nodule ferrihaemoproteins have very similar thermodynamic properties.

3. The three pigments show marked effects of *I* that are also characteristic of mammalian ferri-myoglobin and ferrihaemoglobin. Although the formal charge on the Fe(III) atom of ferrihaemoglobins is +1, the effective charge deduced from the variation of p*K* with *I* at infinite ionic dilution is about -1 on the two major soya-bean pigments and -2 on the third component.

4. The charge properties connected with this ionization are discussed in relation to the structural environment of the haematin nucleus in the nodule pigments.

5. A gradation in the enthalpies and entropies of ionization in the several haemoproteins is discussed.

This research was supported by grants from the National Science Foundation to the University of Pennsylvania.

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Biosynthesis of Lecithin in Brain

PARTICIPATION OF CYTIDINE DIPHOSPHATE CHOLINE AND PHOSPHATIDIC ACID

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(Received 9 July 1962)

Previously it was shown that the phosphorus of [³²P]phosphorylcholine, but not that of α-glycero-[³²P]phosphate, was incorporated into the lecithin of mitochondria and dispersions prepared from rat brain (McMurray, Strickland, Berry & Rossiter, 1957*b*). The incorporation of radioactivity from [³²P]phosphorylcholine was dependent upon the presence of CTP in the reaction mixture. Subsequently, similar rat-brain preparations were shown to incorporate the radioactivity of CDP-choline labelled with ³²P into lecithin (Rossiter, McLeod & Strickland, 1957*a*). The incorporation was increased by the addition of D-αβ-diglyceride prepared enzymically from each of several naturally occurring lecithins. These findings strongly suggested that brain lecithin is formed *in situ* from smaller molecules by reactions similar to those proposed by Kennedy & Weiss (1956) and by Kennedy (1957) for the biosynthesis of phosphatide in fowl liver.

The experiments described in the present paper show that, as previously reported for [³²P]phosphorylcholine, the radioactivity of [¹⁴C]phosphorylcholine is incorporated into lecithin. On the other hand, in contrast with the findings for DL-α-glycero-

[³²P]phosphate, the radioactivity of L-α-[¹⁴C]-glycerophosphate could be recovered from lecithin. Other experiments with ¹⁴C-labelled CDP-choline or [¹⁴C]phosphatidic acid showed that under the appropriate conditions each of these substances may be the precursor of brain lecithin. Experiments of this type indicate that rat-brain preparations are capable of catalysing a sequence of reactions leading to the formation of lecithin.

Preliminary reports on some of these findings have already appeared (Rossiter, McMurray & Strickland, 1957*b*; Rossiter & Strickland, 1959).

METHODS

Tissue preparation and incubation. Rats of the Sprague-Dawley strain (150–250 g.) were decapitated. The cerebral hemispheres were rapidly removed and homogenized in 4 vol. of water at 0° in a Potter-Elvehjem-type homogenizer. Portions of this dispersion were incubated under conditions similar to those described by McMurray, Strickland, Berry & Rossiter (1957*a*). Normally the complete system contained: glucose, 28 mM; ATP (potassium salt), 0.7 mM; NAD, 1 mM; nicotinamide, 40 mM; MgCl₂, 8 mM; tris buffer (pH 7.4), 50 mM; potassium phosphate (pH 7.4), 10 mM. In most experiments an amount of dispersion equivalent to

either 50 mg. or 100 mg. of fresh tissue was added. Other cofactors or labelled intermediates were added as indicated in the Tables. The final volume was either 1.5 or 3 ml. The reaction mixture was incubated for 70 min. in Warburg vessels at 37.5° under an atmosphere of nitrogen.

Lipid extraction and analytical methods. The acid-soluble phosphorus compounds were removed by seven successive extractions with cold 10% trichloroacetic acid containing added 'carrier' (0.05M) appropriate for the labelled intermediate. Phosphatides were extracted with cold ethanol (twice), hot ethanol-ether (3:1, v/v; four times) and ether. The combined extracts were evaporated to dryness at 40–50° with reduced pressure under an atmosphere of nitrogen and the phosphatides were dissolved in light petroleum (b.p. 40–60°). The phosphatides were precipitated by the addition of 8 vol. of dry acetone and a trace of $MgCl_2$ (in ethanol). The precipitate was washed with dry acetone and then extracted with 2 ml. of wet ether and 2 ml. of light petroleum under gentle reflux. The extracts were concentrated to 2 ml. A portion (0.5 ml.) of this extract was plated at infinite thinness on aluminium planchets and dried beneath an infrared lamp. The samples were counted in a windowless gas-flow counter (model D47 A, Nuclear-Chicago Corp., Chicago, Ill., U.S.A.). A second portion (0.2–0.5 ml.) was ashed in 60% (w/v) perchloric acid and the inorganic phosphate released was estimated by the method of Ernster, Zetterström & Lindberg (1950). Specific radioactivity is expressed as counts/min./μmole of phosphate.

In some experiments the lipid extract was evaporated to dryness and the residue subjected to mild alkaline treatment followed by separation of the water-soluble hydrolysis products by two-dimensional paper chromatography as described by Dawson (1954). By this method glycerolphosphorylcholine derived from lecithin, glycerolphosphoryl-ethanolamine derived from phosphatidylethanolamine, glycerolphosphorylserine derived from phosphatidylserine, glycerolphosphorylinositol derived from phosphatidylinositol, and glycerophosphate derived in the main from phosphatidic acid, were separated. The methods of location, elution and determination of specific radioactivity of these areas have been outlined by McMurray *et al.* (1957b).

Phosphatidic acid phosphatidase (L-α-phosphatidic phosphohydrolase). Activity of the enzyme was measured either in dispersions or in particle preparations of rat brain. The dispersion (equivalent to 100 mg. of fresh tissue) or the particle preparation (representing 300 mg. of fresh tissue) was incubated in tris buffer (pH 7.4), 50 mM, containing phosphatidic acid (2–4 μmoles/3 ml.) at 37°. Immediately after the addition of the tissues, 1 ml. of incubation mixture was added to 1 ml. of 20% trichloroacetic acid previously cooled at 0°. After 60 min. a second sample was taken. After centrifuging, portions of the supernatant were analysed for inorganic phosphate by the method of Ernster *et al.* (1950).

The particle preparations were obtained by differential centrifuging of dispersions (1:10) of rat brain in 0.25M-sucrose and 1 mM-EDTA. After preliminary centrifuging for 10 min. at 1000g, a mitochondrial fraction was obtained by centrifuging at 8000g for 20 min. with the high-speed attachment of an International refrigerated centrifuge (model PR-1, International Equipment, Boston, Mass., U.S.A.). A microsomal pellet was obtained from the supernatant by centrifuging at 54 000g for 60 min. in the no. 40 rotor of the Spinco model L preparative ultracentrifuge

(Spinco Division, Beckman Instruments Inc., Belmont, Calif., U.S.A.). Pellets of mixed particles were obtained by omission of the intermediate centrifuging step. Each fraction or pellet was washed by resuspension in sucrose-EDTA, followed by centrifuging. The washed particles were suspended in sucrose-EDTA before addition to the incubation vessels.

Labelled intermediates. (a) [^{14}C]Phosphorylcholine was synthesized by the method of Riley (1944). The reaction mixture, consisting of 0.73 g. of crystalline H_3PO_4 , 0.31 g. of [1,2- $^{14}C_2$]choline bromide (1 mc) and 0.48 g. of dry choline chloride, was heated for 10 hr. at 150–170° under reduced pressure. [^{14}C]Phosphorylcholine of specific radioactivity 15.5×10^4 counts/min./μmole was isolated as described by McMurray *et al.* (1957b).

(b) Cytidine diphosphate [^{14}C]choline was prepared according to the method of Kennedy (1956). The specific radioactivity was 15.5×10^4 counts/min./μmole.

(c) DL-α-[^{14}C]Glycerophosphate was prepared as described by Strickland & Rossiter (1962). The specific radioactivity was 1.8×10^5 counts/min./μmole.

(d) L-α-[^{14}C]Glycerophosphate was prepared biologically from randomly labelled [^{14}C]glucose as described by Stewart & Strickland (1961). The specific radioactivity was 9.5×10^4 counts/min./μmole.

(e) [^{14}C]Phosphatidic acid was prepared biologically by the removal of choline from [^{14}C]lecithin. The latter was prepared by incubation of rat-liver slices (500 mg.) in a Krebs-Ringer bicarbonate medium containing glucose and [1- ^{14}C]glycerol (25 μC in 5.3 μmoles) for 4 hr. at 38° under oxygen + carbon dioxide (95:5). Slices were washed with medium containing inactive glycerol, pooled and blended in chloroform-methanol (2:1, v/v). The phosphatides were extracted and purified by the procedure of Folch, Lees & Sloane-Stanley (1957). [^{14}C]Lecithin was isolated from the mixed phosphatide fraction by chromatography on a silicic acid column by the procedure described by Hanahan, Dittmer & Warashina (1957). A typical preparation yielded 70 mg. of [^{14}C]lecithin from 10 g. of liver.

[^{14}C]Phosphatidic acid was prepared from [^{14}C]lecithin by the enzymic removal of choline by phospholipase D (phosphatidylcholine phosphatidohydrolase) from carrot chromoplasts (Kates, 1954, 1955). [^{14}C]Lecithin was shaken with the chromoplasts in ether and acetate buffer, pH 5.7, for 2 hr. at room temperature. The reaction mixture was acidified with N-perchloric acid and filtered through a sintered-glass funnel. The residue was washed with ether until the filtrate was colourless. The ether phase that separated from the filtrate was washed twice with water and evaporated almost to dryness. The residue was taken up in 5 ml. of chloroform and applied to a silicic acid column, which was eluted stepwise with chloroform, chloroform-methanol (4:1, v/v) and chloroform-methanol (3:2, v/v). The carotenes were removed by the chloroform, the phosphatidic acid, free of colour, was eluted by the first mixed solvent and the lecithin that had not reacted appeared with the second. The fractions containing phosphatidic acid were pooled and the solvent was removed. A yield of 50 mg. of [^{14}C]phosphatidic acid was obtained from 70 mg. of [^{14}C]lecithin. The product had an ester:phosphorus ratio of 2.0 and negligible nitrogen. Ester was estimated by the method of Rapport & Alonzo (1955). Radioactivity in the fatty acids obtained by saponification represented only 10% of the total, showing that the major part of the label had

entered the glycerol moiety of the lecithin and hence the phosphatidic acid derived from it. Two preparations were obtained with specific radioactivities of 18 800 and 33 800 counts/min./ μ mole.

Other materials. Synthetic D- α -dimyristin, D- α -dipalmitin, D- α -distearin, D- α -diolein and L- α -diolein were kindly provided by Dr E. Baer, Banting and Best Department of Medical Research, University of Toronto. Natural α -diglycerides were prepared from purified lecithin (egg and ox spinal cord) by the method of Hanahan & Vercamer (1954), by using the phospholipase C (phosphatidylcholine cholinephosphohydrolase) of *Clostridium perfringens* (kindly supplied by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., U.S.A.). The lecithins were purified as outlined by Rossiter *et al.* (1957a). Diglycerides were normally emulsified in Tween 20 [polyoxyethylene sorbitan monolaurate, Atlas Powder Company (Canada) Ltd., Brantford, Ontario] at a final concentration of 0.025 %.

Synthetic L- α -dioleoylglycerophosphoric acid and L- α -distearoylglycerophosphoric acid were generously supplied by Dr E. Baer. Phosphatidic acid was prepared from purified lecithins (egg and ox spinal cord) by the action of phospholipase D from carrot chromoplasts (Kates, 1954, 1955). The phosphatidic acids were suspended in water by homogenization, if necessary, before addition to the incubation vessels.

CoA, the sodium salt of ATP, NAD and the sodium salt of CTP were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. The sodium salt of CDP-choline was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Formation of lecithin from phosphorylcholine.

Table 1 shows that [14 C]phosphorylcholine was incorporated into the lipid of a dispersion prepared from rat brain and that there was a requirement for CTP, but not for CoA. When the reaction mixture was emulsified with Tween 20 there was a slight increase in the labelling, possibly due to an increase in the availability to the enzyme system of endogenous acceptor for phosphorylcholine. There was no labelling when the [14 C]phosphorylcholine was replaced by [14 C]choline. When the labelled lipids were hydrolysed by Dawson's (1954) procedure, the radioactivity was confined to glycerolphosphorylcholine derived from lecithin (specific radioactivity 930 and 840 counts/min./ μ mole of phosphate in two experiments). These results confirm the previous findings for [32 P]phosphorylcholine (McMurray *et al.* 1957b).

Formation of lecithin from cytidine diphosphate choline. Lecithin synthesis from phosphorylcholine in fowl liver has been shown (Kennedy & Weiss, 1956; Borkenhagen & Kennedy, 1957; Weiss, Smith & Kennedy, 1958) to follow the pathways in reactions (1) and (2).

CTP + phosphorylcholine $\rightleftharpoons$ CDP-choline + pyrophosphate (1)

CDP-choline + D- α -diglyceride $\rightleftharpoons$ L- α -lecithin + CMP (2)

Evidence that the same reactions occur in brain has been adduced by Kennedy & Weiss (1956) and Rossiter *et al.* (1957a). This hypothesis is supported by the results reported in Table 2, which shows that radioactivity from CDP-[14 C]choline was incorporated into the phosphatide fraction of a rat-brain dispersion and that the incorporation was increased by the addition of natural diglycerides. A coupled energy source was not necessary for the incorporation, since a tris buffer medium was adequate, provided that Mg $^{2+}$ ions were present (Expt. 3). All of the radioactivity (4650, 4310 and 4500 counts/min./ μ mole of phosphate in three experiments) was found in glycerolphosphorylcholine derived from lecithin, in agreement with the previous finding of Rossiter *et al.* (1957a) that

Table 1. *Incorporation of radioactivity from [14 C]-phosphorylcholine into the phosphatides of dispersions of rat brain*

Incubation was carried out in complete medium with either [14 C]phosphorylcholine, 3 μ moles (4.65×10^5 counts/min.), or [14 C]choline, 3 μ moles (1.65×10^6 counts/min.). CoA (0.05 mM), CTP (0.7 mM) and Tween 20 (final concn., 0.025 %) were added as shown. Dispersion was equivalent to 50 mg. of fresh rat brain.

Additions	Specific radioactivity of phosphatides (counts/min./ μ mole of P)	
	Expt. 1	Expt. 2
[14 C]Phosphorylcholine	16	34
CoA, [14 C]phosphorylcholine	19	28
CTP, [14 C]phosphorylcholine	210	290
CTP, Tween 20, [14 C]phosphorylcholine	220	340
CTP, [14 C]choline	0	0

Table 2. *Incorporation of radioactivity from CDP-[14 C]choline into the phosphatides of dispersions of rat brain*

Unless otherwise specified, incubation was carried out in complete medium with CDP-[14 C]choline, 0.5 μ mole (7.75×10^4 counts/min.). Dispersion was equivalent to 50 mg. of fresh rat brain. Diglycerides (1 μ mole) were emulsified in Tween 20 (0.025 % final concn.).

Additions	Specific radioactivity of phosphatides (counts/min./ μ mole of P)	
	Expt. 1	Expt. 2
0.025 % Tween 20	530	870
Diglyceride from egg lecithin	780	1020
Diglyceride from ox spinal-cord lecithin	—	1360
	Expt. 3	
Tris buffer only (replacing complete medium)	28	
Tris buffer only + 12 μ moles of MgCl $_2$	1050	

Table 3. *Effect of $\alpha\beta$ -diglycerides on the incorporation of the radioactivity from CDP-[^{14}C]choline into the phosphatides of dispersions of rat brain*

Incubation was carried out in this buffer (pH 7.4), 50 mM, with MgCl_2 , 8 mM, and CDP-[^{14}C]choline, 0.5 μmole (7.75×10^4 counts/min.). Dispersion was equivalent to 50 mg. of fresh rat brain. Diglycerides were emulsified in Tween 20 (0.025% final concn.).

Additions	Ratio of specific radioactivity (D- $\alpha\beta$ -dimyristin = 100)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Mean ratio
None	121	—	—	101	111
D- $\alpha\beta$ -Dimyristin	100	100	100	100	100
D- $\alpha\beta$ -Dipalmitin	121	83	108	105	104
D- $\alpha\beta$ -Distearin	193	98	91	114	124
D- $\alpha\beta$ -Diolein	207	152	134	121	154
L- $\alpha\beta$ -Diolein	—	94	83	94	90
Diglyceride from egg lecithin	—	182	126	129	146

phosphorylcholine from [^{32}P]CDP-choline enters lecithin intact.

Role of diglyceride. McMurray *et al.* (1957*b*) suggested that in brain homogenates lecithin is formed by the transfer of phosphorylcholine from CDP-choline to a suitable acceptor, to form lecithin. Kennedy & Weiss (1956) showed that, for the liver enzyme, D- $\alpha\beta$ -diglyceride was such an acceptor. At this time McMurray *et al.* (1957*b*) were unable to demonstrate any significant increase in the incorporation of labelled phosphorylcholine into lecithin on the addition of D- $\alpha\beta$ -diglyceride. It is probable that the concentration of the emulsifying agent used to suspend the insoluble diglyceride was sufficient to inhibit the enzymic production of ATP, which is necessary for the formation of lecithin from phosphorylcholine. When CDP-choline is substituted for phosphorylcholine, however, a coupled energy source is no longer required. As noted previously for [^{32}P]CDP-choline (Rossiter *et al.* 1957*a*) and confirmed for CDP-[^{14}C]choline by the results reported in Table 2, the addition of suitable diglycerides caused a consistent stimulation of the incorporation of radioactivity from labelled CDP-choline into lecithin. Furthermore, it was found that neither synthetic nor natural L- α -phosphatidic acids caused this stimulation. Experiments such as these led to the conclusion that in the brain system also, as in liver, D- $\alpha\beta$ -diglyceride, but not L- α -phosphatidic acid, is the acceptor of phosphorylcholine.

When synthetic D- $\alpha\beta$ -diglycerides were added, the results were somewhat irregular (Table 3). In general, dimyristin, dipalmitin and distearin did not consistently stimulate the incorporation of radioactivity from CDP-[^{14}C]choline into lecithin, whereas diolein caused a large and consistent stimulation. These findings emphasize the importance of the nature of the constituent fatty acids of the added diglycerides. Apparently the specificity of rat-brain phosphorylcholine-glyceride transferase (CDP-choline-1,2-diglyceride choline phosphotransferase) is such that natural diglycerides and D- $\alpha\beta$ -

diolein are more active than the saturated D- $\alpha\beta$ -diglycerides. Strickland (1962) has reported that all three saturated diglycerides, in addition to D- $\alpha\beta$ -diolein, were effective substrates for the enzyme, diglyceride kinase (ATP-diglyceride phosphotransferase) in similar rat-brain preparations, suggesting that the effects reported in Table 3 are the result of the specificity of the enzyme and not merely the result of differences in the solubility of the diglycerides.

The stimulation of the formation of lecithin brought about by the addition of D- $\alpha\beta$ -diolein was similar to that produced by the same amount of diglyceride prepared from egg lecithin (Table 3). Under the conditions of the experiment no greater incorporation was achieved when amounts of egg diglyceride larger than 1 μmole were added.

Whereas D- $\alpha\beta$ -diolein stimulated the labelling of lipid from CDP-[^{14}C]choline, the stereoisomer L- $\alpha\beta$ -diolein was consistently without effect (Table 3). Weiss, Kennedy & Kiyasu (1960) reported that L- $\alpha\beta$ -diolein was less effective than D- $\alpha\beta$ -diolein in the liver-enzyme system. Since D- $\alpha\beta$ -diglyceride will give rise to L-lecithin, these results are consistent with the currently held view that all natural lecithins, and other glycerophosphatides, are derivatives of L- α -glycerophosphate. Strickland (1962) reported that D- $\alpha\beta$ -diolein, but not L- $\alpha\beta$ -diolein, was an effective substrate for the diglyceride kinase of rat brain.

Formation of phosphatidic acid. Kornberg & Pricer (1952*a*, *b*, 1953*a*, *b*) and Kennedy (1953) have established that in liver preparations L- α -glycerophosphate may give rise to phosphatidic acid according to reactions (3) and (4).

Similar reactions probably occur in brain (McMurray *et al.* 1957*b*; Vignais & Zabin, 1958; Vignais, Gallagher & Zabin, 1958). McMurray *et al.*

(1957*b*) showed that radioactivity from DL- α -glycero[32 P]phosphate is incorporated into phosphatidic acid by rat-brain preparations, provided that ATP and CoA are added.

Table 4 shows that, with DL- α -[14 C]glycerophosphate, CoA greatly increased the incorporation of radioactivity into the phosphatide fraction, whereas the addition of CTP was without effect. When the hydrolysis products of the mixed labelled lipids were examined by the method of Dawson (1954), glycerophosphate (derived from phosphatidic acid) and glycerolphosphorylinositol (derived from phosphatidylinositol) were both labelled and, in addition, a small but consistent amount of radioactivity was recovered from glycerolphosphorylcholine derived from lecithin. In the previous experiments radioactivity from DL- α -glycero[32 P]phosphate was never detected in lecithin (McMurray *et al.* 1957*b*).

Table 5 shows the results of experiments in which L- α -[14 C]glycerophosphate replaced synthetic DL- α -[14 C]glycerophosphate. Although most of the radioactivity was recovered either from the phosphatidic acid or from the phosphatidylinositol, a certain amount of radioactivity was again detected in the lecithin. Considerably more counts were present in this fraction than the modest specific activity figures would suggest, for the concentration of lecithin in the preparation was much greater than that of either phosphatidylinositol or phosphatidic acid. The specific radioactivity of the lecithin was increased by the addition of unlabelled CDP-choline (1 mM) to the reaction mixture. The significance of this finding, and the observation reported in Table 4 that CTP increased the labelling of total phosphatide from DL- α -[14 C]glycerophosphate in the presence of CoA, is discussed below.

Formation of diglyceride by the dephosphorylation of phosphatidic acid. Smith, Weiss & Kennedy (1957) showed that pigeon liver contained an enzyme, phosphatidic acid phosphatase (L- α -phosphatidic phosphohydrolase), that catalysed the liberation of inorganic phosphate from phosphatidic acid (reaction 5).

Although Smith *et al.* (1957) showed the presence of phosphatidic acid phosphatase in rat brain, McMurray *et al.* (1957*b*) reported that they were unable to demonstrate any significant dephosphorylation of several synthetic L- α -phosphatidic acids by various rat-brain preparations. Table 6 shows that, when synthetic L- α -phosphatidic acids were replaced by natural phosphatidic acids, active dephosphorylation was observed. Mg $^{2+}$ ions at a concentration of 8 mM produced a 50–75% inhibition of the dephosphorylation (Table 7), suggesting that the brain enzyme is inhibited by Mg $^{2+}$ ion, as is the liver enzyme described by Smith *et al.* (1957).

The inhibition of brain phosphatidic acid phosphatase probably accounts for the accumulation of phosphatidic acid in brain-tissue preparations (Dawson, 1954; McMurray *et al.* 1957*b*). Apparently only small amounts of phosphatidic acid occur in the brains of freshly killed animals (Dawson, 1955; Hokin & Hokin, 1958).

Role of phosphatidic acid. If phosphatidic acid is a precursor of D- $\alpha\beta$ -diglycerides, the acceptor of phosphorylcholine from CDP-choline in brain preparations, it should be possible to observe a stimulation of the formation of lecithin from CDP-choline after the addition of phosphatidic acid to such preparations, provided that the conditions are appropriate for the dephosphorylation of phosphatidic acid by phosphatidic acid phosphatase. Such a situation was indeed reported by Rodbell & Hanahan (1955) for the incorporation of [32 P]phosphorylcholine into the lecithin of guinea-pig-liver mitochondria. Subsequently Smith *et al.* (1957) showed that the addition of phosphatidic acid to a rat-liver preparation caused an increase in the incorporation of the radioactivity from CDP-[14 C]-

Table 4. *Incorporation of radioactivity from DL- α -[14 C]glycerophosphate into the phosphatides of dispersions of rat brain*

Incubation was carried out in complete medium with DL- α -[14 C]glycerophosphate, 8 μ moles (1.4×10^6 counts/min.). CoA (0.05 mM) and CTP (0.7 mM) were added as shown. Dispersion was equivalent to 50 mg. of fresh rat brain.

Additions	Specific radioactivity of phosphatides (counts/min./ μ mole of P)		
	Expt. 1*	Expt. 2	Expt. 3
—	87	130	110
CoA	340	320	200
CTP	—	100	70
CoA, CTP	—	370	330

* DL- α -[14 C]Glycerophosphate (2 μ moles) added.

Table 5. *Incorporation of radioactivity from L- α -[14 C]glycerophosphate into the individual phosphatides of dispersions of rat brain*

Incubation was carried out in complete medium with L- α -[14 C]glycerophosphate, 3 μ moles (2.85×10^5 counts/min.). Dispersion was equivalent to 60 mg. of fresh rat brain. Phosphatides were examined by the hydrolysis procedure of Dawson (1954).

Fraction	Specific radioactivity (counts/min./ μ mole of P)	
	Expt. 1	Expt. 2
Total phosphatide	150	160
Inositol phosphatide	1330	1090
Phosphatidic acid	1490	1490
Lecithin	30	40
Lecithin (incubated in the presence of 1 mM-CDP-choline)	70	152

choline into lecithin, provided that the phosphatidic acid was first incubated in a magnesium-free medium to allow dephosphorylation of the phosphatidic acid to diglyceride.

In the experiments reported in Table 8 a dispersion of rat brain was incubated at 37° for 60 min. in tris buffer containing no Mg²⁺ ions, with and without the addition of natural phosphatidic acids. After this preliminary incubation, CDP-[¹⁴C]choline and sufficient magnesium chloride to make the final concentration 8 mM were added and the reaction mixture was incubated at 37° for a further 60 min. to permit the formation of lecithin according to reaction (2). The addition of the phosphatidic acid preparation stimulated the incorporation of radioactivity from CDP-[¹⁴C]choline into the lecithin. When Mg²⁺ ions (8 mM) were present during the preliminary incubation, the stimulatory effect of

the added phosphatidic acid was much less (Table 8, Expts. 3 and 5), presumably because the Mg²⁺ ion inhibited the phosphatidic acid phosphatase. It is thus seen that under appropriate conditions L- α -phosphatidic acid, like D- α -diglyceride, can stimulate the formation of lecithin in brain preparations.

When [¹⁴C]phosphatidic acid was added to a homogenate preparation of rat brain, radioactivity was recovered from glycerolphosphorylcholine derived from lecithin (Table 9). In the presence of added CTP the incorporation of radioactivity into lecithin was considerably increased, but the increase was greater when CTP was replaced by CDP-choline.

These experiments provide direct proof that radioactivity from [¹⁴C]phosphatidic acid may be incorporated into the lecithin of brain dispersions.

Table 6. *Dephosphorylation of various preparations of phosphatidic acid by rat brain*

Incubation was in Mg-free tris buffer (50 mM), pH 7.4, for 60 min. at 37°. Phosphatidic acid was added as indicated. Dispersion was equivalent to 100 mg. of fresh tissue, or particle preparation was equivalent to 300 mg. of fresh tissue. Final volume, 3 ml.

Phosphatidic acid	Amount added (μ moles)	Tissue preparation	Release of inorganic P	
			(μ g./60 min./ml.)	(% hydrolysis)
L- α -Distearoylglycerophosphoric acid	2	Mitochondria	0.7	3
			0.9	4
	2	Microsomes	0.2	1
			1.2	6
	4	Water dispersion	0	0
			0	0
L- α -Dioleoylglycerophosphoric acid	2	Mitochondria + microsomes	0.5	2
	2	Water dispersion	0	0
	4	Water dispersion	0.6	1
			2.0	5
Mixed (from egg lecithin)	4	Mitochondria + microsomes	8.9	22
	4	Water dispersion	9.0	22
			11.3	27
Mixed (from ox spinal-cord lecithin)	4	Water dispersion	11.1	27
			6.8	17
			14.8	36
	6	Water dispersion	15.4	26

Table 7. *Effect of magnesium chloride on the dephosphorylation of phosphatidic acid by rat-brain preparation*

Conditions were as described in Table 6.

Tissue preparation	Natural phosphatidic acid (4 μ moles)	MgCl ₂ (8 mM)	Release of inorganic P	
			(μ g./60 min./ml.)	(% inhibition)
Mitochondria + microsomes	Egg	—	8.9	0
		Added	2.5	72
Water dispersion	Egg	—	9.0	0
		Added	2.3	75
Water dispersion	Ox spinal cord	—	6.8	0
		Added	3.5	49
Water dispersion	Ox spinal cord	—	10.2	0
		Added	2.5	75

Table 8. *Effect of preincubation with phosphatidic acid, with and without addition of magnesium chloride, on the subsequent incorporation of radioactivity from CDP-[¹⁴C]choline into the lecithin of dispersions of rat brain*

Dispersions equivalent to 100 mg. of fresh rat brain were preincubated in tris buffer, pH 7.4, at 37°. After 60 min. MgCl₂ (final concn. 8 mM in all vessels), Tween 20 (final concn. 0.025%) and CDP-[¹⁴C]choline (0.5 μ mole, 7.75×10^4 counts/min.) were added and incubation was continued for 60 min.

Additions during preincubation	Specific radioactivity of lecithin (counts/min./ μ mole of P)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
None	1020	1480	470	1600	310
(a) Phosphatidic acid from egg lecithin (4 μ moles)	1820	1890	1010	—	—
(b) (a) plus MgCl ₂ (8 mM)	—	—	810	—	—
(c) Phosphatidic acid from ox spinal-cord lecithin (4 μ moles)	—	—	1690	2710	1130
(d) (c) plus MgCl ₂ (8 mM)	—	—	—	—	510

Table 9. *Effect of CTP and CDP-choline on the incorporation of radioactivity from [¹⁴C]phosphatidic acid into the lecithin of dispersions of rat brain*

Incubation was carried out in complete medium with [¹⁴C]phosphatidic acid, 1.25 μ moles (23 400 counts/min. in Expt. 1 and 42 100 counts/min. in Expt. 2). CTP and CDP-choline were added as shown. Dispersion was equivalent to 60 mg. of fresh rat brain.

Additions	Specific radioactivity of lecithin (counts/min./ μ mole of P)	
	Expt. 1	Expt. 2
None	9	140*
CTP	28	190
CDP-choline	65	250

* This value is high, from slight contamination of added [¹⁴C]phosphatidic acid with [¹⁴C]lecithin.

DISCUSSION

The experiments reported here strengthen previous conclusions that reaction (1) (Table 1), reaction (2) (Tables 2 and 3), reactions (3) and (4) (Tables 4 and 5) and reaction (5) (Tables 6 and 7) take place in preparations of rat brain.

Doubts have been expressed about two points in the biosynthesis of lecithin in brain: (a) the nature of the substance that accepts the phosphorylcholine provided by CDP-choline and (b) the role of phosphatidic acid (McMurray *et al.* 1957*b*). With regard to the first point, the acceptor was difficult to characterize because brain preparations contain endogenous lipid and, presumably, endogenous acceptor. Another difficulty is that diglycerides, the most likely acceptors, are only sparingly soluble in water. The experiments reported in Tables 2 and 3 make it clear that in brain, as in liver (Weiss & Kennedy, 1956), D- α -diglyceride and not L- α -phosphatidic acid is the acceptor. The finding that in brain, as in liver (Smith *et al.* 1957), phosphatidic acid may be dephosphorylated to yield diglyceride

(Tables 6 and 7) elucidates the second point; phosphatidic acid is the precursor for diglyceride. The observations that phosphatidic acid, but not lecithin, is labelled from inorganic ³²P-labelled phosphate (Dawson, 1954) or α -glycero[³²P]phosphate (McMurray *et al.* 1957*b*) are thus explained, since phosphorus incorporated into phosphatidic acid is lost as inorganic phosphate. When the source of radioactivity is α -[¹⁴C]glycerophosphate, however, with brain (Tables 4 and 5) and liver preparations (Strickland & Rossiter, 1962), counts may be recovered from lecithin.

Although each of the reactions (1)–(5) may be demonstrated in brain preparations, it is of importance to determine whether these preparations can catalyse the reactions in an ordered sequence. Evidence that this is, in fact, possible is outlined below.

First, it was shown that when phosphatidic acid is incubated under appropriate conditions (absence of Mg²⁺ ions), the subsequent formation of lecithin from CDP-[¹⁴C]choline is stimulated. A reasonable interpretation of these findings is that the phosphatidic acid gives rise to diglyceride (reaction 5) during the course of the preliminary incubation and that during the subsequent incubation this diglyceride stimulates the formation of lecithin by reaction (2).

Secondly, the finding that phosphatidic acid can give rise to diglyceride, the precursor of lecithin, even when the concentration of Mg²⁺ ions is not optimum for dephosphorylation, explains why the addition of CTP in the presence of CoA stimulates the incorporation of the radioactivity of DL- α -[¹⁴C]-glycerophosphate into the total phosphatides of rat brain (Table 4) and why the addition of unlabelled CDP-choline stimulates the labelling of lecithin from L- α -[¹⁴C]glycerophosphate (Table 5). In both instances phosphatidic acid, newly formed by reactions (3) and (4), is partly degraded to yield labelled diglyceride. The formation of lecithin from

this labelled diglyceride is greatly stimulated by the addition of the unlabelled cytosine nucleotides (reactions 1 and 2). A similar stimulation of lecithin formation by unlabelled CDP-choline was reported by Strickland & Rossiter (1962) for rat-liver mitochondria. In this instance the addition of CDP-choline to a preparation containing phosphatidic acid freshly labelled from α -[^{14}C]glycerophosphate was able to direct lipid synthesis from the formation of triglyceride to the formation of lecithin.

Finally, perhaps the most convincing evidence that lecithin is formed by the proposed series of reactions in brain dispersions is the observation that unlabelled CDP-choline could stimulate the incorporation of [^{14}C]phosphatidic acid into lecithin (Table 9). Again it is suggested that the labelled phosphatidic acid is dephosphorylated to yield diglyceride. In the presence of CDP-choline the labelled diglyceride is incorporated into lecithin.

In the foregoing consideration of the biosynthesis of lecithin in brain, no mention has been made of the well-known fact that lecithins from different sources vary considerably in the nature of their constituent fatty acids and that the fatty acids esterified at the α' -position of the glycerol molecule are different from those esterified at the β -position (Hanahan, 1954*a, b*; Rhodes & Lea, 1956; Tattre, 1959; Hanahan, Brockerhoff & Barron, 1960). In metabolic experiments, saturated fatty acids tend to exchange more readily with saturated fatty acids, which are esterified principally in the α' -position, whereas unsaturated fatty acids tend to exchange more readily with unsaturated fatty acids, which are esterified principally in the β -position (Hanahan & Blomstrand, 1956; Rhodes, 1958; Blomstrand, Dahlback & Linder, 1959; Hawke, 1959). If the metabolic pathways described above have a general application, the positional configuration must be initiated at the stage where L- α -glycerophosphate is esterified (reaction 4) and it must be maintained throughout the D- $\alpha\beta$ -diglyceride stage.

Although the experiments reported here provide good evidence for the view that in brain lecithin may be formed there from smaller molecules by a sequence of reactions similar to that postulated by Kennedy & Weiss (1956) for liver tissue, it should be stressed that these may not be the only pathways. For example, phosphatidic acid (Hokin & Hokin, 1959; Strickland, 1957, 1962) and lyso-phosphatidic acid (Hokin & Hokin, 1960) may be formed in brain preparations by the transfer of phosphate from ATP to D- $\alpha\beta$ -diglyceride and monoglyceride respectively. In addition, the reports of Bremer & Greenberg (1961) that lecithin may be formed in rat-liver microsomes by the transfer of methyl groups from *S*-adenosyl-methionine to phosphatidylethanolamine and of

Lands (1961) that lecithin may be formed by the acylation of lysolecithin in brain preparations serve to emphasize the metabolic versatility of tissue preparations.

SUMMARY

1. In dispersions of rat brain the incorporation of radioactivity from [^{14}C]phosphorylcholine into the phosphatide fraction was increased by the addition of cytidine triphosphate to the incubation medium, but not by the addition of coenzyme A. Examination of the hydrolysis products of the labelled phosphatides showed that the radioactivity was confined to the lecithin fraction.

2. In similar dispersions the incorporation of radioactivity from cytidine diphosphate [^{14}C]choline into the phosphatide fraction was increased by the addition of enzymically prepared diglycerides from naturally occurring lecithins, by the addition of synthetic D- $\alpha\beta$ -diolein, but not by the addition of L- $\alpha\beta$ -diolein. Again the radioactivity was confined to the lecithin fraction.

3. The incorporation of radioactivity from DL- α -[^{14}C]glycerophosphate into the phosphatide fraction was increased by the addition of coenzyme A, but not by the addition of cytidine triphosphate. Radioactivity from L- α -[^{14}C]glycerophosphate was recovered in phosphatidic acid, phosphatidylinositol and lecithin. The incorporation of radioactivity into lecithin was stimulated by the addition of non-labelled cytidine diphosphate choline.

4. Phosphatidic acids were dephosphorylated to yield inorganic phosphate in preparations from rat brain. The dephosphorylation was inhibited by the presence of Mg^{2+} ions in the reaction medium.

5. Phosphatidic acids incubated in magnesium-free medium stimulated the subsequent incorporation of radioactivity from cytidine diphosphate [^{14}C]choline into lecithin.

6. The addition of non-labelled cytidine diphosphate choline stimulated the incorporation of biologically prepared [^{14}C]phosphatidic acid into lecithin.

7. The results are discussed in relation to the biosynthesis of lecithin in brain.

The work was aided by grants from the Medical Research Council of Canada. Assistance rendered by Mr H. Bishop and Mrs S. Boyadjian is gratefully acknowledged.

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Biosynthesis of Phosphatidylinositol in Rat Brain

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(Received 9 July 1962)

The investigations of Folch (1949) and Folch & LeBaron (1953) revealed the presence of two forms of lipid-bound inositol in brain, referred to as diphosphoinositide and phosphatidopeptide. Inositol diphosphate was reported to be a major hydrolysis product of these lipids. More recent work, however, has indicated that the brain diphosphoinositide of Folch is more heterogeneous than was hitherto suspected (Hörhammer, Wagner & Richter, 1959; Grado & Ballou, 1961; Tomlinson & Ballou, 1961; Brockerhoff & Ballou, 1961; Ellis & Hawthorne,

1961; Dawson & Dittmer, 1961). It now seems clear that, in addition to diphosphoinositide, brain contains a monophosphoinositide (Hörhammer, Wagner & Richter, 1958; Hokin & Hokin, 1958; Dawson, 1960; Hörhammer, Wagner & Hözl, 1960; Brockerhoff & Ballou, 1961; Dittmer & Dawson, 1961) and a triphosphoinositide (Brockerhoff & Ballou, 1961; Ellis & Hawthorne, 1961; Dittmer & Dawson, 1961). The structures of the mono-, di- and tri-phosphoinositides of brain have been firmly established and may be designated respectively as