	44	56
	Mean	98	56	44
Stearic acid	C	99	85	15
	D	98	60	40
	E	98	72	28
	G	94	86	14
	Mean	97	76	24
Oleic acid	B	99	58	42
	C	100	56	44
	D	96	48	52
	Mean	98	54	46
Linoleic acid	C	100	61	39
	D	97	51	49
	E	99	55	45
	Mean	99	56	44

Table 5. *Distribution of ^{14}C -labelled fatty acids incorporated into phospholipids of respiring slices of rat cerebrum, between the 1- and 2-positions of ethanolamine phospholipid*

Conditions and expression of results are as described in Table 4 for lecithin.

Acid	Expt.	Hydrolysis (%)	Distribution (%)	
			Position 1	Position 2
Palmitic acid	B	84	56	44
	D	90	63	37
	Mean	87	60	40
Stearic acid	D	95	82	18
	E	93	85	15
	G	94	86	14
	Mean	94	84	16
Oleic acid	B	71	57	43
	D	90	52	48
	Mean	80	54	46
Linoleic acid	D	69	44	56
	E	57	54	46
	Mean	63	49	51

fractions. This value also is significantly different ($P < 0.01$) from those for 1-labelling by the other acids.

In two experiments the effect of incubating slices in the presence of linoleic acid and stearic acid together was studied to see whether in either lipid fraction the positional pattern of labelling could be altered. In the presence of equimolar concentrations of unlabelled linoleic acid and [^{14}C]stearic acid (total acid $1\text{ }\mu\text{mole/flask}$) there was only a very slight increase (5%) in the labelling of the 1-position in either lipid fraction. The proportion of [^{14}C]linoleic acid in the 2-position of lecithin was also only slightly increased (9%) by the presence of unlabelled stearic acid in the incubation medium. In the ethanolamine phospholipid fractions, on the other hand, unlabelled stearic acid markedly changed the distribution of [^{14}C]linoleic acid, 78% of the labelling now being in the 2-position and only 22% in the 1-position, compared with 51 and 49% respectively when [^{14}C]linoleic acid was the only substrate (Table 5).

DISCUSSION

These experiments have demonstrated that slices of adult rat cerebrum can incorporate long-chain fatty acids into phospholipids by an energy-dependent process. An appreciable degree of incorporation of up to $234\text{ }\mu\text{moles}$ of acid/g. of tissue/hr. was found when fatty acids were present in the incubation medium at a concentration of 0.33 mM , a concentration that is of the same order as that of the plasma free fatty acids of man and rats (Fletcher, Gloster & Harris, 1965; Olivecrona,

1962). From the results in Table 1, with [^{14}C]oleic acid as the substrate, it is clear that labelling of phospholipids is almost entirely confined to the lecithin and ethanolamine phospholipid fractions, the former being the most active. Studies on the enzymic acylation of exogenous lysophosphatides by nervous tissues of the rat (Webster, 1965) had previously shown that added 1-acyl-lysolecithin was a considerably more active long-chain acyl-group acceptor than was 1-acyl-lysophosphatidyl-ethanolamine in the presence of brain homogenates reinforced with ATP and CoA, and also that acylation of lysophosphatidylserine in this system could not be detected.

The phospholipase A of brain was shown to bring about the hydrolysis of lecithin, phosphatidyl-ethanolamine and phosphatidylserine, yielding the corresponding lyso derivatives (Gallai-Hatchard *et al.* 1962), although no evidence was available at that time about the site of action of this brain enzyme on the 1- and 2-linked fatty acids of the diacylglycerophosphatide substrates; the structure of the lyso derivatives formed was therefore unknown. More recently Ansell & Spanner (1965) have described a Mg^{2+} ion-dependent vinyl ether-cleaving enzyme in brain, splitting ethanolamine plasmalogen to give lysophosphatidylethanolamine with the 2-acyl structure. It would seem from these reports that brain tissue has enzymes able to produce a variety of lyso compounds from its constituent glycerophosphatides, in addition to enzyme systems capable of acylating these compounds; moreover, the presence of markedly less lysolecithin in slices incubated in oxygen than was found after incubation in nitrogen is consistent with reacylation

of endogenous lysolecithin occurring under aerobic conditions (Webster & Thompson, 1964).

In the present experiments the dependence of incorporation of [^{14}C]oleic acid into phospholipids of brain on oxidative metabolism, and the pattern of labelling in lecithin and ethanolamine phospholipid fractions, which closely resembles that found previously with brain homogenates in the presence of added lysophosphatides (Webster, 1965), support the view that the incorporation of labelled acids was brought about by acylation of endogenously derived lyso compounds. Some indication of this was also found with rat-brain mitochondrial preparations (Webster & Alpern, 1964). Although fatty acid labelling of lecithin and ethanolamine phospholipid via phosphatidic acid formation and the cytidine-dependent pathway described by Kennedy & Weiss (1956) cannot be entirely excluded in these experiments, the results reported by Strickland, Subrahmanyam, Pritchard, Thompson & Rossiter (1963), for phospholipid labelling from L-3- ^{14}C glycerophosphate, by homogenates of adult rat brain reinforced with ATP and other cofactors, show that specific activity was highest in phosphatidic acid; calculations from the data reported suggest that on a tissue-weight basis the amount of [^{14}C]glycerophosphate incorporated into lecithin under their conditions is about 10%, or less, of the amount of ^{14}C -labelled fatty acid incorporated into this lipid in the present studies with brain slices. Scherphof & van Deenen (1966), investigating phospholipid metabolism in rat-liver mitochondria and microsomes, found direct evidence that labelling of lecithin and ethanolamine phospholipid from [^{14}C]palmitic acid resulted mainly from acylation of the corresponding lyso derivatives, rather than via phosphatidic acid formation. It is considered most likely that the acylation pathway is the major one, and perhaps the only one, concerned in the present experiments.

It follows from this view that the lyso derivatives of lecithin and ethanolamine phosphatide produced within the slices during incubation must have been mixtures of the 1- and 2-acyl isomers, since, with all the ^{14}C -labelled fatty acids tested, radioactivity was found in both positions in the lecithin and ethanolamine phospholipid fractions isolated after incubation. Part of the 2-acyl isomer of lysophosphatidylethanolamine could have arisen from ethanolamine plasmalogen breakdown by the enzyme described by Ansell & Spanner (1965), but there is also now direct evidence that phospholipase activity in several tissues results in the formation of both isomeric lyso derivatives from diacylglycerophosphatides. Thus the hydrolysis of lecithin by liver, spleen and lung (van den Bosch & van Deenen, 1964), and also by pancreas from several species (van den Bosch, Postema, de Haas

& van Deenen, 1965), was shown to yield both 1- and 2-acyl-lysolecithin. The question arising from these findings whether one enzyme or a combination of enzymes with different positional specificities is concerned in the hydrolysis of diacylglycerophosphatides was also discussed by van den Bosch and his colleagues.

It has been reported that both the extent of lysophosphatide acylation by different acids and the distribution of incorporated acid between the 1- and 2-positions varied with the proportions of 1- and 2-acyl isomers of lysolecithin (Lands & Merkl, 1963) or lysophosphatidylethanolamine (Merkl & Lands, 1963) added to rat-liver microsomal preparations. It seems probable therefore that the differing degrees of incorporation of the four radioactive acids and also differences in their intramolecular distribution in lecithin and ethanolamine phospholipids in brain partly reflect the pattern of lyso compounds produced in the slices during incubation. Lands and his colleagues also found that stearic acid is esterified more actively at the 1-position and linoleic acid at the 2-position of glycerophosphatides by liver microsomes. A similar specificity as regards stearic acid is apparent in brain (Tables 4 and 5), and the finding of more active labelling of ethanolamine phospholipid than of lecithin by stearic acid suggests a relatively greater formation of 2-acyl-lysophosphatidylethanolamine than of 2-acyl-lysolecithin; the former lyso compound being formed possibly from both ethanolamine plasmalogen and phosphatidylethanolamine. There was, however, no clear indication of specificity for esterification of palmitic acid, oleic acid or even linoleic acid at any one position in either lipid fraction of brain (Tables 4 and 5). The experiments with mixtures of acids were therefore carried out to investigate this apparent lack of specificity further. The results showed that [^{14}C]linoleic acid, in the presence of unlabelled stearic acid, was preferentially esterified at the 2-position in both lipids, especially in ethanolamine phospholipid. Thus brain resembles liver also as regards the position of esterification of lyso compounds with this unsaturated acid.

It is not known whether labelling of ethanolamine phospholipid fractions is confined to phosphatidylethanolamine. A possible explanation for the incomplete hydrolysis by venom of ethanolamine phospholipid fractions, particularly those labelled with radioactive linoleic acid or oleic acid (Table 5), is that some of the unsaturated acids may have been incorporated into the 2-position of ethanolamine plasmalogen by acylation of ethanolamine lysoplasmalogen. Although the 2-linked fatty acids of plasmalogens are split off less actively than those of diacylglycerophosphatides by phospholipase A preparations from pancreas (Magee,

Gallai-Hatchard, Sanders & Thompson, 1962) and venoms (Gottfried & Rapport, 1962), there is as yet no direct evidence that phospholipases in other tissues can hydrolyse the ester linkage in these lipids and yield lysoplasmalogens (Dawson, 1966).

It would appear likely from the foregoing discussion that several different hydrolytic enzymes, giving rise to various isomeric lyso compounds, are present in brain tissue, and as Lands & Hart (1965) have emphasized a number of different acyl-transferases may be involved in lipid synthesis by the acylation pathway. Little is yet known, however, of the distribution and activities of all these enzymes in various regions of the nervous system and more information on these aspects is required.

Apart from possible biological variations in the different enzymes concerned in ^{14}C -labelled fatty acid uptake in the present study, some experimental variations may have arisen from the relatively more complex system represented by brain slices, in contrast with homogenates or isolated subcellular fractions; for example, variation in the proportions of grey and white matter in the slices in different experiments may have contributed to the spread of values seen in Tables 2 and 3.

In relation to the physiological significance of fatty acid incorporation by the acylation pathway, Lands and his colleagues (Lands, 1960; Robertson & Lands, 1962; Lands & Hart, 1964), discussing their work with liver, originally pointed out the probable roles of phospholipase A and lysophosphatide-acylating enzymes in the independent turnover of fatty acids in glycerophosphatides and thus in determining the asymmetrical distribution of different long-chain fatty acids esterified at the 1- and 2-positions. The present experiments and our previous studies on brain support the view that turnover of fatty acids, brought about by similar enzyme systems, occurs also in nervous tissues. O'Brien, Fillerup & Mead (1964) also conclude, from their studies on the composition of human brain lipids, that reacylation after synthesis *de novo* must be considered as one possible mechanism to account for the marked differences in fatty acid composition existing between the major glycerophosphatide classes. In a recent review O'Brien (1965) has pointed out that stability of membrane structures in the nervous system, as elsewhere, may depend in part on the fatty acid composition of the constituent lipids. Whether some abnormality in one or more of the enzymes discussed above could be concerned in the pathogenesis of neurological disease, by causing alteration in fatty acid composition in lipids and hence perhaps instability of membrane structures, is a question for further study.

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