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# Biosynthesis of Phosphatidylinositol in Rat Brain

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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

1-phosphatidyl-L-myoinositol, 1-phosphatidyl-L-myoinositol 4-phosphate and 1-phosphatidyl-L-myoinositol 4,5-diphosphate (Brockerhoff & Ballou, 1961; Dawson & Dittmer, 1961).

This investigation is primarily concerned with the biosynthesis in brain preparations of the monophosphoinositide (1-phosphatidyl-L-myoinositol). This lipid may readily be removed from brain tissue by extraction with mixtures of chloroform and methanol. Under these conditions the triphosphoinositide, and possibly the diphosphoinositide, remains bound to protein and is not readily extracted (Dittmer & Dawson, 1961).

Experiments with labelled precursors have revealed that, compared with other lipids, the inositol-containing phospholipids of brain are metabolically quite active. Dawson (1954*a*, *b*) showed that the inositol lipid of guinea-pig brain is labelled metabolically from radioactive inorganic phosphate. This observation was also confirmed by McMurray, Strickland, Berry & Rossiter (1957) for water homogenate and mitochondrial preparations of rat brain. Similar observations have been made for brain slices starting with inorganic $^{32}$P-labelled phosphate, [1-$^{14}$C]glycerol and [$^{3}$H]inositol (Magee, Berry & Rossiter, 1956; Pritchard, 1958; Hokin & Hokin, 1958). McMurray *et al.* (1957) also showed that actively metabolizing homogenates and mitochondrial preparations of rat brain readily incorporated [$^{32}$P]-α-glycerophosphate into inositol phosphatide as well as into phosphatidic acid. CTP was found to increase the labelling of the phospholipids from inorganic $^{32}$P-labelled phosphate, but it decreased the labelling from [$^{32}$P]-α-glycerophosphate.

Pathways leading to the synthesis of phosphatidylinositol in other tissues have been proposed by Agranoff, Bradley & Brady (1958) (kidney) and Paulus & Kennedy (1960) (liver). In both schemes the liponucleotide, CDP-diglyceride, was implicated. It was suggested that this novel intermediate participated in reaction of equation (1).

$$\text{CDP-diglyceride} + \text{inositol} \rightarrow \text{phosphatidylinositol} + \text{CMP} \quad (1)$$

However, the reactions proposed for the formation of CDP-diglyceride differed. Agranoff *et al.* (1958) suggested that the CMP in CDP-choline is transferred from phosphorylcholine to phosphatidic acid, whereas Paulus & Kennedy (1960) suggested that CTP and phosphatidic acid react to yield CDP-diglyceride and pyrophosphate as shown in reaction (2).

$$\text{CTP} + \text{phosphatidic acid} \rightarrow \text{CDP-diglyceride} + \text{pyrophosphate} \quad (2)$$

In this paper some observations are presented on the formation of phosphatidylinositol from [$^{3}$H]-inositol and [$^{14}$C]phosphatidic acid in rat-brain preparations. Certain aspects of this work have been reported in preliminary communications (Thompson, Strickland & Rossiter, 1959; Thompson, Subrahmanyam & Strickland, 1960; Strickland, Thompson, Subrahmanyam & Rossiter, 1960).

## METHODS

*Tissue incubation and extraction.* Male rats of the Sprague–Dawley strain (100–250 g.) were decapitated and the cerebral hemispheres were rapidly removed. The tissue was dispersed in 3·15 parts of water at 0° in a Potter–Elvehjem hand homogenizer. In some experiments microsomes were prepared by differential centrifuging of homogenates of rat brain prepared in 0·25M-sucrose at 0°. Nuclear and mitochondrial fractions were sedimented by successively centrifuging at 1000*g* for 10 min. and 12 000*g* for 10 min. (with the high-speed attachment of PR-1 International refrigerated centrifuge, International Equipment Co., Boston, Mass., U.S.A.). Microsomes were obtained by centrifuging the supernatant at 100 000*g* for 30 min. (with Spinco model L preparative ultracentrifuge). Mixed particles were obtained by elimination of the centrifuging step used to sediment the mitochondrial fraction. Pellets of either particle preparation were suspended in 0·25M-sucrose (equivalent of 1–1·2 g. of brain/3·0 ml.) before incubation. In two experiments the microsome preparation was treated with 1% Cutscum ('di-isoacetylphenoxypolyethoxyethanol'; Fisher Scientific Co., Toronto, Ontario, Canada), after which it was centrifuged at 100 000*g* for 30 min.

In experiments with [$^{3}$H]inositol as labelled precursor, portions of the dispersion representing 12 mg. wet wt. of brain were pipetted into 12 ml. Pyrex centrifuge tubes containing tris buffer (pH 7·4), 50 mM, and potassium phosphate (pH 7·4), 10 mM. Other substances were added dissolved in water and neutralized when necessary. The final volume was 0·3 ml. The vessels were capped and incubated in a shaking bath at 37·5° for 60 min. in an atmosphere of air. At the end of the incubation period, the reaction was stopped and lipids were extracted by the addition of 5·7 ml. of chloroform–methanol (2:1, v/v). In experiments with [$^{14}$C]phosphatidic acid as labelled precursor, portions of the brain dispersion representing 120 mg. wet wt. of tissue were incubated in a similar medium at 37·5° for 60 min. The final volume was 3·0 ml. At the end of the incubation period the vessels were chilled in an ice bath, after which the contents were transferred to chilled 6 ml. centrifuge tubes with the aid of a further 2·0 ml. of ice-cold buffer. The samples were centrifuged at 0° for 30 min. at 12 000*g* in the International refrigerated centrifuge (model PR-1) with the high-speed-head attachment. The supernatant fluid was discarded and lipids were then extracted from the residue with chloroform–methanol (2:1, v/v).

The chloroform–methanol extracts from both procedures were purified by the method of Folch, Lees & Sloane-Stanley (1957). In the [$^{3}$H]inositol experiments unlabelled carrier inositol (0·09%) was added to the 'upper-phase solvent' (chloroform–methanol–water; 3:48:47, by vol.) containing 0·02% $CaCl_2$. After five washes with this modified 'upper-phase solvent' radioactive contamination in unincubated samples was found to be negligible.

Lipid P was determined as inorganic phosphate by the method of Ernster, Zetterström & Lindberg (1950), after ashing with 60% (v/v) $HClO_4$.

*Chromatography.* Whatman 3MM filter papers were impregnated with silicic acid by the procedure of Marinetti, Erbland & Kochen (1957). Portions of the lipid extract containing 10–15 $\mu$g. of lipid P were chromatographed in the solvent system di-isobutyl ketone–acetic acid–water (40:25:5, by vol.), ascending for 20 hr. at room temperature. The chromatograms were stained with Rhodamine 6G solution and the spots were located by examination of the damp chromatograms under u.v. light. Inositol phosphatide, lecithin and phosphatidic acid were identified by comparison of their mobilities with authentic samples of these lipids. The serine and ethanolamine phosphatides were identified by dipping the chromatograms in ninhydrin (0·2% in acetone) and drying at 90°.

Other samples of phospholipids (representing 240 mg. of original tissue) were hydrolysed in methanolic 0·2M-NaOH for 15 min. at 37° (Dawson, 1954*b*). The water-soluble hydrolysis products were separated by two-dimensional paper chromatography with phenol–$NH_3$ and *tert.*-butanol–trichloroacetic acid solvents. By this method glycerolphosphorylinositol (derived from inositol phosphatide) was separated from glycerophosphate (derived from phosphatidic acid) and glycerolphosphorylcholine, glycerolphosphorylethanolamine and glycerolphosphorylserine, derived from phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine respectively. The compounds were located by spraying with the phosphorus reagent of Wade & Morgan (1953). Glycerolphosphorylethanolamine and glycerolphosphorylserine were further identified by treatment of the chromatograms with ninhydrin. Radioactive areas located by radioautography were cut out with scissors and the phosphate ester was eluted from the paper with water for determination of P content and specific radioactivity.

*Measurement of radioactivity.* Suitable portions of $^3H$- or $^{14}C$-labelled phospholipids were plated on aluminium planchets and dried beneath an infrared light. The samples were then treated with 0·2 ml. of methanol to obtain uniform distribution of the lipids on the planchet, and dried again. Radioactivity was measured in a windowless gas-flow counter (model D47A, Nuclear–Chicago Inc.). Samples of $^3H$-labelled lipids weighing less than 10 $\mu$g. (determined on the basis of lipid P) were counted satisfactorily by this procedure. No correction was made for self-absorption, since under these conditions it was found to reduce the counting rate by not more than 5%. The $^{14}C$-labelled samples were counted at infinite thinness.

*Labelled intermediates.* Myoinositol was tritiated by exposure to tritium gas (New England Nuclear Corp., Boston, Mass., U.S.A.). To remove exchangeable tritium the crude product was dissolved in water. The solution was filtered and evaporated to dryness under reduced pressure at room temperature. The process was repeated several times and the inositol was finally crystallized from aqueous ethanol to constant specific radioactivity. The purified product had a specific activity of $5 \times 10^7$ counts/min./$\mu$mole. In the experiments 0·125 $\mu$mole of [$^3H$]inositol containing $6{\cdot}25 \times 10^6$ counts/min. was added to a final volume of 0·3 ml. in each incubation vessel.

Two preparations of [$^{14}C$]phosphatidic acid (labelled principally in the glycerol portion of the molecule) of specific radioactivities 18 800 and 33 800 counts/min./$\mu$mole were prepared biologically (Strickland, Subrahmanyam, Pritchard, Thompson & Rossiter, 1963). In the experiments 1·25 $\mu$moles of [$^{14}C$]phosphatidic acid containing 23 400 or 42 100 counts/min. were added to a final volume of 3 ml. in each vessel.

*Other materials.* Synthetic D-$\alpha\beta$-diolein, D-$\alpha\beta$-distearin and dioleoyl- and distearoyl-L-$\alpha$-glycerophosphoric acid were generously supplied by Dr E. Baer, Banting and Best Department of Medical Research, University of Toronto. Natural phosphatidic acid was prepared by the action of carrot phospholipase D (phosphatidylcholine phosphatidohydrolase) on ox spinal-cord lecithin according to the method of Kates (1955).

DL-Dipalmitoyl-$\alpha$-glycerophosphoric acid was prepared by a method combining features of those described by Baer (1951) and Paulus & Kennedy (1960). DL-Dipalmitin (1·0 g.), prepared according to the procedure of Howe & Malkin (1951), was dissolved in dry pyridine (25 ml.) and phosphorylated by the slow addition of 3 moles of $POCl_3$ at 0°. After 30 min. the reaction mixture was allowed to attain room temperature and the excess of pyridine was removed on a rotary evaporator. The contents of the flask were shaken with 30 ml. of ice-cold water and the pH was adjusted to 2–3 with $H_2SO_4$ or HCl. The turbid aqueous layer was extracted three times with 1–2 vol. of ether. The extract of lipid-soluble material was dried over anhydrous $Na_2SO_4$ and the ether was removed by evaporation. The residue was dissolved in the minimal amount (approx. 20 ml.) of hot acetone and the precipitate that formed on cooling was collected and dried under reduced pressure at room temperature. After a second precipitation from hot acetone, 0·44 g. of product was obtained. By analysis this product was found to contain 4·47% of P (theoretical, 4·78) and had an ester:P ratio 2·08. Ester was estimated by the method of Rapport & Alonzo (1955).

CDP-diglyceride was synthesized by causing cytidine monophosphoromorpholidate (prepared by the method of Moffatt & Khorana, 1961) to react with DL-dipalmitoyl-$\alpha$-glycerophosphoric acid, as described by Agranoff & Bradley (1961). The product was isolated by following the procedure of Paulus & Kennedy (1960). It possessed a P:cytidine:ester ratio 2:0·95:1·90.

CoA, NAD, ATP, CTP, CDP, CMP and CDP-choline were purchased either from Pabst Laboratories, Milwaukee, Wis., U.S.A., or from Sigma Chemical Co., St Louis, Mo., U.S.A.

## RESULTS

### *Incorporation of* [$^3H$]*inositol*

*Cofactors.* Table 1 shows that the cofactor requirements for the incorporation of [$^3H$]inositol into the lipids of brain dispersions are relatively simple. The addition of CTP caused an increase in the lipid labelling, but a much greater increase was observed in the presence of added magnesium chloride. It was found that maximum stimulation occurred when the concentration of $Mg^{2+}$ ions was 16 mM. This concentration of $Mg^{2+}$ ions could be replaced by 2 mM-$Mn^{2+}$ ions.

Table 1 also shows that supplementation of the system with glucose, ATP and NAD, cofactors

necessary to promote glycolysis, with the concomitant production of ATP (McMurray *et al.* 1957), did not increase the incorporation. Although these additional cofactors caused some increase in the absence of CTP, they were ineffective when CTP was present. These findings are in contrast with the demonstration by McMurray *et al.* (1957) of exacting requirements for the labelling of lipids from inorganic $^{32}P$-labelled phosphate and [$^{32}P$]-α-glycerophosphate in similar water dispersions of rat brain. Optimum incorporation of these labelled precursors were shown to be dependent upon the maintenance of an active system for the generation of ATP.

*Nucleotide requirement.* In brain dispersions concentrations of CTP as low as 0·01 mM stimulated the incorporation of [$^3H$]inositol into lipid. This effect was optimum at 1 mM-CTP.

Other cytosine nucleotides, CDP, CMP and CDP-choline also stimulated lipid labelling, although their effects were less than that of CTP, when tested at a concentration of 1 mM (Table 2). The triphosphates of guanosine, inosine, adenosine and uridine were relatively ineffective compared with the cytosine nucleotides. Thus the requirement of nucleotides for the incorporation of [$^3H$]inositol into the lipids of dispersions of rat brain appears to be limited to the nucleotides of cytosine, of which group CTP was found to be the most effective.

*Effect of phosphatidic acids and diglycerides.* Table 3 shows that natural phosphatidic acid stimulated the incorporation of [$^3H$]inositol into the lipids of rat-brain dispersions. This stimulatory effect of phosphatidic acid was observed only when phosphatidic acid was added as a dispersion in the detergent Tween 20 [polyoxyethylene sorbitan monolaurate, Atlas Powder Co. (Canada) Ltd., Brantford, Ontario]. Suspensions of phosphatidic acid in water were ineffective in this system. Presumably emulsification of the phosphatidic acid by Tween 20 facilitated the penetration of the lipid to the active surface of the enzyme catalysing the incorporation of [$^3H$]inositol. At the same concentration synthetic distearoyl- and dioleoyl-L-α-glycerophosphoric acid also stimulated lipid labelling, but to a lesser extent. The corresponding synthetic diglycerides, D-αβ-distearin and D-αβ-diolein, were ineffective. It may be assumed therefore that the effect of phosphatidic acid is not the result of its conversion into diglyceride.

In the experiments shown in Table 3, NAD and nicotinamide were added to the reaction mixture because a greater phosphatidic acid effect was observed in their presence. AMP, ATP and other pyridine nucleotides, such as the 3-acetylpyridine analogue of NAD, $NADH_2$, NADP and $NADPH_2$, produced similar effects. The combination of ATP and NAD caused no more stimulation than NAD

Table 1. *Effect of cofactors on the incorporation of* [$^3H$]*inositol into the lipids of a dispersion of rat brain*

Incubation was with buffer containing [$^3H$]inositol, 0·125 μmole ($6·25 \times 10^6$ counts/min.). $MgCl_2$ (8 mM), CTP (0·7 mM), glucose (28 mM), ATP (0·5 mM), NAD (1 mM) and nicotinamide (40 mM) were added as indicated.

| | Specific radioactivity of lipids (counts/min./μg. of P) | | | |
|---|---|---|---|---|
| | Expt. 1 | | Expt. 2 | |
| Additions | CTP absent | CTP present | CTP absent | CTP present |
| None | 20 | 200 | 10 | 130 |
| $MgCl_2$ | 140 | 1250 | 140 | 1030 |
| $MgCl_2$, glucose, ATP, NAD and nicotinamide | 610 | 1050 | 510 | 830 |

Table 2. *Effect of various nucleotides on the incorporation of* [$^3H$]*inositol into the lipids of a dispersion of rat brain*

Incubation was with buffer, $MgCl_2$ (16 mM) and [$^3H$]-inositol, 0·125 μmole ($6·25 \times 10^6$ counts/min.). Nucleotides were added as indicated.

| | Specific radioactivity of lipids (counts/min./μg. of P) | | | |
|---|---|---|---|---|
| Nucleotide (1 mM) | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
| None | 440 | 230 | 450 | 390 |
| CTP | 4640 | 3110 | 4940 | 3810 |
| CDP | 4300 | 2720 | — | — |
| CMP | 3780 | 1720 | — | — |
| CDP-choline | 3320 | 1730 | 3640 | 3250 |
| UTP | — | — | 780 | 690 |
| ATP | — | — | 710 | 650 |
| ITP | — | — | 580 | 550 |
| GTP | — | — | 610 | 450 |

Table 3. *Effect of phosphatidic acids and diglycerides on the incorporation of* [$^3H$]*inositol into the lipids of a dispersion of rat brain*

Incubation was with buffer, $MgCl_2$ (16 mM), CTP (1 mM), Tween 20 (0·025 mg./ml.), NAD (1 mM), nicotinamide (40 mM) and [$^3H$]inositol, 0·125 μmole ($6·25 \times 10^6$ counts/min.). Phosphatidic acid and diglycerides (each 0·125 μmole) were added as indicated.

| | Specific radioactivity of lipids (counts/min./μg. of P) | | |
|---|---|---|---|
| Additions | Expt. 1 | Expt. 2 | Expt. 3 |
| None | 5 430 | 3 140 | 4 920 |
| Natural phosphatidic acid | 10 800 | — | — |
| Distearoyl-L-α-glycero-phosphoric acid | 8 210 | 5 780 | — |
| Dioleoyl-L-α-glycero-phosphoric acid | 6 850 | — | 6 360 |
| D-αβ-Distearin | — | 3 150 | — |
| D-αβ-Diolein | — | — | 5 220 |

alone. Since the stimulatory effect of phosphatidic acids could be demonstrated quite easily in the absence of these substances, it may be assumed that the latter have a non-specific and indirect action which is not, of itself, an integral part of the mechanism of the incorporation of [$^3$H]inositol.

In the experiments presented in Table 2, all of the cytosine nucleotides tested caused some degree of stimulation. Paulus & Kennedy (1960), in reporting similar findings for a liver enzyme, suggested that there is an enzymic exchange of free inositol with the indigenous monophosphoinositide of the enzyme preparation. In the experiments described in Table 4, however, phosphatidic acid stimulated the incorporation of [$^3$H]inositol in the presence of CTP, but not in the presence of CDP-choline. These findings suggest that, whereas CDP-choline or other cytosine nucleotides may stimulate an enzymic exchange of [$^3$H]inositol, CTP is necessary for the formation of inositol phosphatide from phosphatidic acid (see below, Table 6).

First attempts to determine the importance of CDP-diglyceride in the formation of inositol phosphatide in brain were not successful (Table 5, Expts. 1 and 2); CDP-diglyceride was not as active as CTP, or even CDP-choline, in stimulating the incorporation of [$^3$H]inositol into the lipid fraction of a dispersion of rat brain (Expt. 1). In a mixed particle preparation CDP-diglyceride was still not as effective as CTP (Expt. 2). With untreated microsomes CDP-diglyceride was slightly more active than CTP, but treatment of microsomes with Cutscum, as described by Agranoff & Bradley (1961) for kidney particles, yielded a solubilized enzyme preparation which demonstrated a much more specific requirement for CDP-diglyceride (an increase of 250–300-fold) than for CTP (an increase of 20–40-fold). Treatment with Cutscum greatly increased the incorporation when CDP-diglyceride was present (increase of six- to eight-fold) but had little or no effect in the presence of CTP (Table 5, Expts. 3 and 4). This observation suggests that the action of the detergent enables the added CDP-diglyceride to reach the active sites of the enzyme.

*Identification of labelled inositol phosphatide.* Portions of the $^3$H-labelled lipids (containing 10–15 $\mu$g. of P) were chromatographed on silicic acid-impregnated paper by the method of Marinetti *et al.* (1957). The separated lipids were eluted from appropriate sections of the paper at 37° with three successive portions (5 ml.) of chloroform–methanol–water (75:25:2, by vol.) (Hokin & Hokin, 1958) and examined for radioactivity. On five separate runs 75–90 % of the total applied radioactivity was recovered from the area corresponding to authentic liver phosphatidylinositol. No radioactivity was recovered from any other areas of the chromatograms.

A portion of the extract of labelled lipid was taken almost to dryness and hydrolysed in the presence of 1·0–2·0 ml. of 6 N-hydrochloric acid in a boiling-water bath for 10 min. The hydrolysate was extracted with chloroform and the water-soluble

Table 4. *Effect of CTP, CDP-choline and phosphatidic acid on the incorporation of* [$^3$H]*inositol into the lipids of a dispersion of rat brain*

Incubation was with buffer, $MgCl_2$ (16 mM), Tween 20 (0·025 mg./ml.) and [$^3$H]inositol, 0·125 $\mu$mole ($6{\cdot}25 \times 10^6$ counts/min.). CTP, CDP-choline (each 1 mM) and phosphatidic acid (0·125 $\mu$mole) were added as indicated.

| Additions | Specific radioactivity of lipids (counts/min./$\mu$g. of P) Expt. 1 | Expt. 2 |
|---|---|---|
| None | 360 | 190 |
| CTP | 2840 | 1600 |
| CTP, natural phosphatidic acid | 3710 | 3040 |
| CDP-choline | 1970 | 1120 |
| CDP-choline, natural phosphatidic acid | 1380 | 930 |

Table 5. *Effect of CDP-diglyceride on the incorporation of* [$^3$H]*inositol into the lipids of rat brain*

Incubation was with buffer, $MgCl_2$ (16 mM) and [$^3$H]-inositol, 0·125 $\mu$mole ($6{\cdot}25 \times 10^6$ counts/min.). Cytosine nucleotides (1 mM) were added as indicated.

| | Radioactivity incorporated into the lipid fraction (counts/min./sample) | |
|---|---|---|
| | Dispersion. | Mixed particle preparation (equivalent to 36 mg. of fresh tissue). |
| Cytosine nucleotide | Expt. 1 | Expt. 2 |
| — | 8 720 | 1 440 |
| CTP | 31 100 | 31 600 |
| CDP-choline | 25 200 | — |
| CDP-diglyceride | 12 900 | 20 700 |
| | Microsomes (equivalent to 36 mg. of fresh tissue) | |
| | Expt. 3 | Expt. 4 |
| — | 360 | 838 |
| CTP | 6 540 | 4 880 |
| CDP-diglyceride | 7 970 | 10 410 |
| | Supernatant from microsomes after treatment with 1 % Cutscum (derived from 36 mg. of fresh tissue) | |
| | Expt. 3 | Expt. 4 |
| — | 240 | 224 |
| CTP | 4 370 | 7 490 |
| CDP-diglyceride | 64 400 | 64 100 |

products were applied to 5 cm. × 72 cm. strips of Whatman 3MM paper and subjected to electrophoresis in 0·02 M-potassium lactate buffer (pH 3·6) at 600 v for 6·5 hr. Authentic inositol monophosphate, α-glycerophosphate and orthophosphate were clearly separated. Appropriate strips of the electrophoretograms were eluted with water. Approximately 50 % of the total applied radioactivity was found to migrate with inositol monophosphate. In addition, an unidentified spot with low electrophoretic mobility contained from 4 to 20 % of the total radioactivity. No radioactivity was recovered from any other areas. This finding, taken in conjunction with the chromatographic evidence, strongly suggests that the labelled lipid was phosphatidylinositol.

*Incorporation of* [$^{14}$C]*phosphatidic acid*

The stimulatory effect of phosphatidic acid on the incorporation of [$^{3}$H]inositol into the lipids of rat-brain dispersions in the presence of CTP suggested that phosphatidic acid might be an intermediate in the formation of brain phosphatidylinositol.

To test this possibility [$^{14}$C]phosphatidic acid was added to dispersions of rat brain and the labelled lipids were examined by the method of Dawson (1954*b*). Radioautographs prepared from chromatograms of the deacylated lipids showed radioactivity in glycerophosphate (from the added [$^{14}$C]phosphatidic acid), glycerolphosphorylcholine (from lecithin) and in free glycerol. No radioactivity was detected in glycerolphosphorylethanolamine (from phosphatidylethanolamine), glycerolphosphorylserine (from phosphatidylserine) or glycerolphosphorylinositol (from phosphatidylinositol). Addition of CTP caused an increase in the radioactivity of glycerolphosphorylinositol, with no increase for the other deacylated products. On the other hand, addition of CDP-choline did not affect the radioactivity in glycerolphosphorylinositol, but it caused a considerable increase in the radioactivity of glycerolphosphorylcholine. In three experiments with [$^{14}$C]phosphatidic acid the glycerolphosphorylinositol was eluted for determination of specific radioactivity. Incorporation was greatly stimulated by the addition of CTP, but CDP-choline had little effect (Table 6). This is in marked contrast with the finding for the incorporation of [$^{14}$C]phosphatidic acid into lecithin, where CDP-choline was more effective than CTP (Strickland *et al.* 1963).

These results suggest that phosphatidic acid is a precursor of phosphatidylinositol and that CTP, but not CDP-choline, is necessary for the conversion.

Table 6. *Effect of CTP and CDP-choline on the incorporation of radioactivity from* [$^{14}$C]*phosphatidic acid into the phosphatidylinositol of a dispersion of rat brain*

Incubation was with buffer, $MgCl_2$ (16 mM), [$^{14}$C]phosphatidic acid (1·25 μmoles), inositol (1·25 μmoles) and Tween 20 (0·025 mg./ml.). CTP and CDP-choline (each 1 mM) were added as indicated. The specific radioactivity of [$^{14}$C]phosphatidic acid was 23 400 counts/min./μmole in Expt. 1 and 42 100 counts/min./μmole in Expts. 2 and 3. NAD (2 mM) was added in Expt. 3.

| Addition | Specific radioactivity of phosphatidylinositol (counts/min./μmole) | | |
|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 |
| None | 160 | 48 | 0 |
| CTP | 1100 | 2330 | 3160 |
| CDP-choline | 190 | 250 | — |

## DISCUSSION

The experiments show that phosphatidylinositol is readily formed from [$^{3}$H]inositol and [$^{14}$C]phosphatidic acid in brain dispersions. Previously it had been shown that this lipid is readily labelled from inorganic $^{32}$P-labelled phosphate (Dawson, 1954*a*, *b*; McMurray *et al.* 1957), [$^{32}$P]-α-glycerophosphate (McMurray *et al.* 1957), [1-$^{14}$C]glycerol (Hokin & Hokin, 1958; Pritchard, 1958) and [2-$^{3}$H]-inositol (Hokin & Hokin, 1958) in various brain preparations.

The present experiments with [$^{3}$H]inositol, in particular those in which CDP-diglyceride was added, provide good evidence that this liponucleotide is an intermediate in the formation of brain phosphatidylinositol, presumably combining with inositol according to reaction (1). The experiments with [$^{14}$C]phosphatidic acid, in which CTP but not CDP-choline stimulated the incorporation of radioactivity into phosphatidylinositol, provide evidence that in brain dispersions CDP-diglyceride is formed by reaction (2), as suggested by Paulus & Kennedy (1960) for liver tissue. The experiments provide no evidence that the cytidylyl group of CDP-diglyceride is derived from CDP-choline, as suggested by Agranoff *et al.* (1958) for kidney tissue. According to reactions (1) and (2), the phosphorus of brain phosphatidylinositol is derived from phosphatidic acid, which is readily labelled from inorganic $^{32}$P-labelled phosphate in brain dispersions (Dawson, 1954*a*, *b*; McMurray *et al.* 1957). In contrast, the phosphorus of brain phosphatidylcholine is derived from phosphorylcholine, which is poorly labelled from inorganic $^{32}$P-labelled phosphate in brain dispersions (McMurray *et al.* 1957).

In experiments reported by Thompson *et al.* (1960) and Strickland *et al.* (1960) dispersions of rat brain supplemented with $Mg^{2+}$ ions, CoA and ATP were found to support the labelling of both mono-

phosphoinositide and phosphatidic acid from L-α-glycerophosphate labelled either with $^{32}P$ or with $^{14}C$. In these experiments the incorporation of L-α-glycerophosphate into monophosphoinositide was stimulated by the addition of CDP-choline, but not by the addition of CTP. At the same time the addition of CDP-choline reduced the labelling of phosphatidic acid from L-α-glycerophosphate to a point where the specific radioactivity of the monophosphoinositide greatly exceeded that of the phosphatidic acid, which suggested that L-α-glycerophosphate may be incorporated into monophosphoinositide of brain by a pathway that does not involve phosphatidic acid as an intermediate. This possibility is under further investigation.

## SUMMARY

1. In dispersions of rat brain the incorporation of [$^3H$]inositol into the lipid fraction was increased by the addition of cytidine triphosphate to the incubating medium. Other cytosine nucleotides, such as cytidine di- and mono-phosphate and cytidine diphosphate choline, also increased the incorporation, but to a lesser extent. Nucleoside triphosphates other than cytidine triphosphate were ineffective.

2. In the presence of cytidine triphosphate, the incorporation of [$^3H$]inositol into the lipid of the rat-brain dispersion was stimulated by the addition of L-α-phosphatidic acids, but not by the addition of D-αβ-diglycerides. The stimulation brought about by L-α-phosphatidic acids was not observed when the cytidine triphosphate was replaced by cytidine diphosphate choline.

3. The incorporation of [$^3H$]inositol into the lipid of a microsome preparation treated with the detergent Cutscum was stimulated (by a factor of 20–40) by the addition of cytidine triphosphate, but was stimulated much more (by a factor of 250–300) by the addition of the liponucleotide, cytidine diphosphate diglyceride.

4. Evidence is presented to support the view that the [$^3H$]inositol was incorporated into a monophosphoinositide, probably phosphatidylinositol.

5. Radioactivity from [$^{14}C$]phosphatidic acid, prepared biologically, was incorporated into phosphatidylinositol of dispersions of rat brain. The incorporation was stimulated by the addition of cytidine triphosphate, but not by the addition of cytidine diphosphate choline.

6. These results are discussed in relation to the biosynthesis of phosphatidylinositol in brain and other tissues.

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