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Studies on the Acylation of Lysolecithin by Rat Brain

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Evidence has been obtained recently indicating that lysolecithin is a normal minor constituent of nervous tissue (Thompson, Niemi & Webster, 1960; Webster & Thompson, 1962). Previous reports from several other Laboratories have described the presence of small amounts of this lipid in extracts from various other mammalian tissues (see Webster & Thompson, 1962, for references).

In view of the structural and functional changes produced by adding lysolecithin or other lysophosphatides to tissue preparations, which have been

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the subject of numerous studies in recent years (see Webster, 1961, for references), the origin, further metabolism and physiological significance of lysolecithin in tissues is of considerable interest. The question whether pathological disturbances such as certain diseases of the nervous system might result from abnormalities in the metabolism of lysophosphatides *in vivo* is a further reason for obtaining more information about the biochemistry of these lipids.

Phospholipase A-like activity has recently been demonstrated in preparations of human and of rat brain which can hydrolyse added diacylphosphatides with the liberation of a free fatty acid and the

accumulation of the corresponding lyso derivative (Gallai-Hatchard, Magee, Thompson & Webster, 1962); previous studies showed that brain also contains a phospholipase B, which further degrades lysolecithin to water-soluble phosphorus compounds (Marples & Thompson, 1960). Robertson & Lands (1962) have also reported the hydrolysis of lecithin by homogenates of several rat tissues including brain.

However, in addition to these hydrolytic enzymes, which could be concerned with the production and degradation of lysophosphatides *in vivo*, Lands (1960) described an enzyme system in rat liver that catalyses the acylation of lysolecithin to form lecithin, and which utilizes acyl-CoA as an intermediate in this reaction. Pieringer & Hokin (1962) and Keenan & Hokin (1962) reported the formation of phosphatidic acid and of inositol phosphatide respectively from the corresponding lyso derivatives by preparations of guinea-pig brain. It was therefore of interest to know whether nervous tissues could acylate lysolecithin to form lecithin, and it has now been found that rat brain possesses considerable capacity for carrying out this acylation.

This paper describes the results of an investigation into some of the properties of the enzyme system concerned in this reaction. Some preliminary experiments in this work have been briefly reported (Webster, 1962).

EXPERIMENTAL

Chemicals. Lysolecithin was prepared from ovelcithin (Marples, Thompson & Webster, 1959).

[Me-¹⁴C]Choline-labelled lecithin was obtained from rat liver (Gallai-Hatchard *et al.* 1962), and labelled lysolecithin was prepared from this by the procedure used for ovolysolecithin. The specific radioactivities of different preparations of these lipids were between 0.07 and 0.10 $\mu\text{Ci}/\mu\text{mole}$.

All lipid preparations were examined by silicic acid-impregnated-paper chromatography (see below) and each gave a single spot with the correct R_f value for the pure substance. Lipids were stored in chloroform solution in a deep-freeze.

Palmitoyl-CoA was synthesized, by using palmitic anhydride, by the method of Vignais & Zabin (1958). Phosphorylcholine (Riley, 1944) and glycerylphosphorylcholine (Tattre & McArthur, 1955) were also prepared in this Laboratory.

¹⁴C-labelled fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks., and were stored in a deep-freeze as solutions in benzene containing, nominally, 1 $\mu\text{Ci}/100 \mu\text{l}$. Coenzyme A (75%) and ATP (crystalline sodium salt) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany, and oleic acid (redistilled) and palmitic acid from British Drug Houses Ltd.

All other reagents were of A.R. grade; solvents were redistilled before use, the chloroform being immediately restabilized with 2% (by vol.) of methanol.

Enzyme preparations. Adult rats (200–250 g. body wt.) were killed by stunning and decapitation and the cerebral hemispheres immediately removed, weighed and homogenized.

For the preparation of subcellular fractions, tissues were homogenized in 9 vol. of ice-cold 0.25 M-sucrose soln. containing 1 mM-EDTA, in a modification (Webster & Smith, 1964) of the homogenizer described by Aldridge, Emery & Street (1960). Centrifuging of the homogenate for 10 min. at 1000g was used to remove nuclei and unbroken cells. The supernatant from this separation was then centrifuged for 20 min. at 10 000g to obtain a mitochondrial fraction and a second supernatant; the latter was then centrifuged at 21 000g for 60 min. to give a microsomal fraction and a final supernatant. These manipulations were all carried out at 0° in a MSE High-Speed 13 refrigerated centrifuge (Measuring and Scientific Equipment Ltd.). The particulate fractions were immediately resuspended in sufficient ice-cold sucrose-EDTA solution to make 1 ml. of suspension equivalent to 1 g. of fresh tissue.

In some experiments 10% (w/v) homogenates of rat cerebral hemispheres were prepared in water.

Incubation. The required amounts of chloroform or benzene solutions of each lipid substrate were pipetted into glass-stoppered test tubes and the solvents removed by warming in a stream of nitrogen. Aqueous solutions of the other reagents were added and the lipids emulsified. The enzyme preparation was then added and the tubes were incubated at 38° for the required times. The final volume of the incubation mixture was 0.92 ml. The reaction was stopped and the lipids extracted by shaking with 3.68 ml. (4 vol.) of chloroform-methanol (2:1, v/v).

Assay of enzyme activity. The tubes were centrifuged to separate the two liquid phases and the lower layer was transferred to another tube. Solvents were removed by warming in a stream of nitrogen and the lipids were redissolved in a small volume of chloroform (usually 0.4 ml.). Suitable portions were chromatographed on silicic acid-impregnated paper as described by Webster & Thompson (1962). A radioactive lecithin standard was run on each paper. The lecithin spots were cut out and the surface radioactivity was counted with a mica end-window counter.

The counting rates of the standard solutions of radioactive lecithin and of the labelled substrate (¹⁴C-labelled fatty acid or [¹⁴C]lysolecithin) used in each experiment were compared directly by plating out suitable portions on 1.5 cm. planchets at infinite thinness, and counting as described above.

RESULTS

Accuracy of the method used for quantitative assessment of enzymic activity. Slight differences which were observed in the size and compactness of the stained spots of radioactive lipid on different paper chromatograms were considered to be due to such factors as minor variations in the degree of impregnation with silicic acid and in the composition of the developing solvent mixture. To minimize any effects which such variations might have on the surface-counting rate of the lipid spots, portions of a standard solution of [¹⁴C]lecithin

were always run on each sheet of chromatography paper and the counting rate of the radioactive lecithin spots from total lipid extracts was referred to that of the standard spots on the same chromatogram. When assessed in this way, close agreement was evident between results obtained from different chromatograms of the same lipid extract. In 20 experiments in which duplicate portions of lipid extract were run on separate sheets, each with a portion of standard, the deviation of the duplicate results from their mean was calculated and expressed as a percentage of the corresponding mean; the average of the values so obtained was only 4.84 (S.E. 0.97).

The counting rates of the radioactive lecithin spots from a total lipid extract of rat brain, when plotted against the amounts of extract applied to the chromatogram, showed a linear response over the range of amounts covering the experiments in this paper. The method thus provided a reasonably accurate means of assessing quantitatively the degree of ^{14}C -labelling of lecithin in these experiments.

In preliminary experiments (Webster, 1962) in which preparations of rat-brain mitochondria were incubated for 3 hr. at 38° with ^{14}C lysolecithin in the presence of CoA, ATP and Mg^{2+} ions and either oleic acid or palmitic acid, 13.6 and 11.4% respectively of the radioactivity was found in the lecithin fraction. Omission of fatty acid, CoA and ATP reduced the amount of radioactivity appearing in the lecithin fraction, after incubation, to 6.5% of the amount added initially as ^{14}C lysolecithin.

The addition of increasing amounts of palmitoyl-CoA also progressively stimulated the incorporation of radioactivity from ^{14}C lysolecithin into the lecithin fraction in the presence of brain mitochondria; no such incorporation was found, however, if the brain-enzyme preparation was omitted.

To obtain further evidence identifying the newly formed radioactive lipid as lecithin, the fraction containing it, after incubation of the brain-mitochondrial preparation with ^{14}C lysolecithin and palmitoyl-CoA, was isolated on a silicic acid column (Webster & Thompson, 1962). A silicic acid-impregnated-paper chromatogram of this fraction showed a single radioactive spot with R_f of lecithin. Treatment of the fraction with *Agkistrodon piscivorus piscivorus* venom caused complete disappearance of lecithin and its replacement by a lipid with R_f of lysolecithin, containing 70% of the radioactivity originally present in the lecithin.

Because rat brain is known to contain enzymes capable of degrading lysolecithin, an experiment was carried out to determine whether addition of any of the possible choline-containing degradation

products affected the incorporation of radioactivity from ^{14}C lysolecithin into lecithin. In the presence of glycerylphosphorylcholine, phosphorylcholine or choline, in concentrations equal to that of the ^{14}C lysolecithin (1.30 mM), 14.0, 9.7 and 12.4% respectively of the radioactivity was found in lecithin after 3 hr. at 38° , compared with 11.9% with no such additions. These values, which are not considered to differ significantly from each other, support the view that the complete ^{14}C lysolecithin molecule is concerned in the incorporation of radioactivity into the lecithin; conversely, it seems unlikely that incorporation of possible labelled degradation products of ^{14}C lysolecithin contributes significantly to the formation of labelled lecithin in these experiments. The results so far obtained thus indicate that rat brain can form lecithin by acylation of lysolecithin with acyl-CoA as an intermediate.

Previous experiments also showed (Webster, 1962) that, when rat-brain mitochondria were incubated with unlabelled lysolecithin and ^{14}C oleic acid or ^{14}C palmitic acid in a system otherwise similar to that used in the experiments with ^{14}C lysolecithin, about 2% of the radioactivity of the substrate appeared in lecithin after 3 hr.; the difference between this figure and that obtained when lysolecithin was the labelled substrate was attributed to the utilization of endogenous fatty acid in the latter system. The incorporation of ^{14}C -labelled fatty acid was stimulated six- to seven-fold, however, by adding sodium fluoride (50 mM final concentration) to the incubation medium. Since the specific radioactivity of the labelled substrate could be made much higher by using ^{14}C -labelled fatty acids, and thus allow a more accurate assessment of lecithin formation than was possible with ^{14}C lysolecithin, it was decided to use ^{14}C -labelled fatty acid as the substrate for further detailed study of the properties of the lysolecithin-acylating system in rat brain and to investigate first the effects of variation in concentration of the different constituents of the incubation medium on its activity.

Effect of fluoride. The effect of variation in the concentration of sodium fluoride in the incubation medium on the formation of labelled lecithin from lysolecithin and ^{14}C oleic acid by preparations of rat-brain mitochondria is shown in Table 1. Progressive stimulation was observed as the concentration was increased up to 81 mM, and with this amount of fluoride the radioactivity found in the lecithin after 3 hr. incubation at 38° corresponded to the conversion of 19% of the substrate. At higher concentrations slight further stimulation occurred and reached a maximum value at 136 mM, when an average of 21.3% of the substrate radioactivity was present in the lecithin.

Effect of added metal ions. The results of an experiment to determine the requirement of the lysolecithin-acylating system in rat brain for added magnesium are shown in Table 2. The medium in this experiment contained the previously determined optimum concentration of sodium fluoride (136 mM). Only a low activity was found in the absence of added magnesium, but marked stimulation occurred with a final concentration of 1.1 mM. Maximal activity was found with concentrations of magnesium between 4.4 and 17.4 mM. The enzyme preparation in this experiment was less active than those used for the experiments described in Table 1; subsequent work with other preparations, has, however, confirmed the findings set out in Table 2.

In other experiments carried out with homogenates of rat cerebrum as enzyme source (see below) a similar requirement for added magnesium was found. Manganese and cobalt also stimulated

incorporation of [14 C]oleic acid into lecithin, although at 4.4 mM final concentration manganese was only 82 % and cobalt only 74 % as effective as magnesium. At a higher concentration (35 mM) both manganese and cobalt were inhibitory whereas magnesium still caused nearly maximal activation. Calcium was found to be slightly inhibitory to the lysolecithin-acylating system, even at a final concentration of 4.4 mM.

Requirement for ATP and CoA. The requirement of the lysolecithin-acylating system in preparations of rat-brain mitochondria for added ATP is shown in Fig. 1. The values plotted are the means of results from several experiments in which the enzyme was incubated for 1 hr. in a system similar to that described in Table 1, but containing 136 mM-sodium fluoride and various amounts of ATP. It can be seen that in the absence of added ATP there was negligible incorporation of radioactivity from [14 C]oleic acid into lecithin. Marked stimulation occurred even with small amounts of ATP and the optimum concentration was about 9 mM; concentrations above 13 mM were inhibitory.

In contrast with the absolute requirement for ATP, appreciable acylating activity was observed without the addition of CoA to the system. The activity was, however, consistently increased 1.5- to 2-fold by adding CoA to a final concentration of 0.033 mM. No further significant effect was found with concentrations above this; nevertheless a final concentration of 0.066 mM-CoA was regularly used to ensure that an adequate amount was present.

Table 1. *Effect of fluoride concentration on the acylation of lysolecithin with [14 C]oleic acid by preparations of rat-brain mitochondria*

Results are expressed as the percentage of the initial substrate radioactivity found in lecithin after 3 hr. incubation at 38°. The incubation system contained 1.2 μ moles of [14 C]oleic acid (1.0 μ C), 1.2 μ moles of lysolecithin, 40 μ moles of KH_2PO_4 buffer (pH 7.4), 4 μ moles of MgCl_2 , 0.06 μ mole of CoA, 2 μ moles of ATP, 0.4 ml. of mitochondrial suspension and NaF as indicated, in a final volume of 0.92 ml.

Concn. of NaF (mM)	No. of observations	[14 C]Lecithin (mean values)
0	7	1.8
14	2	3.8
27	2	7.2
54	5	14.4
81	2	19.0
109	3	19.8
136	6	21.3
163	2	20.0
190	2	18.7

Table 2. *Effect of magnesium concentration on the acylation of lysolecithin with [14 C]oleic acid by preparations of rat-brain mitochondria*

Conditions of incubation were the same as described in Table 1 with 136 mM-NaF, but with MgCl_2 as indicated. Results are expressed as described for Table 1.

Concn. of MgCl_2 (mM)	[14 C]Lecithin
0	1.4
1.1	9.0
2.2	12.8
4.4	13.2
8.7	13.6
17.4	13.5
34.8	12.1

Fig. 1. Dependence of the lysolecithin-acylating activity of preparations of rat-brain mitochondria upon added ATP concentration. The incubation system was the same as in Table 1, with 136 mM-NaF, but with ATP as indicated. Incubation was carried out for 1 hr. at 38°.

No increase in activity of the acylating system resulted from adding GSH or cysteine (1.1 mM final concentration) either in the presence or absence of added CoA. With 11 mM-GSH or cysteine, in the absence of added CoA, inhibition of acylating activity, amounting to 51 and 65% respectively, was found in one experiment.

Effect of varying substrate concentrations. A number of experiments were carried out to determine the effects of varying concentrations of fatty acid and lysolecithin on the acylating activity of preparations of rat-brain mitochondria. The incubation medium contained optimum amounts of cofactors and sodium fluoride (136 mM) and each tube contained 0.5 μ C of carrier-free [14 C]oleic acid. In one pair of experiments different amounts of carrier oleic acid and a constant amount of lysolecithin (1.2 μ moles) were added; in a second pair of experiments the amount of carrier oleic acid was kept constant (1.2 μ moles/tube) and the amount of added lysolecithin was varied. Results were calculated both as a percentage of and as μ m-moles of radioactive substrate incorporated into lecithin after incubation for 1 hr. at 38°.

It can be seen from Table 3 that in the absence of carrier oleic acid utilization of the small amount of labelled fatty acid was almost complete, 92.7% of it being incorporated into lecithin in 1 hr. With the addition of increasing amounts of carrier oleic acid to the system it was evident that there was greater total incorporation of oleic acid into lecithin in the presence of higher concentrations of the fatty acid. This point is shown by expressing the results as μ m-moles of oleic acid incorporated into lecithin (Table 3, column 3); the amount of newly formed lecithin reached a maximum with 1.2 μ moles of oleic acid/tube and thereafter remained approximately constant with up to 4.8 μ moles/tube. When 9.6 μ moles of oleic acid were added, significant inhibition of the reaction occurred.

When the amount of oleic acid was kept constant (1.2 μ moles/tube) and the amount of lysolecithin added was varied, a similar dependence of lecithin labelling from [14 C]oleic acid upon substrate concentration was seen (Table 4). When no lysolecithin was added to the system an average of 64 μ m-moles of newly formed lecithin was found. The amount of lecithin formed increased when lysolecithin was added to the medium and again maximum values were reached in the presence of between 1.2 and 4.8 μ moles of added lysolecithin in each tube. Marked inhibition of the acylation reaction occurred, accompanied by visible optical clearing of the incubation medium, when larger amounts of lysolecithin were used.

Effect of pH. When the activity of the acylating system was studied over a range of values between pH 5.5 and 7.8 in phosphate buffer, with prepara-

tions of rat-brain mitochondria as the enzyme source, no sharp pH optimum was found. The activity was very low below pH 6.0, but then rose steadily as the pH was increased to 7.0; between pH 7.0 and 7.8 a slight further rise occurred (Fig. 2). A similar pattern, over the same pH range, was found also in one experiment carried out in the citric acid-phosphate-boric acid-diethylbarbituric acid buffer of Johnson & Lindsey (1939); between pH 7.8 and 9.0 the activity remained approximately constant. The absolute values of the enzyme activity in this buffer were somewhat lower, however, at pH 7.4, than in phosphate.

Although in both the buffers studied the activity of the acylation reaction was slightly greater at pH 7.8 than at pH 7.4, all the experiments

Table 3. *Effect of variation in oleic acid concentration on the acylation of lysolecithin by preparations of rat-brain mitochondria*

The incubation system contained 1.2 μ moles of lysolecithin, 40 μ moles of KH_2PO_4 buffer (pH 7.4), 4 μ moles of MgCl_2 , 0.06 μ mole of CoA, 8.0 μ moles of ATP, 125 μ moles of NaF, 0.5 μ C of [14 C]oleic acid and various amounts of carrier oleic acid; 0.4 ml. of mitochondrial suspension was added to each tube. (Specific activity of carrier-free [14 C]oleic acid was 88 μ C/ μ mole.) All tubes contained a final volume of 0.92 ml. and were incubated at 38° for 1 hr.

Carrier oleic acid/tube (μ moles)	Incorporation of [14 C]oleic acid into lecithin	
	(%)	(μ m-moles/tube)
0	92.7	5
0.1	65.7	69
0.3	45.7	139
0.6	39.4	238
0.9	29.6	268
1.2	27.8	335
2.4	12.2	293
4.8	6.0	288
9.6	2.3	221

Table 4. *Effect of variation in the concentration of lysolecithin on its acylation with [14 C]oleic acid by preparations of rat-brain mitochondria*

Conditions of incubation were as described in Table 3 except that each tube contained 1.2 μ moles of carrier oleic acid and the amount of lysolecithin indicated.

Lysolecithin/tube (μ moles)	Incorporation of [14 C]oleic acid into lecithin	
	(%)	(μ m-moles/tube)
0	5.3	64
0.6	23.0	277
1.2	40.6	489
2.4	43.2	520
4.8	42.2	509
9.6	26.5	320
38.4	8.8	106

described in this paper were done at the latter more physiological pH value.

Two experiments were carried out to determine whether lecithin formation by the acylation pathway is associated especially with any particular cell structure in rat cerebrum. Cell fractions were prepared by centrifuging homogenates of the tissue in sucrose-EDTA solution as described above. Samples, equivalent to 50 mg. of tissue, of the fractions obtained at each stage of the separation procedure were incubated in a system containing optimum concentrations of each constituent.

It can be seen from Table 5 that appreciable acylating activity was present in the whole-tissue homogenate, in both the particulate fractions and in the final supernatant fraction. Since the particulate fractions were not washed and re-

centrifuged before use, it is recognized that separation was not complete and that some degree of contamination of the mitochondrial and final supernatant fractions with microsomes was likely. Nevertheless, since the activity of the mitochondrial fraction in both experiments was greater than that of the corresponding microsomal fraction, it is improbable that the acylating activity of the former fraction was entirely due to microsomal contamination. The activity of the final supernatant fractions, on the other hand, may have been largely due to the presence of microsomes since only 21000g for 60 min. was applied for their isolation. Recovery of the homogenate activity in the two particulate and final supernatant fractions derived from it averaged 115% for the two experiments.

In view of the complexity of the overall reaction and the crude nature of the enzyme preparations used for these experiments, detailed kinetic studies were not carried out. However, a limited investigation was made of the effects of variation in the amount of enzyme preparation and the duration of incubation. With preparations of rat-brain mitochondria, in amounts equivalent to 54 mg. of fresh cerebrum/ml. in the incubation medium described in Table 5, the rate of radioactive lecithin formation was almost linear for up to 3 hr.; when greater amounts of this enzyme preparation were used the reaction rate departed increasingly from linearity when measured after 1 and 3 hr. incubation. In three experiments with sucrose-EDTA homogenates of rat cerebrum as enzyme source, the reaction was linear for up to 1 hr. with 11 mg. of fresh tissue/ml., but the rate fell off progressively before this time in tubes containing 27 and 54 mg. of fresh tissue/ml.; after 30 min. incubation the mean rate of lecithin formation, with 11 mg. of fresh tissue/ml., corresponded to 8.4 μ moles/g. of fresh tissue/hr., but to only 5.8 μ moles/g./hr. with 54 mg. of fresh tissue/ml. of incubation medium. The activity of the enzyme system in rat cerebrum was found to be fully as active in homogenates prepared in water as in homogenates in sucrose-EDTA solution. No significant loss of activity was evident in either type of homogenate after storage for 24 hr. at 0-4°. Cerebral hemispheres that had been stored intact for 24 hr. at 0-4° and then homogenized either in water or in sucrose-EDTA solution showed no loss of activity when compared with the corresponding hemisphere assayed immediately after death of the animal.

Fig. 2. Variation of lysolecithin-acylating activity of preparations of rat-brain mitochondria with pH of the medium. The incubation system was as described in Table 3 except that the pH of the phosphate buffer (40 μ moles/tube) was varied as indicated. Values plotted are the mean results of two experiments.

Table 5. Intracellular distribution of lysolecithin-acylating activity in rat cerebrum

Subcellular fractions were prepared as described in the Experimental section. Amounts of each fraction equivalent to 50 mg. of fresh tissue were incubated at 38° for 1 hr. in the system described in Table 3, containing 1.2 μ moles of carrier oleic acid/tube. Values given are the mean results of two experiments.

Tissue fraction	Percentage incorporation of [14 C]oleic acid into lecithin	Percentage of homogenate activity
Homogenate	25.2	100
Nuclear fraction	6.6	26.2
First supernatant	20.8	—
Mitochondrial fraction	9.0	35.7
Second supernatant	15.0	—
Microsomal fraction	7.1	28.2
Final supernatant	6.2	24.6

DISCUSSION

The results described above, together with the preliminary findings already reported (Webster, 1962), provide good evidence that rat brain can

form lecithin from lysolecithin and a long-chain fatty acid by the pathway described by Lands (1960) in liver. In this reaction the fatty acid is first activated by the formation of its CoA derivative and then enzymically transferred to lysolecithin; the main purpose of this paper is to determine optimum conditions for the measurement of the overall reaction in nervous tissue as a prelude to studying its possible physiological importance.

Although Kornberg & Pricer (1953) originally found little or no evidence of long-chain fatty acid activation by tissues other than liver, it was shown later by Vignais, Gallagher & Zabin (1958) that this reaction could be brought about by rat brain in the presence of added ATP and CoA. The dependence of lecithin formation in the present experiments upon added ATP (Fig. 1) and its stimulation by added CoA clearly confirm that brain has considerable capacity for activating long-chain fatty acids. The optimum concentration of ATP required for the brain-enzyme system in the present study was about 9 mM; this is about three times as great as the optimum concentration reported by Lands (1960) for rat-liver microsomes. The CoA requirement, on the other hand, is similar for the two tissues. The brain-enzyme system appears also to have an absolute requirement for added bivalent cations, and of those tested Mg^{2+} ions (Table 2) appeared to be the most effective, although Mn^{2+} or Co^{2+} ions in suitable concentrations stimulated the reaction almost as actively.

The marked enhancement of the overall reaction by fluoride is most probably explained by an inhibitory action on adenosine-triphosphatase activity. According to Järnefelt (1962) the adenosine-triphosphatase activity of rat brain exceeds 1000 μ moles/g./hr., and it is likely therefore that it would be capable of rapidly reducing the ATP concentration of the system in the present experiments. Although 136 mM-fluoride was found to be necessary for optimum activity and was always used in the present work, considerable stimulation of the reaction was produced by 54 mM-fluoride. Lower concentrations, however, had much less effect, and Aldridge (1962) found that 10 mM-sodium fluoride had a relatively small inhibitory action on rat-brain microsomal adenosine triphosphatase.

Experiments on the effect of variation of substrate concentration showed that, when carrier-free [^{14}C]oleic acid was incubated with lysolecithin and a preparation of rat-brain mitochondria, 93% (Table 3) of the initial substrate radioactivity was incorporated into lecithin in 1 hr. This high percentage suggests that small amounts of fatty acid can be efficiently utilized by rat brain for the acylation of lysolecithin. The amount of labelled

lecithin formed was maximal under the conditions of these experiments, however, when carrier oleic acid was added to the system to give a final concentration of 1.3 μ moles/ml.

The finding (Table 4) that 64 μ m-moles of [^{14}C]lecithin were formed when the optimum amount of [^{14}C]oleic acid was incubated with mitochondria in the absence of added lysolecithin would seem to indicate that under these experimental conditions endogenous lysolecithin is acylated to form lecithin. Previous studies (Webster & Thompson, 1962) have shown that the lysolecithin content of fresh rat cerebrum is of a similar order, namely about 100 μ m-moles/g. Added lysolecithin stimulated the production of labelled lecithin and again maximal activity was found in these experiments with about 1.3 mM-lysolecithin present in the system.

As already stated, no detailed kinetic studies have yet been carried out on the formation of lecithin from lysolecithin and fatty acid by the enzyme system present in brain. Apart from adenosine-triphosphatase activity, other enzymes have also been described in brain that might be expected to compete at various stages in the sequence of reactions concerned. For example, Srere, Seubert & Lynen (1959) and Vignais & Zabin (1958) reported that rat brain contains enzymes which can hydrolyse acyl-CoA and acyl-adenylate derivatives, and which can catalyse the transfer of the acyl group to other acceptors such as GSH. It may be that the inhibition of lecithin formation by high concentrations of GSH, which was noted earlier, is due to its competing with lysolecithin as an acyl acceptor, although no direct evidence for this possibility was obtained.

Despite the crude nature of the enzyme preparations, when small amounts of a whole-brain homogenate were used as enzyme source to obtain linear rates of reaction for up to 1 hr., the results obtained showed that the rate of lecithin formation by the acylation pathway corresponded to about 8 μ moles/g. of fresh tissue/hr. This finding indicates that rat brain possesses considerable potential for carrying out this reaction under the conditions used in these investigations.

The acylating activity appeared to be fairly uniformly distributed among the four subcellular fractions that were prepared from rat cerebrum. As already pointed out, however, part of the activity of the mitochondrial and final supernatant fractions especially may be attributable to microsomal contamination, and it is possible therefore that most of the activity of whole brain is located in the microsomes. Nevertheless, for the reasons already given, the results obtained are consistent with the view that a considerable proportion of the whole-brain activity is present in mitochondria.

Since the original description of the lysolecithin-acylating system in rat liver by Lands (1960), evidence has been reported that this reaction also occurs in human red blood cells (Oliveira & Vaughan, 1962; Mulder, De Gier & Van Deenen, 1963) and in the aortic wall of certain animal species (Stein & Stein, 1962); it may be that further search will show an even more general distribution throughout the tissues. Phospholipase A activity, hydrolysing diacylglycerophosphatides with the production of the corresponding lyso derivatives and free fatty acids, has now also been demonstrated in a number of tissues, including brain (Robertson & Lands, 1962; Gallai-Hatchard *et al.* 1962). Lands (1960) and Robertson & Lands (1962) have discussed the possible importance of these two enzyme systems, acting in a cyclical manner, in relation to the independent turnover of fatty acids in the lecithin molecule. They point out that the combined effect could be a factor in determining the positional asymmetry of saturated and unsaturated fatty acids on the α - and β -carbon atoms of the glycerol moiety, which is now well established for many naturally occurring lecithins (Tattrie, 1959; Hanahan, 1960; Brockerhoff, Ackman & Hoyle, 1963).

The demonstration of these two enzyme activities in brain is consistent with such a cycle being present also in this tissue, although direct evidence for this hypothesis is still lacking. The physiological significance of these enzyme systems in relation to both lysophosphatide and fatty acid metabolism in nervous tissues is still uncertain. With regard to a possible pathological significance it may be relevant that Baker, Thompson & Zilkha (1963) have recently put forward evidence suggesting that the fatty acid composition of lecithin from brains of patients with multiple sclerosis is abnormal.

SUMMARY

1. Rat-brain homogenates and subcellular fractions prepared from them have been shown to be capable of actively forming lecithin from added lysolecithin and long-chain fatty acids.

2. Some of the conditions affecting the activity of the enzyme system concerned in the reaction have been investigated by following the formation of [14 C]lecithin from [Me - 14 C]choline-labelled lysolecithin and unlabelled fatty acids or from [14 C]-oleic acid and unlabelled lysolecithin.

3. The incorporation of [14 C]oleic acid into lecithin by mitochondrial preparations is markedly dependent upon the addition of ATP, CoA, Mg^{2+} and F^- ions to the incubation system; palmitoyl-CoA also stimulates the reaction with [14 C]lyso-

lecithin as substrate. The pathway concerned in this formation of lecithin would thus seem to be similar to that described by Lands (1960) in liver.

4. The possible physiological significance of this reaction in brain is discussed.

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