Incorporation of ^{32}P -labelled Intermediates into the Phospholipids of Cell-free Preparations of Rat Brain

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In previous papers (McMurray, Strickland, Berry & Rossiter, 1957*b*; McMurray, Berry & Rossiter, 1957*a*) two systems capable of supporting the incorporation of inorganic ^{32}P into the phospholipids of rat-brain dispersions were described: (a) an anaerobic system (best demonstrated in hypotonic dispersions), which supported the labelling of lipid P under optimum conditions for glycolysis (McMurray *et al.* 1957*b*), and (b) an aerobic system (best shown in mitochondria isolated from isotonic dispersions), which supported the labelling of lipid P under optimum conditions for oxidative phosphorylation (McMurray *et al.* 1957*a*). The effects of metabolic inhibitors such as 2:4-dinitrophenol (DNP) and fluoride on the specific radioactivity and concentration of adenosine triphosphate (ATP) in each of these two systems suggested that the labelling of phospholipid was dependent upon both the prior labelling of ATP and the maintenance of adequate concentrations of this substance (McMurray *et al.* 1957*a*).

In order to elucidate some of the intermediate reactions concerned with the labelling of phospholipid from inorganic ^{32}P , a study was made of the incorporation of ^{32}P -labelled adenosine triphosphate (AT^{32}P), phosphorylcholine (^{32}PCh) and α -glycerophosphate ($\alpha\text{-G}^{32}\text{P}$) into the lipid P of the two phosphorylating systems. The distribution of radioactivity in the hydrolysis products of individual phospholipids, separated by the chromatographic method of Dawson (1954*b*), suggested that ^{32}PCh and $\alpha\text{-G}^{32}\text{P}$ were incorporated into lecithin and phosphatidic acid respectively. Inorganic ^{32}P appeared to be incorporated chiefly into phosphatidic acid and diphosphoinositide.

A preliminary account of some of this work has appeared (McMurray, Berry & Strickland, 1956).

METHODS

Dispersions of rat brain prepared in water at 0° were incubated in Warburg flasks at 37.5° for 70 min. in an atmosphere of N_2 . Details of the preparation of the dispersions

were given by McMurray *et al.* (1957*b*). The 'reinforced' incubation medium used in these studies contained: glucose, 0.028*M*; potassium ATP, 0.0007*M*; diphosphopyridine nucleotide, 0.001*M*; nicotinamide, 0.04*M*; MgCl_2 , 0.008*M*; 2-amino-2-hydroxymethylpropane-1:3-diol buffer, pH 7.4, 0.05*M*; potassium phosphate (containing 120 μC of ^{32}P), 0.01*M*. The total volume was 3 ml.

Mitochondria obtained from dispersions of rat brain prepared in 0.25*M*-sucrose were incubated in Warburg vessels for 70 min. at 37.5° in an atmosphere of O_2 . Oxygen consumption was measured during the last 60 min. of incubation. Details of the preparation of the mitochondria were given by McMurray *et al.* (1957*a*). The mitochondria were incubated in a medium containing: sodium pyruvate, 0.013*M*; sodium malate, 0.002*M*; adenosine 5'-phosphate (AMP), 0.0025*M*; MgCl_2 , 0.008*M*; NaF , 0.012*M*; glycylglycine buffer, pH 7.4, 0.015*M*, and sodium phosphate (containing 120 μC of ^{32}P), 0.01*M*. The total volume was 3 ml.

When measured, the concentration and the specific radioactivity of ATP were determined by the chromatographic procedures of Krebs & Hems (1953). Usually the lipid P was extracted and purified for specific radioactivity determinations as described by McMurray *et al.* (1957*b*).

In some experiments a number of samples were pooled and portions of the lipid extract, representing 200–300 mg. of original tissue, were taken to dryness under reduced pressure at 50° . The residue was extracted in CHCl_3 -methanol (1:1, v/v) and the extract was carried through the mild alkaline-hydrolysis procedure of Dawson (1954*b*). The water-soluble hydrolysis products were purified and subjected to two-dimensional paper chromatography with the phenol-aq. NH_3 soln. and *tert*-butanol-trichloroacetic acid solvents as described by Dawson (1954*b*). By this method glycerophosphate (GP), glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), glycerylphosphorylserine (GPS), a hydrolysis product of diphosphoinositide (IP) and other unidentified P-containing compounds were separated. Dawson (1954*b*) suggested that the GP is derived from phosphatidic acid, but it is possible that some GP also may arise from diphosphoinositide (Folch & LeBaron, 1956; Hawthorne, 1955) or some other lipid. The GPC, GPE and GPS are derived from phosphatidylcholine (lecithin), phosphatidylethanolamine and phosphatidylserine respectively. The spots were located by radioautography and by spraying the chromatograms either with the phosphorus reagent of Hanes & Isherwood (1949) or with ninhydrin reagent. The spots containing GP, GPC and GPE were identified by comparing their R_F values with those of authentic compounds. Acid hydrolysis of the GPC, GPE and GPS yielded GP and choline, ethanolamine and serine respectively, which were identified chromatographically. The material from each spot was eluted with water and ashed, and the concentration and specific radioactivity of the total P were determined.

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Labelled intermediates

Radioactive adenosine triphosphate. The AT³²P was prepared biosynthetically by a modification of the method used in the metabolic studies. Water dispersions of rat brain were incubated for 40 min. at 37.5° in the medium described above. The incubation was carried out aerobically in the presence of 1 mc of inorganic ³²P and 0.01 M-KF. The total trichloroacetic acid (TCA)-soluble fraction from four samples was separated chromatographically (in 50 portions of 250 μ l.) by the method of Krebs & Hems (1953). The chromatograms were washed with ether to remove the TCA and the spots containing ATP were eluted in a total volume of 40 ml. The yield was 6 mg. of ATP (computed from Passay), with a specific radioactivity of 10⁵ counts/min./ μ g. of P.

In the anaerobic-homogenate experiments the final concentration of ATP was 0.0007 M, with a specific radioactivity of 10⁴ counts/min./ μ g. of P. In the aerobic mitochondrial experiments the final concentration was 0.0025 M, with a specific radioactivity of 3 $\times$ 10³ counts/min./ μ g. of P.

Preparation of radioactive phosphorylcholine (³²PCh). This was synthesized by the method of Riley (1944a). The reaction mixture, consisting of 0.5 g. of crystalline H₃PO₄ containing 17 mc of ³²P and 0.5 g. of dry choline chloride, was heated for 10 hr. at 155° under reduced pressure. The inorganic P was removed by precipitation with Ca(OH)₂ at pH 8.9, and the calcium salt of ³²PCh was precipitated at -20° from the supernatant by the addition of an equal volume of ethanol. The yield was 0.15 g. of crystalline product, with a specific radioactivity of 1.5 $\times$ 10⁴ counts/min./ μ g. of P [Found: N, 4.27 (Kjeldahl); P, 9.36%; N/P, 0.995 (atom/atom). Calcium salt of PCh requires N, 4.25; P, 9.38%; N/P, 1.00].

Calcium was removed from a solution of ³²PCh by precipitation with oxalate. In both the homogenate and mitochondrial experiments a portion (0.3 ml.) of the solution was added to each vessel to give a final concentration of 0.002 M-PCh, with a specific radioactivity of 1.5 $\times$ 10⁴ counts/min./ μ g. of P.

Preparation of radioactive α -glycerophosphate. A method similar to that described by Bailly & Gaumé (1924) was used for the preparation of α -G³²P. A mixture of 5 ml. of m- α -glycerochlorohydrin (Eastman Kodak Co. Rochester,

N.Y.) and 5 ml. of m-Na₂PO₄, containing 17 mc of ³²P, was allowed to react at room temperature for 50 hr. Inorganic P was removed and α -G³²P was precipitated as the calcium salt by the method described above for ³²PCh. The yield was 0.75 g. of fine amorphous powder, with a specific radioactivity of 1.5 $\times$ 10⁴ counts/min./ μ g. of P (Found: P, 10.2. Calcium salt of GP requires P, 10.1%). The ester P was 96–100% α -GP, with negligible amounts of β -GP, as determined by the periodate method of Burmaster (1946).

Calcium was removed from a solution of α -G³²P by precipitation with oxalate. In both the homogenate and mitochondrial experiments a portion (0.6 ml.) of the calcium-free solution was added to each vessel to give a final concentration of 0.004 M- α -GP with a specific radioactivity of 1.5 $\times$ 10⁴ counts/min./ μ g. of P.

Reagents

Coenzyme A (CoA), ATP and cytidine triphosphate (CTP) were obtained from Pabst Laboratories, Milwaukee, Wisconsin.

The GPE was a gift from Dr E. Baer, Banting and Best Department of Medical Research, University of Toronto, and the GPC was a gift from Dr C. S. McArthur, Department of Biochemistry, University of Saskatchewan.

The origin of other reagents used was given by McMurray *et al.* (1957b).

RESULTS

Inorganic ³²P

Coenzyme A. Table 1 shows that the addition of either CoA (5 $\times$ 10⁻⁵ M) or glutathione (2.5 $\times$ 10⁻² M) to the water dispersion of rat brain caused an increase in the anaerobic labelling of phospholipid from inorganic ³²P (Expts. 1 and 2). When the two compounds were added together, the increase was greater than that obtained when either substance was added singly. The increased labelling of phospholipid observed in the presence of CoA and glutathione was found not to be accompanied by any significant increases either in the rate of glycolysis or in the labelling and concentration of ATP.

Table 1. *Effect of coenzyme A and glutathione on the specific radioactivity of the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria*

Incubated as described in Methods with 120 μ c of inorganic ³²P (final concn., 0.01 M). Specific radioactivity is expressed as counts/min./ μ g. of P.

System	Water dispersion (gas phase, N ₂)		Mitochondria (gas phase, O ₂)	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Control	30.5	23.7	55.3	72.6
CoA (5 $\times$ 10 ⁻⁵ M)	60.0	40.1	57.8	73.1
Glutathione (2.5 $\times$ 10 ⁻² M)	56.6	32.1	50.8	—
CoA + glutathione	87.0	44.4	61.0	53.1
 % of the control				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
CoA	197	169	105	101
Glutathione	186	136	92	—
CoA + glutathione	287	188	110	73

Kornberg & Pricer (1952) reported that the addition of CoA stimulated the incorporation of α -G³²P into the lipid P of an extract of rat liver. Subsequently the same workers (Kornberg & Pricer, 1953*a, b*) showed that the addition of cysteine potentiated the activating effect of CoA, presumably by preventing auto-oxidation of the essential thiol group. In our experiments glutathione was added in place of cysteine for this purpose, after it had been shown that cysteine inhibited the oxygen consumption, glycolysis and the ³²P incorporation in the brain preparations.

In the aerobic-mitochondrial system the addition of CoA or glutathione did not similarly stimulate the incorporation of inorganic ³²P into phospholipid (Table 1, Expts. 3 and 4). In Expt. 4 the addition of glutathione and CoA caused a decrease in the specific radioactivity of the lipid P. Dawson (1953) reported that the addition of CoA to respiring KCl dispersions of guinea-pig brain did not affect the incorporation of inorganic ³²P into phospholipid.

The observation that CoA increased the labelling of lipid P from inorganic ³²P in the water dispersions, but not in mitochondria, is quite consistent with the less exacting cofactor requirements demonstrated previously for labelling of mitochondrial phospho-

lipids (McMurray *et al.* 1957*a*). Schneider (1953) reported that CoA is present in isolated mitochondria, and it is conceivable that the concentration of CoA within the mitochondrion is optimum for phospholipid labelling. On the other hand, it seems likely that in the water dispersions, in which the mitochondria are damaged both morphologically and metabolically, the CoA had diffused from sites of metabolic activity in the damaged particles.

Cytidine triphosphate. The recent researches of Kennedy & Weiss (1955, 1956) disclosed the role of CTP as a cofactor in the synthesis of lecithin by liver-mitochondrial preparations. Table 2 indicates that the addition of CTP, in concentrations as low as 3.5×10^{-5} M, increased the incorporation of inorganic ³²P into the lipid P of both the anaerobic hypotonic dispersions and mitochondrial preparations of rat brain. With 3.5×10^{-4} M-CTP the increase was of the order of 70–80% for both systems. These increases were found not to be associated with significant increases in glycolysis or respiration, or increases in the labelling or concentration of ATP.

Table 3 shows the effects of adding CoA or CTP upon the specific radioactivities of the hydrolysis products of the phospholipids labelled in each of the two systems. In the anaerobic system (Expt. 1), the

Table 2. *Effect of cytidine triphosphate on the specific radioactivity of the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria*

Incubated as described in Methods with 120 μ C of inorganic ³²P (final concn., 0.01 M).

CTP concn. (M)	Water dispersion (gas phase, N ₂)		Mitochondria (gas phase, O ₂)	
	Specific radioactivity (counts/min./ μ g. of P)	% of the control	Specific radioactivity (counts/min./ μ g. of P)	% of the control
0	52.9	100	23.8	100
7.0×10^{-6}	56.7	107	18.4	77
3.5×10^{-5}	75.7	143	28.6	120
7.0×10^{-5}	73.3	139	30.0	126
3.5×10^{-4}	94.8	179	41.0	172
7.0×10^{-4}	90.4	171	50.7	213

Table 3. *Effect of coenzyme A and cytidine triphosphate on the specific radioactivity of the hydrolysis products of the individual phospholipids from a water dispersion of rat brain and from rat-brain mitochondria*

Incubated as described in Methods with 120 μ C of inorganic ³²P (final concn., 0.01 M). GPS, GPE, GPC, GP and IP were separated by two-dimensional paper chromatography (Dawson, 1954*b*). Specific radioactivity is expressed as counts/min./ μ g. of P.

Hydrolysis product	Expt. 1. Water dispersion (gas phase, N ₂)			Expt. 2. Mitochondria (gas phase, O ₂)		
	Control	CoA (5×10^{-5} M)	CTP (7×10^{-4} M)	Control	CoA (5×10^{-5} M)	CTP (3.5×10^{-4} M)
GPC	2.1	1.8	4.3	0.4	0.4	4.6
GPE	1.7	2.3	7.5	1.5	1.5	2.9
GPS	15.1	19.9	26.1	9.1	9.3	12.9
GP	1170	1360	680	1480	1620	1520
IP	1440	1980	2430	530	472	1860
Total lipid P	69.0	89.2	92.9	98.0	95.5	172

GP (presumably from phosphatidic acid or diphosphoinositide) and IP (from diphosphoinositide) of the control samples had specific radioactivities many times greater than that of the total lipid P. Moreover, most of the ³²P incorporated into the phospholipids was recovered from the GP and IP, despite the low concentrations of these compounds. As characterized by their respective hydrolysis products (GPC, GPE and GPS), the phosphoglycerides that contribute most of the P to the phospholipid extract were not significantly labelled. In most cases, the observed counts/min. were no more than twice the background counting rate, so that the recorded specific activities of GPC, GPE and GPS may be regarded as negligible. Similar results were obtained with the mitochondrial system, but here the specific activity of the IP was relatively lower (Expt. 2, Table 3). The results are in accord with reports of Dawson (1954*a, b*) that phosphatidic acid and diphosphoinositide were strongly labelled in KCl dispersions of guinea-pig brain incubated aerobically in the presence of inorganic ³²P, with no appreciable incorporation into the major phosphoglycerides.

In the water dispersion the addition of CoA caused an increase in the anaerobic labelling of the total lipid P (Expt. 1, Table 3). This increase was almost exclusively associated with similar increases in the specific radioactivities of GP and IP. The changes in the labelling of the GPC, GPE and GPS were so small that they contributed negligible radioactivity to the increased labelling of the total lipid P observed in the presence of CoA. In contrast with the observations obtained with water dispersions of rat brain, CoA was found to have no significant effect on

the labelling of phospholipids in mitochondria (Expt. 2, Table 3). This confirms the results previously reported in Table 1.

With both systems the increase in labelling of lipid P observed after the addition of CTP was largely the result of an increase in the specific radioactivity of the IP compound (Table 3). The labelling of GPC, GPE and GPS was also increased by the addition of CTP, but the specific radioactivities of these derivatives of the phosphoglycerides were so small that the increases were of doubtful significance.

Glycerophosphate and glycerol. Table 4 shows that the addition of α -GP in a concentration of 0.001M to the hypotonic dispersion did not affect the anaerobic labelling of the lipid P (Expt. 1). At a concentration of 0.005M both α -GP and β -GP slightly decreased the labelling (Expt. 2). The addition of glycerol was without significant effect. Dawson (1953) reported that the addition of α -GP, β -GP (0.0025M) or glycerol (0.008M) did not significantly affect the labelling of lipid P from inorganic ³²P in KCl dispersions of guinea-pig brain under aerobic conditions.

In brain mitochondria (Expts. 3 and 4) the addition of α -GP (0.005M) caused a 30–40% decrease in the ³²P labelling of lipid P. In comparison, the effects of β -GP and glycerol were small. Kennedy (1953) reported that in rat-liver mitochondria the inhibitory effect of α -GP was much greater and was observed at lower concentrations (0.001M). Presumably the decrease in the labelling of phospholipid is due to a dilution of the activity of α -G³²P formed from inorganic ³²P. Kennedy (1953) also reported that the addition of glycerol (0.00017M) increased the labelling of phospholipid in rat-liver mitochondria. None of the compounds tested was found to affect the anaerobic glycolysis of the water dispersions or the oxygen consumption of the mitochondria.

Other experiments showed that the addition of known components of brain phospholipids (choline, serine, ethanolamine or inositol) did not affect the anaerobic labelling of lipid P in the water dispersions to any significant degree. Dawson (1953) reported similar negative findings in aerobic experiments with KCl dispersions of guinea-pig brain.

Table 4. *Effect of α - and β -glycerophosphate and glycerol on the specific radioactivity of the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria*

Incubated as described in Methods with 120 μ C of inorganic ³²P (final concn., 0.01M). Concn. of GP and glycerol, 0.001M (Expt. 1), 0.005M (Expts. 2–4). Specific radioactivity is expressed as counts/min./ μ g. of P.

Additions	Water dispersion (gas phase, N ₂)		Mitochondria (gas phase, O ₂)	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
None	22.6	35.3	105	45.8
α -GP	23.4	29.6	71.3	28.2
β -GP	—	31.1	91.1	46.2
Glycerol	25.8	35.8	112	44.0
% of the control		% of the control		
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
α -GP	104	84	68	62
β -GP	—	88	87	101
Glycerol	114	102	107	97

³²P-Labelled intermediates

The labelling of a phospholipid from a suspected precursor labelled with ³²P is of itself insufficient evidence that the compound is a precursor of the phospholipid. When ³²P-labelled phosphoryl-ethanolamine (Chargaff & Keston, 1940) or ³²PCh (Riley, 1944*b*) was administered to animals *in vivo*, the liver phospholipid was labelled; however, in these experiments the labelled compounds were hydrolysed to inorganic ³²P, which could, in turn,

have been incorporated into the phospholipids by reactions not necessarily involving phosphoryl-ethanolamine or PCh.

In the experiments *in vitro* described below, the specific radioactivity of the inorganic P of the TCA extract was determined after incubation of the tissue with ^{32}P -labelled compounds. In this way a relative specific activity (specific radioactivity of lipid P/specific radioactivity of inorganic P) was computed. If the relative specific activity had been found to be the same when the radioactivity was added either as the ^{32}P -labelled suspected precursor or as inorganic ^{32}P , then the radioactivity appearing in the phospholipid must have been derived from the inorganic ^{32}P arising from the breakdown of the ^{32}P -labelled compound. If, on the other hand, the relative specific radioactivity of the phospholipid from the samples containing the labelled precursor had been found to be greater than that from the samples containing added inorganic ^{32}P , it could be argued that some of the ^{32}P in the phospholipid had been derived from the labelled compound *per se*, without prior breakdown to inorganic ^{32}P .

The results presented in Table 5 demonstrate that the ^{32}P from AT^{32}P , ^{32}PCh and $\alpha\text{-G}^{32}\text{P}$ was in-

corporated into the phospholipids of the anaerobic water dispersion and mitochondria of brain. For each compound, and in each of the two systems, the relative specific radioactivity of the lipid P was greater when the labelled intermediate was the source of the radioactivity than when the radioactivity was derived from inorganic ^{32}P . From the considerations presented above, it may be concluded that the labelling of phospholipid from the ^{32}P -labelled suspected intermediates did not proceed solely from the inorganic ^{32}P arising from hydrolysis of the compounds.

^{32}P -Labelled phosphorylcholine. Table 6 shows that in both the anaerobic water dispersion and aerobic mitochondrial system there was virtually no incorporation of ^{32}PCh into lipid P in the absence of CTP. With the addition of CTP good labelling was achieved. As shown previously (Table 2), the addition of CTP also stimulated the incorporation of inorganic ^{32}P into phospholipid in both brain preparations, but the increase was smaller than that observed with ^{32}PCh . Furthermore, with inorganic ^{32}P considerable incorporation was observed in the absence of CTP. The addition of CoA, which increased the labelling of lipid P from inorganic ^{32}P

Table 5. Incorporation of ^{32}P -labelled intermediates into the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria

Incubated as described in Methods with $120\text{ }\mu\text{C}$ of inorganic ^{32}P , $12\text{ }\mu\text{C}$ of AT^{32}P , $18\text{ }\mu\text{C}$ of ^{32}PCh or $36\text{ }\mu\text{C}$ of $\alpha\text{-G}^{32}\text{P}$. In the ^{32}PCh experiments CTP was added, $7.0 \times 10^{-4}\text{ M}$ for water dispersion and $3.5 \times 10^{-4}\text{ M}$ for mitochondria.

Labelled precursor	Concn. of precursor (M)	Water dispersion (gas phase, N_2)		Mitochondria (gas phase, O_2)	
		Specific radioactivity (counts/min./ $\mu\text{g. of P}$)	$10^3 \times$ Relative specific radioactivity†	Specific radioactivity (counts/min./ $\mu\text{g. of P}$)	$10^3 \times$ Relative specific radioactivity†
Inorg. ^{32}P	1×10^{-3}	24.4	1.4	48.5	3.2
AT^{32}P	7×10^{-4}	8.6	6.7	5.3	8.2
Inorg. ^{32}P	1×10^{-3}	137	7.4	112	6.5
^{32}PCh	2×10^{-3}	18.9	2590	20.8	1060
Inorg. ^{32}P	1×10^{-3}	69.0	2.7	97.9	3.8
$\alpha\text{-G}^{32}\text{P}$	4×10^{-3}	11.7	36.6	24.9	93.0

* Concn. of ATP in mitochondrial experiment, $2.5 \times 10^{-3}\text{ M}$.

† Relative specific radioactivity = specific radioactivity of lipid P/specific radioactivity of inorganic P.

Table 6. Effect of cytidine triphosphate on the incorporation of ^{32}PCh into the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria

Incubated as described in Methods with $18\text{ }\mu\text{C}$ of ^{32}PCh (final concn., $2 \times 10^{-3}\text{ M}$). Specific radioactivity is expressed as counts/min./ $\mu\text{g. of P}$. CTP, $7.0 \times 10^{-4}\text{ M}$ for water dispersion and $3.5 \times 10^{-4}\text{ M}$ for mitochondria. CoA, $5 \times 10^{-3}\text{ M}$ for both systems.

System	Water dispersion (gas phase, N_2)				Mitochondria (gas phase, O_2)	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
Control	1.7	2.2	3.6	3.4	0	1.7
CTP	32.9	63.8	50.9	49.8	33.1	31.7
CoA	1.5	1.9	—	—	—	—
CTP + CoA	24.1	—	—	—	—	—

in the anaerobic system, somewhat decreased the labelling from ³²PCh, when it was added alone or together with CTP.

It was observed that the requirement for added CTP in the incorporation of ³²PCh into phospholipid in the anaerobic water homogenate was not satisfied by replacing CTP with the triphosphates of uridine (UTP), inosine (ITP) or guanosine (GTP). This is in agreement with the findings of Kennedy & Weiss (1955), who first showed an absolute requirement for CTP and no requirement for CoA in the labelling of lecithin from ³²PCh by lyophilized liver mitochondria.

The distribution of radioactivity in the hydrolysis products of the labelled phospholipids from a hypotonic dispersion that had been incubated anaerobically with ³²PCh is shown in the radioautographs reproduced in Fig. 1. A very small amount of radioactivity was recovered from the GPC derived from the lecithin of control samples (Fig. 1a). Labelling of the GPC was greatly increased by the addition of CTP (Fig. 1b). An unknown compound (3) was labelled to a lesser extent in the latter samples. Similar results were obtained with phosphorylating mitochondria. The data presented in Table 7, Expt. 1, demonstrate

Fig. 1. Radioautographs of chromatograms of hydrolysis products of labelled phospholipids. Water dispersion of rat brain was incubated anaerobically with ³²PCh as described in Methods. (a), No CTP; (b), CTP, 7×10^{-4} M. GPC, Glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine; GPS, glycerylphosphorylserine; GP, glycerophosphate; IP, hydrolysis product of diphosphoinositide; 3, unidentified compound.

Table 7. Specific radioactivity of hydrolysis products of phospholipids from a water dispersion of rat brain and from rat-brain mitochondria incubated with ³²PCh and with α -G³²P

Incubated as described in Methods. Hydrolysis products of the phospholipids were separated by two-dimensional paper chromatography (Dawson, 1954b). Specific radioactivity is expressed as counts/min./ μ g. of P.

Hydrolysis product	Expt. 1. ³² PCh (18 μ C; final concn., 2×10^{-3} M)			
	Water dispersion (gas phase, N ₂)		Mitochondria (gas phase, O ₂)	
	Control	CTP (7×10^{-4} M)	Control	CTP (3.5×10^{-4} M)
GPC	15.8	285	1.8	75.5
GP IP	Expt. 2. α -G ³² P (36 μ C; final concn., 4×10^{-3} M)			
	Water dispersion (gas phase, N ₂)		Mitochondria (gas phase, O ₂)	
	Control	CoA (5×10^{-5} M)	Control	CoA (5×10^{-5} M)
GP	107	147	577	2385
IP	236	255	56.9	61.8

that in both the anaerobic and aerobic systems the addition of CTP greatly increased (20–40 times) the incorporation of ^{32}PCh into the GPC. The radioactivity in other phospholipid hydrolysis products (GPE, GPS, GP and IP) was less than one-half of the background counting rate and was considered to be negligible. This is in contrast with the experiments with inorganic ^{32}P , where the increase in the labelling of lipid P brought about by the addition of CTP was largely the result of an increase in the specific radioactivity of IP (Table 3).

The fact that the label from ^{32}PCh was incorporated almost exclusively into the lecithin in both the anaerobic and aerobic systems indicates that ^{32}PCh , in contrast with inorganic ^{32}P , is not a precursor of phosphatidic acid or diphosphoinositide.

^{32}P -Labelled α -glycerophosphate. The addition of CTP caused a slight decrease in the labelling of lipid P from α -G ^{32}P in both the water dispersion and the mitochondrial system (Table 8). The concentration of CTP was the same as that which caused a considerable increase in labelling when the radioactivity was derived from inorganic ^{32}P (Tables 2 and 3). On the other hand, the addition of CoA, which caused an increase in the incorporation of inorganic ^{32}P into lipid P in the anaerobic, but not in the aerobic system, caused an increase in the labelling from α -G ^{32}P in both systems. Kornberg & Pricer (1952) showed that the incorporation of the label from α -G ^{32}P into the phospholipids of a soluble enzyme preparation from liver dispersions was considerably increased by the addition of CoA.

Fig. 2a shows that in the aerobic-mitochondrial system most of the radioactivity of the α -G ^{32}P was recovered from the GP. The increased radioactivity caused by the addition of CoA was almost all in the GP, with lesser amounts in the IP and unknown compound 1 (Fig. 2b). Similar results were obtained with the anaerobic hypotonic dispersion. From Expt. 2, Table 7, it may be seen that the GP and IP were both strongly labelled from α -G ^{32}P in the anaerobic water dispersions. The IP was less radioactive than the GP in the mitochondrial samples. In both the aerobic and anaerobic systems, the specific radioactivity of GP, but not of IP, was increased by the addition of CoA, the

Table 8. *Effect of cytidine triphosphate and coenzyme A on the incorporation of α -G ^{32}P into the lipid phosphorus of a water dispersion of rat brain and of rat-brain mitochondria*

Incubated as described in Methods with $36\text{ }\mu\text{C}$ of α -G ^{32}P (final concn., $4 \times 10^{-3}\text{ M}$).

System	Specific radioactivity of lipid P (counts/min./ $\mu\text{g. of P}$)		
	Water dispersion (gas phase, N_2)	Mitochondria (gas phase, O_2)	
		Expt. 1	Expt. 2
Control	13.4	21.0	25.4
CTP ($3.5 \times 10^{-4}\text{ M}$)	8.2	19.1	19.0*
Coenzyme A ($5 \times 10^{-5}\text{ M}$)	35.5	59.0	89.2

* CTP concentration was $1.7 \times 10^{-4}\text{ M}$.

Fig. 2. Radioautographs of chromatograms of hydrolysis products of labelled phospholipids. Rat-brain mitochondria were incubated aerobically with α -G ^{32}P as described in Methods. (a), No CoA; (b), CoA, $5 \times 10^{-5}\text{ M}$. GPC, Glycerylphosphorylcholine; GPE, glycerylphosphorylethanolamine; GPS, glycerylphosphorylserine; GP, glycerophosphate; IP, hydrolysis product of diphosphoinositide; 1, unidentified compound.

Table 9. *Effect of potassium fluoride and iodoacetate on the incorporation of ³²PCh and α-G³²P into the lipid phosphorus of a water dispersion of rat brain*

Incubated as described in Methods with ³²PCh or α-G³²P. Gas phase, N₂. CTP, 7×10^{-4} M, was added to samples with ³²PCh.

System	Expt. 1. ³² PCh (18 μC; final concn., 2×10^{-5} M). Specific radioactivity of lipid P		Expt. 2. α-G ³² P (36 μC; final concn., 4×10^{-5} M). Specific radioactivity of lipid P	
	(counts/min./ μg. of P)	% of the control	(counts/min./ μg. of P)	% of the control
Control	18.9	100	13.4	100
KF (10^{-2} M)	9.9	53	2.9	22
Iodoacetate (10^{-3} M)	5.9	31	0.5	4

Table 10. *Effect of sodium fluoride and 2:4-dinitrophenol on the incorporation of ³²PCh and α-G³²P into the lipid phosphorus of rat-brain mitochondria*

Incubated as described in Methods with ³²PCh or α-G³²P. Gas phase, O₂. CTP, 3.5×10^{-4} M, was added to samples with ³²PCh.

System	Expt. 1. ³² PCh (18 μC; final concn., 2×10^{-5} M). Specific radioactivity of lipid P		Expt. 2. α-G ³² P (36 μC; final concn., 4×10^{-5} M). Specific radioactivity of lipid P	
	(counts/min./ μg. of P)	% of the control	(counts/min./ μg. of P)	% of the control
Control	20.8	100	10.6	100
No NaF	8.8	42	4.3	41
DNP (5×10^{-5} M)	12.6	61	3.5	33

effect of CoA being considerably greater in the aerobic than in the anaerobic system.

The distribution of the label in the individual phospholipids (chiefly in the phosphatidic acid and diphosphoinositide, and not in the major phosphoglycerides) is similar to that reported above for the incorporation of inorganic ³²P (Table 3), and contrasts markedly with the strong labelling of lecithin from ³²PCh. Moreover, CoA, but not CTP, increased the labelling of phosphatidic acid from α-G³²P.

Inhibitors

Experiments described in the earlier papers indicated that in water dispersions of brain (McMurray *et al.* 1957*b*) and in brain mitochondria (McMurray *et al.* 1957*a*) the labelling of lipid P from inorganic ³²P was associated with the labelling of ATP. In addition, the maintenance of an adequate concentration of ATP was necessary. In these experiments the labelling of lipid P and both the labelling and the concentration of ATP were found to be decreased by the addition of fluoride or iodoacetate in the anaerobic system, and by the omission of fluoride, or the addition of DNP, in the aerobic system.

In the experiments reported in Tables 9 and 10 the radioactivity was derived from either ³²PCh or α-G³²P, i.e. the labelling of the lipid P was independent of the presence or absence of the label in

ATP. In both the anaerobic and the aerobic systems the labelling of the lipid P from ³²PCh or α-G³²P was inhibited under conditions where it is known that optimum concentrations of ATP were not maintained.

DISCUSSION

The experiments demonstrate the existence in brain of two reaction sequences involved in the ³²P-labelling of brain phospholipids: (a) the labelling of lecithin from ³²PCh in the presence of a specific cofactor, CTP, and (b) the labelling of phosphatidic acid from α-G³²P, the latter reaction being stimulated by the addition of CoA.

Kornberg & Pricer (1952) suggested that ³²PCh was incorporated as a unit into lecithin by an aqueous extract of liver. More recently, Kennedy & Weiss (1955, 1956) verified that ³²PCh was a precursor of lecithin in rat-liver mitochondria, and exploited the impurities in a commercial preparation of ATP to demonstrate a specific requirement for CTP. The latter investigators postulated that ³²PCh was 'activated', with the formation of a novel intermediate, cytidine diphosphate choline (CMP~³²PCh). Isolation, characterization and synthesis of this intermediate, and the demonstration that the labelled intermediate is incorporated quantitatively into lecithin by the liver-enzyme

system, is good evidence for the occurrence of the following reactions:

where P-P represents pyrophosphate and CMP represents cytidine monophosphate. It appears that a similar activation with CTP occurs in the incorporation of ${}^{32}\text{P}$ -labelled phosphorylethanolamine into phosphatidylethanolamine, and that the reaction may be a general one in the biosynthesis of phospholipids (Kennedy, 1956).

The substance that accepts PCh from $\text{CMP} \sim \text{PCh}$ in reaction (2) was difficult to characterize because the enzyme preparations contained endogenous lipid and, presumably, endogenous acceptor. Another difficulty is that $\alpha\beta$ -diglycerides, the most likely acceptors, are only sparingly soluble in water. Rodbell & Hanahan (1955) showed that the incorporation of ${}^{32}\text{PCh}$ into the lecithin of guinea-pig liver mitochondria was slightly stimulated by the addition of either $\text{D-}\alpha\beta$ -diglyceride or an $\text{L-}\alpha$ -phosphatidic acid. These experiments were carried out without the addition of CTP. However, Weiss & Kennedy (1956) reported that the addition of a mixture of $\text{D-}\alpha\beta$ -diglycerides to a liver-enzyme system greatly stimulated the incorporation of labelled $\text{CMP} \sim \text{PCh}$ into lecithin, and the same group of workers have recently reported a net synthesis of lecithin from $\text{CMP} \sim \text{PCh}$ and $\text{D-}\alpha\beta$ -diglyceride (Weiss, Smith & Kennedy, 1956).

The experiments reported in the present paper, i.e. the labelling of lipid P from ${}^{32}\text{PCh}$ without involving the labelling of inorganic P or ATP, suggest that ${}^{32}\text{PCh}$ is incorporated as a unit into the lipid P of the brain preparations. The almost absolute requirement for CTP is strongly suggestive of an activating mechanism similar to that described by Kennedy & Weiss (1955, 1956). As in the experiments of Kennedy (1956), lecithin, characterized by its hydrolysis product GPC, contained the major part of the radioactivity of the phospholipids.

The requirements for conditions that promote optimum glycolytic phosphorylation (water dispersions) or oxidative phosphorylation (mitochondria) for the optimum incorporation of ${}^{32}\text{PCh}$ into lipid P demonstrate the necessity for maintenance of adequate concentrations of ATP in the labelling of lecithin. As indicated in reactions (1) and (2), the 'activation' of ${}^{32}\text{PCh}$ and its subsequent transfer to lecithin results in the overall conversion of CTP into CMP. In addition, CTP may be broken down by brain phosphatases. A source of ATP is necessary for the restoration of an adequate concentration of CTP. The phosphorylation of CMP or CDP may be brought about by enzymes similar to

those described by Herbert & Potter (1956) for liver tissue, i.e. enzymes catalysing reactions such as

In agreement with the observations of Dawson (1954*a, b*), it was noted that when cell-free brain preparations were incubated with inorganic ${}^{32}\text{P}$, a major portion of the total labelling of the phospholipid was recovered from a fraction provisionally identified as phosphatidic acid. The experiments provide some evidence for the participation of $\alpha\text{-G-}{}^{32}\text{P}$ as an intermediate in the labelling of brain phosphatidic acid from inorganic ${}^{32}\text{P}$, for the addition of unlabelled $\alpha\text{-G-P}$ decreased the specific radioactivity of the lipid P, presumably by dilution of the specific radioactivity of $\alpha\text{-G-}{}^{32}\text{P}$. Similar results were obtained by Kennedy (1953) with liver mitochondria.

The experiments indicate that the radioactivity of $\alpha\text{-G-}{}^{32}\text{P}$ was not incorporated into brain lecithin. However, the $\alpha\text{-G-}{}^{32}\text{P}$ was rapidly incorporated into phosphatidic acid, so that it may be concluded that neither the labelled P of $[{}^{32}\text{P}]\text{phosphatidic acid}$ nor that of $\alpha\text{-G-}{}^{32}\text{P}$ is incorporated into lecithin. Moreover, the addition of CoA, which increased the labelling of phosphatidic acid from inorganic ${}^{32}\text{P}$ and $\alpha\text{-G-}{}^{32}\text{P}$, did not increase the labelling of lecithin from any of the labelled compounds studied.

The finding that the addition of CoA increased the labelling of lipid P from $\alpha\text{-G-}{}^{32}\text{P}$ and inorganic ${}^{32}\text{P}$ was of considerable interest. The participation of coenzyme A in the metabolism of fatty acids has been firmly established (Lynen, 1953, 1954; Mahler, 1953). The possibility that the metabolic synthesis of phospholipid may exhibit a similar requirement for CoA was first demonstrated by Kornberg & Pricer (1952). Subsequently, Kornberg & Pricer (1953*a*) found that the esterification of $\text{L-}\alpha\text{-G-}{}^{32}\text{P}$ by a fatty acid was preceded by the 'activation' of the fatty acid by a thiokinase reaction, with the formation of a thioester of CoA:

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acyl} \sim \text{CoA} + \text{AMP} + \text{P-P}. \quad (4)$$

The reaction is catalysed by a liver enzyme, for which the name fatty acid thiokinase has been suggested. Presumably the adenylyl derivative of the fatty acid is an intermediate in the reaction, as suggested by Berg (1956) for acetic thiokinase.

Kornberg & Pricer (1953*b*) also demonstrated the presence of a second liver-enzyme system capable of esterifying the $\text{L-}\alpha\text{-G-}{}^{32}\text{P}$ with two molecules of the 'activated' fatty acid to form $[{}^{32}\text{P}]\text{L-}\alpha\text{-phosphatidic acid}$

Jedeikin & Weinhouse (1954) and Stansly (1955) have provided confirmatory evidence for such a mechanism in the formation of phosphatidic acid.

observed in the presence of CTP, might suggest the formation of an active inositol-containing intermediate analogous to CMP~PCh. However, further speculation concerning the biosynthesis of brain diphosphoinositide must await its fuller chemical characterization.

SUMMARY

1. In suitably 'reinforced' water dispersions of rat brain the anaerobic labelling of lipid phosphorus from inorganic ^{32}P was increased by the addition of coenzyme A (CoA), glutathione, or cytidine triphosphate (CTP) to the incubation medium. The labelling was not significantly affected by the addition of α -glycerophosphate (α -GP) (0.001M), glycerol, choline, serine, ethanolamine or inositol.

2. In phosphorylating mitochondria prepared from isotonic-sucrose homogenates of rat brain, the labelling of lipid phosphorus from inorganic ^{32}P was increased by the addition of CTP, but not by the addition of CoA or glutathione. The labelling was decreased by the addition of α -GP (0.005M), but not by the addition of β -GP or glycerol.

3. Examination of the hydrolysis products of the labelled phospholipids showed that the increase in labelling produced by the addition of CoA to the anaerobic system (hypotonic dispersion) was almost exclusively the result of an increase in the labelling of GP (presumably from phosphatidic acid) and a hydrolysis product of diphosphoinositide (IP), whereas that produced by the addition of CTP to either system was largely due to an increase in the labelling of IP.

4. In both systems the ^{32}P from radioactive adenosine triphosphate (AT^{32}P), phosphorylcholine (^{32}PCh) and α -G ^{32}P was incorporated into lipid phosphorus without prior breakdown into inorganic ^{32}P .

5. The incorporation of ^{32}PCh into the lipid phosphorus of both systems was almost entirely into lecithin and was greatly stimulated by the addition of CTP, but not by the addition of CoA. The requirement for CTP could not be replaced by uridine, inosine or guanosine triphosphates.

6. The incorporation of α -G ^{32}P into the lipid phosphorus of both systems was stimulated by the addition of CoA, but not by the addition of CTP. Radioactivity of the hydrolysis products derived from the labelled phospholipids was largely in GP and IP. The labelling of GP, but not that of IP, was increased by the addition of CoA.

7. The labelling of lipid phosphorus from both ^{32}PCh and α -G ^{32}P was inhibited in the hypotonic dispersion by conditions that inhibited glycolysis, and in the mitochondria by conditions that inhibited oxidative phosphorylation.

8. The results are discussed in relation to the biosynthesis of phospholipids in brain.

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