The Incorporation of the Phosphate Esters of *N*-Substituted Aminoethanols into the Phospholipids of Brain and Liver

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1. The phosphate esters of dimethylaminoethanol and monomethylaminoethanol can be incorporated from their cytidine diphosphate esters into the phospholipids of brain and liver dispersions; the deoxycytidine nucleotides of the same bases are less effective precursors. 2. The cytidyltransferases of brain and liver are less effective in forming the cytidine diphosphate esters of monomethylaminoethanol and dimethylaminoethanol than those of ethanolamine and choline.

The work of Kennedy and his associates (Kennedy, 1961) has unequivocally shown that phosphorylcholine and phosphorylethanolamine can be incorporated into the phospholipids of tissue preparations by a pathway involving cytidine nucleotides. It has been established that the monoesters first react with CTP to yield the CDP ester, which can then transfer the phosphorylated base to a suitable acceptor molecule, e.g. D-2,3-diglyceride, 2-acyl-1-alkenylglycerol (plasmalogenic diglyceride) or *N*-acylsphingosine (ceramide). Although deoxycytidine can replace cytidine in the nucleotide (Kennedy, Borkenhagen & Smith, 1959; Schneider & Behki, 1963) no other nucleotide base has so far proved effective (Kennedy & Weiss, 1956; Chojnacki & Korzybski, 1963). The requirement for the lipid base is much less specific (Chojnacki, 1964).

Although the enzymic steps in the cytidine pathway are the only ones capable of forming new phosphodiester bonds in phospholipids, other pathways to phosphatidyl-ethanolamine and -choline have been established in liver. Thus, although it has been known for some time that injected L-serine can give rise to ethanolamine in liver (Arnstein, 1951), Bremer, Figard & Greenberg (1960) showed that L-[3-¹⁴C]serine labelled lipid ethanolamine better than the free phosphorylethanolamine or CDP-ethanolamine, suggesting that the last two compounds were not intermediates. Further, Wilson, Gibson & Udenfriend (1960) demonstrated that the labelling of lipid ethanolamine from L-[3-¹⁴C]serine by liver and brain slices was not significantly diminished by adding unlabelled ethanolamine or phosphorylethanolamine. The methylation of phosphatidylethanolamine to phos-

phatidylcholine by *S*-adenosylmethionine *in vivo* was also indicated by the experiments of Bremer *et al.* (1960). So far the introduction of the first methyl group into phosphatidylethanolamine has not been demonstrated *in vitro* though the methylation of phosphatidylmonomethylaminoethanol and phosphatidyl dimethylaminoethanol by *S*-adenosylmethionine in the presence of different liver preparations has been successfully accomplished (Artom & Lofland, 1960; Bremer & Greenberg, 1961; Artom, 1964; Reh binder & Greenberg, 1965).

Methylation of phospholipids does not occur in brain tissue preparations (Chojnacki, Korzybski & Ansell, 1964; Marshall, Chojnacki & Ansell, 1965), which would imply an exclusive dependence on the cytidine pathway for the formation of phosphatidylcholine in this tissue. Further, evidence is available for the operation of the cytidine pathway in the formation of phospholipids containing monomethylaminoethanol and dimethylaminoethanol in mammalian tissues. After injection of 2-dimethylaminoethanol into the rat, the base could be recovered in a lipid-bound form from liver (Artom & Crowder, 1949) and as its free phosphate ester from brain and liver (Ansell & Spanner, 1962). Free dimethylaminoethanol can be incorporated into the lipids of liver homogenates and slices (Artom, 1960) and can be phosphorylated by liver preparations (Artom, 1962) and brain tissue (Ansell & Spanner, 1962). Subsequently it was shown that CDP-dimethylaminoethanol can give rise to phosphatidyl dimethylaminoethanol in brain and liver homogenates (Ansell & Chojnacki, 1962*a*). The present paper deals with the incorporation of monomethylaminoethanol and dimethylaminoethanol via their CDP and dCDP esters into the phospholipids of brain and liver. Some of this work has been briefly reported (Ansell & Chojnacki, 1962*a,b*).

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METHODS

Materials. CMP and dCMP from Sigma Chemical Co., London, S.W. 6, or Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A., were used without further purification. Ethanolamine, 2-dimethylaminoethanol, 2-monomethylaminoethanol and choline chloride were products of British Drug Houses Ltd., Poole, Dorset. Carrier-free [^{32}P]orthophosphate was purchased from The Radiochemical Centre, Amersham, Bucks. Dicyclohexylcarbodi-imide and aluminium oxide ('prepared for chromatography') were products of British Drug Houses Ltd. and were used as purchased.

Synthesis of ^{32}P -labelled phosphorylated bases

The [^{32}P]phosphate esters of choline, monomethylaminoethanol, dimethylaminoethanol and ethanolamine were prepared by methods essentially similar to those used by Riley (1944) and Schmidt (1957) for phosphorylcholine and phosphorylethanolamine. Carrier-free [^{32}P]orthophosphate (1–5 mc) was added to 0.01 ml. of 88% orthophosphoric acid in a small tube (1.5 cm. $\times$ 4.5 cm.) and the solution evaporated to a syrup with an infrared lamp. After cooling, an equimolar amount of the free base (or the chloride in the case of choline) was slowly added and the thick mixture stirred. The tube was then transferred to a pear-shaped flask containing 0.2–0.5 g. of P_2O_5 and the flask connected to a vacuum line. The temperature was raised and the pressure slowly increased or decreased to prevent frothing in the reaction mixture. The cooled reaction mixture was diluted with 2–3 ml. of water and heated at 100° to hydrolyse any pyrophosphate to orthophosphate. At this stage, the yield of phosphate ester was checked by ascending chromatography in propan-2-ol–water–trichloroacetic acid–18 N- NH_3 (75:25:5:0.3, v/v/w/v) (Ebel, 1952). A radioautograph was made to locate the ester and the percentage conversion of [^{32}P]orthophosphate calculated from a quantitative assay of the isotope in the spot. The yields of phosphate ester obtained at 10 mm. Hg pressure were: 20–30% of phosphorylcholine (155°, 6–8 hr.); less than 5% of phosphoryldimethylaminoethanol (110–120°, 6–8 hr.); 5–10% of phosphorylmonomethylaminoethanol (120–130°, 6–8 hr.); more than 50% of phosphorylethanolamine (170°, 3 hr.).

Isolation of [^{32}P]phosphorylcholine and [^{32}P]phosphoryldimethylaminoethanol. The reaction mixture diluted to 5–10 ml. with water was passed through a column (1 cm. $\times$ 10 cm.) of Amberlite IR-120 (NH_4^+ form) to remove the free base, and then through Amberlite IRA-400 (formate form) to remove inorganic phosphate. Any NH_3 could be removed with Dowex 50 (H^+ or pyridinium form) and the final effluent contained only [^{32}P]phosphoryl-base.

Isolation of [^{32}P]phosphorylethanolamine and [^{32}P]phosphorylmonomethylaminoethanol. The pH of the diluted reaction mixture was adjusted to 9–10 with N-NaOH and the mixture was diluted to about 100 ml. with water. The solution was applied to a column (1.5 cm. $\times$ 10 cm.) of Dowex 1 (formate form) resin and washed through with 50 ml. of water. A linear gradient of formic acid (500 ml. of 0.04 M-formic acid in the reservoir and 500 ml. of water in the mixing chamber) was then used to elute the phosphorylated bases. They were mostly eluted as a sharp peak when 100–150 ml. of eluate had passed through the column

at a rate of 40 ml./hr. (cf. Weisburger & Schneider, 1961; Kennedy, 1956; Chojnacki & Korzybski, 1961). After the relevant fractions had been evaporated to dryness *in vacuo*, the radioactive esters were dissolved in water, any free resin particles were removed by centrifugation and the purity of the fraction was checked by paper chromatography in Ebel's (1952) solvent. Single ninhydrin-positive (phosphorylethanolamine only), radioactive, phosphorus-containing spots were obtained.

Synthesis of CDP esters of the substituted ethanolamines

These were prepared from CMP and the [^{32}P]phosphate esters of ethanolamine and the N-methylaminoethanols essentially by the method of Kennedy (1956). Equimolar amounts of CMP and the radioactive ester were dissolved in pyridine–water (7:1, v/v) and dicyclohexylcarbodi-imide (30 mg./mg. of CMP) was added to the clear solution. After 2 days at 37° further dicyclohexylcarbodi-imide (15 mg./mg. of CMP) was added and the process usually repeated after a further 2 days. For the derivatives of ethanolamine and monomethylaminoethanol an incubation period of 6 days was used, though half this time (and two additions of dicyclohexylcarbodi-imide) were adequate for the choline and dimethylaminoethanol derivatives. The coupling efficiency was estimated as 40% for these two compounds, about 20% for the CDP-monomethylaminoethanol and about 5% for CDP-ethanolamine.

After the appropriate reaction time the semi-solid mixture was shaken well with 20 ml. of water and the mixture filtered. The cloudy filtrate was extracted three times with equal volumes of diethyl ether and the clear aqueous phase evaporated *in vacuo* to about half its original volume.

Isolation of CDP-choline and CDP-dimethylaminoethanol. The pH of the ether-free solution was adjusted to 9 with N-NaOH and diluted to 100–120 ml. with water. It was then applied to a column (1.5 cm. $\times$ 10 cm.) of Dowex 1 (X8; formate form), washed through with 50 ml. of water and the components of the mixture were eluted with a linear gradient of formic acid identical with that used for phosphorylethanolamine. Fractions of volume 10 ml. were collected at a rate of 40 ml./hr. and assayed for extinction at 280 m μ and radioactivity. The monoester of the base was eluted first followed by the CDP-choline or CDP-dimethylaminoethanol. After this came unchanged CMP. The fractions containing the required compounds were pooled, evaporated to dryness *in vacuo* at room temperature and, after removal of the fine resin particles, adjusted to pH 7 with 0.1 N-NaOH and diluted for use.

Isolation of CDP-monomethylaminoethanol and CDP-ethanolamine. Radioautographs of paper chromatograms of the reaction mixture in these instances showed that, although there was very little of the desired compound [R_F 0.1 in Ebel's (1952) solvent] very little ^{32}P -labelled monoester remained. A number of unidentified radioactive compounds was, however, present. This is presumably one of the reasons why Kennedy (1956) and Schneider & Rotherham (1958), working with dCMP, and Sanno & Tanaka (1962) obtained such low yields of CDP-ethanolamine by this method. It was therefore necessary to make a prior separation of the desired compound by large-scale paper chromatography (Chojnacki, 1964; Ansell, Chojnacki & Metcalfe, 1965). After the reaction mixture had been

Fig. 1. Chromatography on Dowex 1 (formate form) of the reaction products formed when dicyclohexylcarbodi-imide is allowed to react with dCMP and [^{32}P]phosphorylcholine for 3 days (see the text). A, Unchanged [^{32}P]phosphorylcholine; B, unidentified material; C, dCDP-choline; D, unchanged dCMP. ○, Radioactivity; ●, $E_{280\text{m}\mu}^{1\text{cm}}$.

filtered and washed with ether it was applied to Whatman no. 1 paper as a streak about 1m. in length per 100mg. of CMP originally present in the reaction mixture. After ascending chromatography in Ebel's (1952) solvent, a radioautograph of a small section of the paper was used to locate the required nucleotide. The band containing the desired compound was then cut out, washed with ether to remove the trichloroacetic acid and eluted with water. The pH of the eluate was adjusted to 9. Chromatography on a column of Dowex 1 (formate form) was then carried out in the manner described above. A non-radioactive nucleotide was also eluted from the column in appreciable amounts; this was probably dicytidine pyrophosphate or unchanged CMP.

Syntheses of deoxycytidine analogues. These were carried out exactly as described for the ribonucleotides. Separation of radioactive deoxycytidine nucleotide from unchanged phosphate monoester on Dowex 1 (formate form) was not as good as the ribose analogue but it was possible to isolate, for example, dCDP-choline from the other components of the reaction mixture (Fig. 1).

Miscellaneous methods

Solutions and spots on chromatograms containing radioactive phosphorus were assayed as described by Ansell *et al.* (1965).

Tissue preparations and incubation. Tissue was ground in an ice-cold glass pestle and mortar until a fine paste was obtained. In most experiments this was then diluted with KCl-NaF (Dawson, 1953), and 1ml. of the suspension was added to 2ml. of a mixture containing the compounds indicated in Tables 2 and 3. After a suitable period the contents of the tube were shaken with a volume of chloroform-methanol (2:1, v/v) equivalent to 20 vol. of the incubated suspension. The mixture was quickly heated to the boiling point, filtered and evaporated three times to dryness

in vacuo to break protein-lipid bonds. After dissolving in 10ml. of chloroform-methanol (2:1, v/v) the lipid solution was washed by the method of Folch, Lees & Sloane-Stanley (1957). This washed lipid was then ready for chromatography on alumina or for other investigations. In experiments in which it was desirable to investigate the radioactivity in the aqueous phase, the reaction was stopped with trichloroacetic acid (see the Results section).

For the experiments on cytidylyltransferase activity a 25% homogenate was prepared by diluting the ground tissue with water. This was incubated with CTP, [^{32}P]phosphate esters and other components as indicated in Table 1 in a final volume of 0.4ml. After incubation the reaction was stopped by the addition of an equal volume of ice-cold 10% (w/v) trichloroacetic acid solution. A small volume of the supernatant (20–100 $\mu\text{l.}$), obtained by centrifuging the precipitated homogenate, was subjected to single-dimensional chromatography in Ebel's (1952) solvent in which radioactive CDP esters were clearly separated from the monophosphate esters of the lipid bases. The CDP esters (R_f about 0.1) were located by radioautography on Kodak No-Screen X-ray film, cut out and ashed to determine the ^{32}P content.

The precipitated tissue was washed and the lipids were extracted by the method of Ansell & Dohmen (1956) to determine their ^{32}P content.

RESULTS

Formation of CDP esters of phospholipid bases.

The capacity of tissues to form CDP esters of the various lipid bases was determined by incubating 0.1ml. of 25% homogenate with 0.5 μmole of CTP and 0.2 μmole of the [^{32}P]phosphate ester as indicated in Table 1. After precipitation with trichloroacetic acid, the radioactivity in the CDP ester formed was measured and compared with the radioactivity in the original amount of [^{32}P]phosphate ester of the lipid base added to the reaction mixture. In some experiments the amount of the radioactivity subsequently transferred to lipids was also measured.

The percentages of added phosphate esters converted into the CDP esters and into phospholipids are given in Table 1. In brain the formation of CDP esters in 15min. was slight compared with liver, but significant. The formation of CDP-monomethylaminoethanol and CDP-dimethylaminoethanol was stimulated by the presence of CTP. Small but significant amounts of radioactivity were transferred to lipids, though with both liver and brain there was a more efficient transfer of the esters of choline and ethanolamine.

Incorporation of the radioactivity of labelled nucleotides into phospholipids. Samples of CDP and dCDP esters of the ^{32}P -labelled bases were incubated with 100mg. of a brain-cortex dispersion in a medium similar to that used by Dawson (1953) at 37° in an atmosphere of oxygen + carbon dioxide (95:5). The reaction was stopped with 3ml. of

Table 1. *Transfer of radioactivity of the [32 P]phosphate esters of N-substituted aminoethanols into CDP nucleotides and phospholipids*

Each incubation consisted of 0.1 ml. of a 25% homogenate of tissue, 0.09 ml. of 0.1 M-phosphate buffer, pH 7.6, 0.01 ml. of M-MgCl₂, 0.1 ml. of CTP (0.5 μ mole) and 0.1 ml. of a solution of the [32 P]phosphate ester (0.2 μ mole, 6×10^5 counts/100 sec.). After incubation for the stated time at 37°, the reaction was stopped with 0.4 ml. of 10% trichloroacetic acid.

[32 P]Phosphate ester	Expt. no.	Incubation time (min.)	Transfer of radioactivity from added ester (%)			
			Into brain		Into liver	
			CDP nucleotides	Phospho-lipids	CDP nucleotides	Phospho-lipids
Phosphorylethanolamine	1	15	0.8*	—	9.7	—
	2	60	—	0.04	—	0.92
Phosphorylmonomethylaminoethanol	1	15	0.1*	—	1.3	—
	2	60	—	0.03	—	0.18
Phosphoryldimethylaminoethanol	1	15	0.3*	—	1.2	—
	2	60	—	0.11	—	0.08
Phosphorylcholine	1	15	1.7*	—	2.6	—
	2	60	—	0.49	—	0.89

* Mean of two experiments.

Table 2. *Incorporation of the radioactivity of 0.05 μ mole of labelled nucleotide into the lipids of brain-cortex dispersions (100 mg. of tissue $\equiv$ 115 μ g. of lipid phosphorus)*

The radioactivity of the labelled nucleotide varied from 1.5×10^4 to 8.0×10^4 counts/100 sec. In addition to the labelled nucleotide and the tissue the following constituents were present: NaCl (52.5 mm), KCl (19 mm), KH₂PO₄ (1.18 mm), MgCl₂ (13.6 mm), NaHCO₃ (25 mm), pyruvate (4.93 mm), fumarate (5.4 mm), cytochrome *c* (32 μ M), AMP (1 mm) and NaF (15.5 mm). The final volume was 3 ml. and the gas phase was O₂ + CO₂ (95:5). The incubation time was 1 hr. The tissue was precipitated with trichloroacetic acid and the components were determined as described in the Methods section.

Labelled nucleotide	Radioactivity recovered (% of that added)		
	From medium		
	In phospholipids	Adsorbed on charcoal	Not adsorbed
CDP-choline	44.2	13.3	19.5
dCDP-choline	12.9	43.5	30.0
CDP-dimethylaminoethanol	35.0	—	—
dCDP-dimethylaminoethanol	3.5	—	—
CDP-monoethylaminoethanol	37.4	11.1	28.6
dCDP-monomethylaminoethanol	3.0	32.4	41.7
CDP-ethanolamine	36.0	—	—
dCDP-ethanolamine	0.5	—	—

10% trichloroacetic acid and the acid extract shaken with about 50 mg. of acid-washed charcoal to distinguish between 32 P still bound in a nucleotide and any free [32 P]phosphate ester of the lipid base or inorganic [32 P]orthophosphate. Phospholipids were extracted from the precipitate of lipid and protein by the method of Ansell & Dohmen (1956).

Table 2 shows that 35–44% of the radioactivity present in the CDP esters of all four bases was incorporated into lipids by brain cortex. The incorporation from the dCDP esters was very much less

and negligible for dCDP-ethanolamine. Only about 25% of the radioactivity in the CDP ester was released as a non-nucleotide water-soluble compound; the remainder was either in a lipid form or still as a nucleotide. These experiments extend the earlier findings of Kennedy (1961) to the cytidine compounds of bases other than ethanolamine and choline and show that the deoxycytidine compounds are much less effective agents for transferring phosphorylated bases to phospholipids in the system used.

Table 3. *Incorporation of the radioactivity of labelled nucleotides (0.05 μ mole) into the lipids of brain and liver dispersions (100mg.)*

In addition to the labelled nucleotide (radioactivity varied from 1.5×10^4 to 8.0×10^4 counts/100sec.) and tissue the medium was identical with that in Table 2. After incubation for 1hr. the lipids were extracted with chloroform-methanol (see the Methods section).

Base in nucleotide	Radioactivity in lipid (% of that added)					
	CDP esters			dCDP esters		
	Brain cortex	White matter	Liver	Brain cortex	White matter	Liver
Choline	38.6*	23.0	26.0	11.1*	8.1	5.1
Dimethylaminoethanol	36.3*	22.3	21.0	4.2*	2.4	1.1
Monomethylaminoethanol	36.0*	20.7	20.0	3.4*	1.4	0.5
Ethanolamine	36.0	21.6	20.7	0.5	0.5	0.2

* Mean of two experiments.

Table 3 shows that, on the basis of wet tissue weight, brain cortex was nearly twice as effective as white matter or liver in incorporating the ^{32}P -labelled ester into a lipid-bound form. For all three tissues and all four lipid bases, the percentage transfer was of the same order, indicating a low specificity for the enzymes ethanolamine phosphotransferase (EC 2.7.8.1) and choline phosphotransferase (EC 2.7.8.2). For the deoxycytidine compounds, however, the amount of incorporation was dependent on the degree of methylation of the base. The reason why this should be the determining factor for the deoxy derivatives is obscure.

Fractionation of the phospholipids formed on incubation of labelled nucleotides with brain cortex. There was no reason to suppose that ^{32}P -labelled phospholipids formed from CDP-ethanolamine or CDP-choline in brain homogenates would be other than phosphatidylethanolamine (or the corresponding plasmalogen) and phosphatidylcholine respectively (McMurray, Strickland, Berry & Rossiter, 1957). It was, however, decided to identify the lipids formed from the CDP esters of monomethylaminoethanol and dimethylaminoethanol. The lipids, equivalent to about 70mg. of tissue, were dissolved in chloroform and applied to small columns of 2.5g. of alumina. They were then eluted in a stepwise fashion with chloroform-methanol or chloroform-ethanol mixtures containing increasing amounts of water (Chojnacki & Korzybski, 1964). The radioactivity and phosphorus content of the fractions were determined and the results are shown in Fig. 2.

There were two phosphorus-containing peaks corresponding to the choline- and ethanolamine-containing lipids. This was confirmed by application of the selective hydrolysis technique of Dawson (1960) to these peaks. In the experiments with labelled CDP-ethanolamine and CDP-choline the

radioactivity corresponded to the peak due to the corresponding phospholipid. The radioactive lipids obtained in the experiments with CDP-monomethylaminoethanol and CDP-dimethylaminoethanol were eluted at intermediate points between choline and ethanolamine lipids as indicated in Fig. 2. Very little of the radioactivity was incorporated into phospholipids containing ethanolamine and choline. Judged from the position of elution the radioactive phospholipids were the analogues of phosphatidyl-ethanolamine and -choline. The radioactive lipid in Fig. 2(a) was identified as phosphatidyl-2-dimethylaminoethanol from the following criteria: (1) When the material in the radioactive peak from the alumina column was mixed with synthetic distearoylphosphatidyl-2-dimethylaminoethanol (kindly provided by Professor E. Baer) and resubjected to chromatography on alumina, the elution of the synthetic material coincided with that of the radioactive lipid. (2) Chromatography of the total lipid extract on silicic acid paper in the chloroform-methanol solvent of Lea, Rhodes & Stoll (1955) or the diisobutyl ketone-acetic acid-water solvent of Marinetti (1962) gave only one significantly radioactive spot. Thin-layer chromatography on silica gel G (Merck) gave similar results. (3) When the radioactive peak from the alumina column was freed from glycolipids with Florisil (Ansell & Spanner, 1963) and run on silicic acid papers or thin-layer chromatograms in the presence of increasing amounts of synthetic lipid the size of the radioactive area increased (cf. Hokin & Hokin, 1958). (4) Mild alkaline hydrolysis of the radioactive lipid by the method of Dawson (1960) gave a single water-soluble phosphate ester that behaved chromatographically in phenol-aq. ammonia in the same way as the glycerylphosphoryl ester from the synthetic lipid.

Fig. 2. Chromatography on alumina of the radioactive phospholipids formed by incubating dispersions of rat brain cortex with the CDP esters of lipid bases (see the text). An amount of lipid equivalent to about 70 mg. of tissue was applied to the column in chloroform and eluted in a stepwise fashion as indicated. The radioactive lipids formed from (a) labelled CDP-dimethylaminoethanol, (b) labelled CDP-monomethylaminoethanol and (c) labelled CDP-ethanolamine are shown. A, Choline lipids; B, ethanolamine lipids. O, Radioactivity; ●, phosphorus.

No synthetic phosphatidylmonomethylaminoethanol was available for comparison with the radioactive lipid synthesized from labelled CDP-monomethylaminoethanol. Its formation was assumed by analogy and the fact that it behaved as a homogeneous compound on thin-layer chromatograms and silicic acid papers.

DISCUSSION

The experiments described in this paper show that isolated adult liver and brain tissues can incorporate the labelled phosphate esters of monomethylaminoethanol and dimethylaminoethanol into the corresponding phospholipids by a pathway involving their CDP esters. The observed rate of transfer from the nucleotide to phospholipid of $0.18 \mu\text{mole/g.}$ of brain cortex tissue/hr. was the same as that found for CDP-ethanolamine and CDP-choline. It is assumed that the enzymes responsible for the transfer of phosphorylated bases are identical with or analogous to ethanolamine phosphotransferase and choline phosphotransferase, which appear to be distinct enzymes (Kennedy & Weiss, 1956). In a study with CDP-propanolamine Ansell *et al.* (1965) have shown that this ester is preferentially attacked by ethanolamine phosphotransferase, but there is a low specificity with both enzymes (Chojnacki, 1964).

From the results shown in Table 1 it is clear that ethanolamine phosphate cytidyltransferase (EC 2.7.7.14) and choline phosphate cytidyltransferase (EC 2.7.7.15) are more specific than the phosphotransferases. Very little activity was found towards the phosphate esters of monomethylaminoethanol and dimethylaminoethanol, particularly in brain tissue. Similarly, Ansell *et al.* (1965) have shown that cytidyltransferase activity towards phosphorylpropanolamine was very feeble, though the transfer of the ester from CDP-propanolamine to phospholipids was as good as with CDP-ethanolamine. In short, the incorporation of the phosphate ester of a given base into phospholipids by the cytidine pathway is determined by the specificity exhibited by cytidyltransferases.

Under the conditions used, the dCDP esters of all four lipid bases were less effective substrates for the phosphotransferases than the corresponding cytidine compounds (Table 3). The efficiency of transfer to lipid decreased with the degree of *N*-substitution so that, for example, the incorporation of phosphoryl ethanolamine into phospholipids of brain cortex was only 4–5% of that of phosphorylcholine when the dCDP esters were used as donors. Decreased transfer was not due to increased hydrolysis of the nucleotides in the system. Using a mixture of microsomes and mitochondria,

Kennedy *et al.* (1959) found that dCDP-choline was as effective as CDP-choline in the phosphotransferase reaction, but that, when the ethanolamine analogue was used, the CDP ester was five times as effective. Our observations on the preference for CDP-choline over dCDP-choline are in agreement with the findings of Schneider & Behki (1963), who also used rat-liver homogenates, and Chojnacki & Korzybski (1963), who used tissues from a number of species.

The fact that monomethylaminoethanol and dimethylaminoethanol can be incorporated into phospholipids via the cytidine pathway raises the issue of the importance of such a pathway in phospholipid synthesis in brain and liver. Small but significant amounts of phospholipids containing these bases are found in liver (Bremer & Greenberg, 1959), where they appear to be intermediates in the stepwise methylation to yield phosphatidylcholine (Bremer & Greenberg, 1961). On the other hand, significant amounts of the free bases or their phosphate esters have not so far been detected in animal tissues, though the free phosphate esters occur in choline-requiring mutant strains of *Neurospora crassa* (Wolf & Nye, 1959). In brain, for example, the amount of free dimethylaminoethanol is as low as 40–80 µg./kg. (Honegger & Honegger, 1960) and no detectable amount of its phosphate ester is present (Ansell & Spanner, 1962). In fact, the formation of phosphatidyl-monomethylaminoethanol and -dimethylaminoethanol by the cytidine pathway may be of no significance in brain since their methylation to phosphatidylcholine cannot take place (Chojnacki *et al.* 1964; Marshall *et al.* 1965). It has also been shown that the formation of phosphatidylcholine by stepwise methylation is negligible *in vivo* (Ansell & Spanner, 1965). This suggests, in turn, an exclusive dependence on the cytidine pathway to phosphatidylcholine in brain, though it would not explain the origin of the choline itself. It is possible that some choline derives from serine in the brain as found by Wilson *et al.* (1960), but methylation must be part of this pathway and methylation has not been demonstrated. There is no evidence for a pathway to free choline in brain tissue and it is not impossible that all the brain choline is transferred there from the blood. The work of Groth, Bain & Pfeiffer (1958) has shown that significant amounts of intraperitoneally injected ¹⁴C-labelled choline is found in the acid-soluble and lipid fractions of the brain. This suggests a direct transfer of the base from blood to brain and its subsequent phosphorylation and incorporation into phosphatidylcholine (cf. dimethylaminoethanol; Ansell & Spanner, 1962). The cytidine pathway to phosphatidylcholine, in effect, will predominate in any organ requiring exogenous choline.

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REFERENCES

- Ansell, G. B. & Chojnacki, T. (1962a). *Nature, Lond.*, **196**, 545.
 Ansell, G. B. & Chojnacki, T. (1962b). *Biochem. J.* **85**, 31p.
 Ansell, G. B., Chojnacki, T. & Metcalfe, R. F. (1965). *J. Neurochem.* **12**, 649.
 Ansell, G. B. & Dohmen, H. (1956). *J. Neurochem.* **1**, 150.
 Ansell, G. B. & Spanner, S. (1962). *J. Neurochem.* **9**, 253.
 Ansell, G. B. & Spanner, S. (1963). *J. Neurochem.* **10**, 941.
 Ansell, G. B. & Spanner, S. (1965). *Biochem. J.* **96**, 64p.
 Arnstein, H. R. V. (1951). *Biochem. J.* **48**, 27.
 Artom, C. (1960). *Fed. Proc.* **19**, 233.
 Artom, C. (1962). *Fed. Proc.* **21**, 255.
 Artom, C. (1964). *Biochem. biophys. Res. Commun.* **15**, 201.
 Artom, C. & Crowder, M. (1949). *Fed. Proc.* **8**, 180.
 Artom, C. & Lofland, H. B., jun. (1960). *Biochem. biophys. Res. Commun.* **3**, 244.
 Bremer, J., Figard, P. H. & Greenberg, D. M. (1960). *Biochim. biophys. Acta*, **43**, 477.
 Bremer, J. & Greenberg, D. M. (1959). *Biochim. biophys. Acta*, **35**, 287.
 Bremer, J. & Greenberg, D. M. (1961). *Biochim. biophys. Acta*, **46**, 205.
 Chojnacki, T. (1964). *Acta biochim. polon.* **11**, 11.
 Chojnacki, T. & Korzybski, T. (1961). *Acta biochim. polon.* **8**, 111.
 Chojnacki, T. & Korzybski, T. (1963). *Acta biochim. polon.* **10**, 455.
 Chojnacki, T. & Korzybski, T. (1964). *Acta biochim. polon.* **11**, 341.
 Chojnacki, T., Korzybski, T. & Ansell, G. B. (1964). *Biochem. J.* **90**, 18p.
 Dawson, R. M. C. (1953). *Biochem. J.* **55**, 507.
 Dawson, R. M. C. (1960). *Biochem. J.* **75**, 45.
 Ebel, J. P. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 321.
 Folch, J., Lees, M. & Sloane-Stanley, G. M. (1957). *J. biol. Chem.* **226**, 497.
 Groth, D. P., Bain, J. A. & Pfeiffer, C. C. (1958). *J. Pharmacol.* **124**, 290.
 Honegger, C. G. & Honegger, R. (1960). *Nature, Lond.*, **185**, 530.
 Hokin, L. E. & Hokin, M. R. (1958). *J. biol. Chem.* **233**, 800.
 Kennedy, E. P. (1956). *J. biol. Chem.* **222**, 185.
 Kennedy, E. P. (1961). *Proc. 5th int. Congr. Biochem., Moscow*, vol. 7, p. 113.
 Kennedy, E. P., Borkenhagen, L. F. & Smith, S. W. (1959). *J. biol. Chem.* **234**, 1998.
 Kennedy, E. P. & Weiss, S. B. (1956). *J. biol. Chem.* **222**, 193.

- Lea, C. H., Rhodes, D. N. & Stoll, R. D. (1955). *Biochem. J.* **60**, 353.
- McMurray, W. C., Strickland, K. P., Berry, J. F. & Rossiter, R. J. (1957). *Biochem. J.* **66**, 634.
- Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
- Marshall, E. F., Chojnacki, T. & Ansell, G. B. (1965). *Biochem. J.* **95**, 30r.
- Rehnbinder, D. & Greenberg, D. M. (1965). *Arch. Biochem. Biophys.* **108**, 110.
- Riley, R. F. (1944). *J. Amer. chem. Soc.* **66**, 512.
- Sanno, Y. & Tanaka, K. (1962). *Chem. Pharm. Bull., Tokyo*, **10**, 231.
- Schmidt, G. (1957). In *Methods in Enzymology*, vol. 3, p. 346. Ed. by Kolwick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Schneider, W. C. & Behki, R. M. (1963). *J. biol. Chem.* **238**, 3565.
- Schneider, W. C. & Rotherham, J. (1958). *J. biol. Chem.* **233**, 948.
- Weisburger, J. H. & Schneider, W. C. (1961). *J. org. Chem.* **26**, 1658.
- Wilson, J. D., Gibson, K. D. & Udenfriend, S. (1960). *J. biol. Chem.* **235**, 3539.
- Wolf, B. & Nyc, J. F. (1959). *J. biol. Chem.* **234**, 1068.