The Metabolism of [Me-14C]Choline in the Brain of the Rat in vivo

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[Me-14C]Choline was injected intracerebrally into the adult rat, and its uptake into the lipids and their water-soluble precursors in brain was studied. The radio-activity could be detected only in the choline-containing lipids and was confined to the base choline. The results indicated that initial phosphorylation of the free choline followed by the formation of CDP-choline and the subsequent transfer of the phosphorylcholine to a diglyceride is one of the principal routes by which choline lipids in brain are formed. Further evidence for this was obtained in experiments in which either phosphoryl[Me-14C]choline or [32P]orthophosphate was injected and the radioactivity in the choline-containing water-soluble and lipid-bound components studied.

Several workers have studied the metabolism of lipid components after the intracerebral injection of lipid precursors. Bickerstaffe & Mead (1967) injected [1-14C]palmitaldehyde into rat brains and traced its metabolism in the phospholipids. Kopaczyk & Radin (1965) used [1-14C]lignoceric acid and [1-14C]stearic acid in a study of brain sphingolipid metabolism. Davison & Gregson (1966) gave rats intracerebral injections of inorganic [35S]sulphate to study the metabolic activity of sulphatides in myelin. Other workers injected [32P]orthophosphate (Ansell & Chojnacki, 1964), 3H-labelled *erythro*- and *threo*-sphingosine (Kanfer & Gal, 1966) and [14C]serine (Abdel-Latif & Abood, 1966), and studied their incorporation into the brain lipids.

As far as we are aware no one has reported on the fate of intracerebrally injected labelled choline, although the metabolism of choline lipids, in particular phosphatidylcholine, has been studied extensively in tissues. Groth, Bain & Pfeiffer (1958) studied the fate of both 14C-labelled choline and 14C-labelled 2-dimethylaminoethanol after intra-peritoneal injection, but they examined only the incorporation into the total acid-soluble and lipid-bound choline of the brain. The metabolism of choline in the brain is of interest because choline does not appear to be synthesized in this tissue (Marshall, Chojnacki & Ansell, 1965; Ansell & Spanner, 1967).

In the present work [Me-14C]choline was injected intracerebrally into mature rats and its subsequent metabolism was studied. Preliminary reports of this work have been published (Ansell & Spanner, 1966, 1968).

MATERIALS AND METHODS

Materials. [Me-14C]Choline and [32P]orthophosphate were obtained from The Radiochemical Centre, Amersham, Bucks. Phosphoryl[Me-14C]choline was prepared as described for the 32P-labelled compound by Ansell & Chojnacki (1966). The purity of the radioactive chemicals was checked by paper chromatography followed by radio-autography just before use. All chemicals were of A.R. quality whenever possible. The rats used were females, 230–250g. body wt., and 12–14 weeks old.

Injections. The rats were anaesthetized and the radio-active compounds injected as described by Ansell & Spanner (1967). In the experiments described below, two different amounts of [Me-14C]choline, $0.55\,\mu c$ ($0.017\,\mu$mole) and $1.0\,\mu c$ ($0.03\,\mu$mole), were injected. With [32P]ortho-phosphate, $10\,\mu c$ ($0.32\,\mu$mole) was injected, and with phosphoryl[Me-14C]choline, $0.035\,\mu c$ ($0.029\,\mu$mole) was injected.

Extraction and determination of water-soluble components. The brains were removed, weighed and homogenized with ice-cold trichloroacetic acid (10%, w/v), and the water-soluble components were separated on Dowex 1 (X8; formate form). The elution pattern for the choline-containing compounds was almost the same as that for the analogous ethanolamine compounds described by Ansell & Spanner (1967). The choline was eluted with the water; phosphorylcholine and CDP-choline were then eluted with increasing concentrations of formic acid. The water eluate from the column was evaporated to dryness and the residue dissolved in 1ml. of 5% (w/v) trichloroacetic acid, and the free choline was determined by the method of Long, Odavić & Sargent (1967). This fraction included choline originally present as acetylcholine since this would have been hydrolysed when the pH of the original extract was adjusted to 8·5 before column chromatography. Since the acetylcholine concentration in rat brain ($0.007\,\mu$mole/g. fresh wt.; Richter & Crossland, 1949) is only 4% of the free

choline pool, it was ignored when calculations of the specific radioactivity of free choline were made. The choline- and ethanolamine-containing components of the radioactive peaks, other than free choline, were separated by paper chromatography (Ansell & Spanner, 1967).

Extraction and determination of phospholipids. The brains were removed and weighed, and the lipids were extracted and washed by the method of Folch, Lees & Sloane-Stanley (1957). The choline-containing phospholipids were isolated by chromatography on a column of alumina (Davison & Wajda, 1959). These choline lipids were separated into an alkali-labile fraction (phosphatidylcholine), an alkali-stable acid-labile fraction (choline plasmalogen) and an alkali- and acid-stable fraction (sphingomyelin) by the hydrolytic procedures of Dawson, Hemington & Davenport (1962). In this instance the more prolonged saponification necessary to remove the fatty acid from the 2-position of the ethanolamine plasmalogen (Ansell & Spanner, 1963) was found unnecessary. For experiments in which the determination of choline plasmalogen was not required, the trichloroacetic acid-insoluble residue (see above) was washed twice with water and the lipids were extracted and washed by the method of Folch *et al.* (1957).

The fractions were assayed for radioactivity and phosphorus. Phosphorus determinations were carried out by the method of Ernster, Zetterström & Lindberg (1950), modified where necessary to increase the sensitivity by using half volumes of the reagents.

Assay of radioactivity. The ^{14}C-labelled samples were counted in a Packard liquid-scintillation counter and the ^{32}P-labelled samples in a M6 liquid-counting tube (20th Century Electronics) with standard ancillary equipment. The scintillator used was described by Ansell & Spanner (1967). The counts of the ^{14}C-labelled samples were corrected to 100% efficiency by the channels-ratio method.

RESULTS

Uptake of [Me-^{14}C]choline by whole brain and the distribution in the acid-soluble components. When [Me-^{14}C]choline was injected into the brain of rats in the region of the lateral ventricle, there was a rapid dispersal of the choline. The same amount of radioactivity was found in each hemisphere when measured 30 min. after the injection. There was, however, a rapid loss of total radioactivity from the brain. After only 30 min., 60% of the radioactivity injected as choline had disappeared (Fig. 1). In this, labelled choline differed from [1,2-^{14}C$_2$]-ethanolamine (Ansell & Spanner, 1967), 70% of whose radioactivity could be recovered from the brain as long as 10 hr. after the injection.

There was a rapid transfer of labelled choline from the acid-soluble pool to the lipids (Fig. 1) so that, within 6 hr., 85% of the radioactivity in the brain was in a lipid-bound form. The concentrations of the water-soluble components, choline, phosphorylcholine and CDP-choline, in brain were measured (Table 1) and their respective radioactivities after a number of exchange periods were determined. Fig. 2 shows there that was a rapid
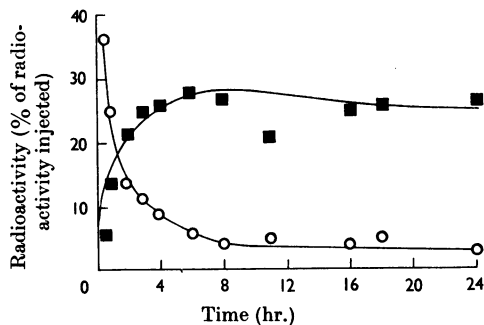

Fig. 1. Percentages of radioactivity injected as [Me-^{14}C]-choline recovered in the brain at various times after injection. ○, Acid-soluble fraction; ■, lipid fraction. Each point represents the mean obtained from two or three animals.
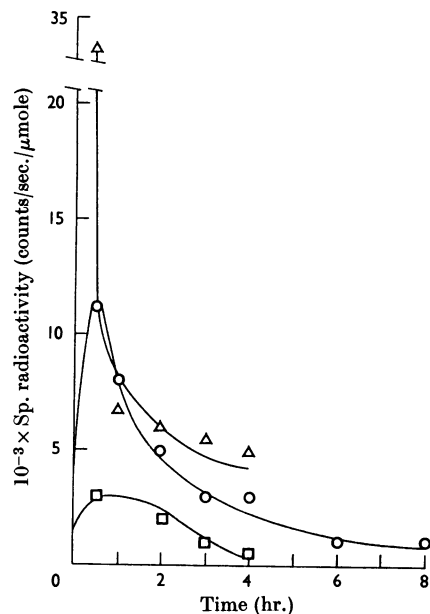

Fig. 2. Specific radioactivities of acid-soluble choline components in the brain at various times after the injection of [Me-^{14}C]choline. △, Choline; ○, phosphorylcholine; □, CDP-choline.

phosphorylation of free choline at a minimum rate of 0·076 μmole/g. of brain/hr. The specific radioactivity–time curve for choline crosses that of phosphorylcholine at a point where the specific radioactivity of phosphorylcholine was maximal, which suggests a direct precursor–product relationship (Zilversmit, Entenman & Fishler, 1943). The CDP-choline was also labelled, but incorporation

Table 1. *Concentration of choline lipids and their water-soluble precursors in rat brain*

Values are given as means ± S.D., with the numbers of determinations in parentheses.

	Concn. (μmoles/g. of brain fresh wt.)	
	Present work	Values in the literature
Choline	0·17 ± 0·01 (5)	0·20*
Phosphorylcholine	0·38 ± 0·05 (10)	0·442†; 0·390‡
CDP-choline	0·05 ± 0·006 (7)	0·03§ (4-week-old animals)
Phosphatidylcholine	14·70 ± 1·50 (39)	—
Choline plasmalogen	0·62 ± 0·08 (9)	0·47¶; 0·22–0·35‖
Sphingomyelin	3·65 ± 0·24 (6)	3·63–3·70‖

* Dr C. Hebb, personal communication (determined by conversion into acetylcholine followed by bioassay).
† Dawson (1955).
‡ Porcellati (1958).
§ Ansell & Bayliss (1961).
‖ Wells & Dittmer (1967).
¶ Webster (1960).

into it was curiously low compared with the labelling of CDP-ethanolamine after the injection of [1,2-$^{14}C_2$]ethanolamine (Ansell & Spanner, 1967).

Incorporation of [Me-^{14}C]choline into the phospholipids of brain. It was shown in Fig. 1 that the lipids of the brain were rapidly labelled after the injection of [Me-^{14}C]choline. The choline-containing fraction eluted from the alumina column contained 100% of the counts recovered in the total lipid. This choline-containing lipid was then hydrolysed in 6N-hydrochloric acid for 3 hr. at 100° under reflux. Fractionation of the water-soluble products on Dowex 1 (X8; OH⁻ form) showed that all the radioactivity in the choline lipids was in the base. Thus in a typical experiment, with a 3 hr. exchange period, the radioactivity (counts/sec./brain) was: 8180 for total lipid, 8000 for the choline lipid and 7825 in the lipid-bound choline. When a sample of the total lipid extract was separated by thin-layer chromatography on silica gel G in chloroform–methanol–water (65:35:4, by vol.) and the radioactive lipids were located by radioautography, only phosphatidylcholine + choline plasmalogen, lysophosphatidylcholine and sphingomyelin were labelled.

Of the three major choline-containing lipids in brain (Table 1), by far the greatest radioactivity at all the times examined was in the phosphatidylcholine (Fig. 3). There was considerable labelling of the choline plasmalogen, but the sphingomyelin showed only a small uptake of the labelled choline (Fig. 3).

Uptake of phosphoryl[Me-^{14}C]choline into brain water-soluble and lipid fractions. When phosphoryl-[Me-^{14}C]choline was injected into the brain there was, as with choline, a rapid loss of the label from the brain. There was a very similar pattern of
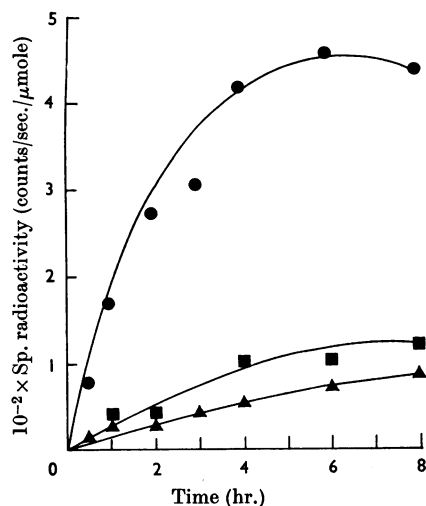

Fig. 3. Specific radioactivities of the choline-containing phospholipids in the brain at various times after the injection of [Me-^{14}C]choline. ●, Phosphatidylcholine; ■, choline plasmalogen; ▲, sphingomyelin.

transfer of water-soluble radioactivity to lipid components, though at a somewhat lower rate (Fig. 4). This may have been due, partly, to the initial breakdown of the phosphorylcholine on injection that is shown in Fig. 5. After this initial hydrolysis of injected phosphorylcholine, there was a rapid equilibration between the specific radioactivity of the choline and the phosphorylcholine (Fig. 5), as was shown after the injection of [Me-^{14}C]-choline (Fig. 2).

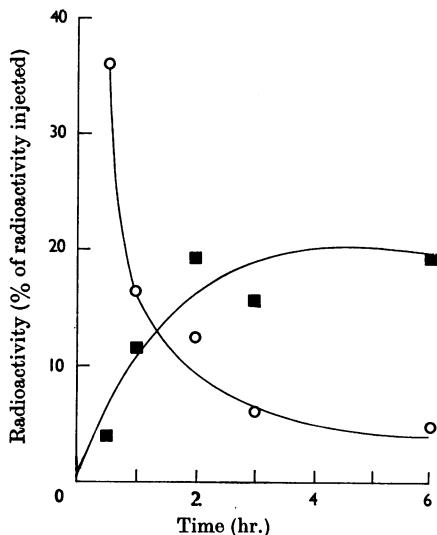*Comparison of the specific radioactivities of phosphorylcholine and of phosphatidylcholine after the*

Fig. 4. Percentages of radioactivity injected as phosphoryl[Me-^{14}C]choline recovered in the brain. ○, Acid-soluble choline; ■, lipid-bound choline.

Fig. 6. Specific radioactivities of the brain CDP-choline (□) and phosphatidylcholine (●) at various times after the injection of [Me-^{14}C]choline.
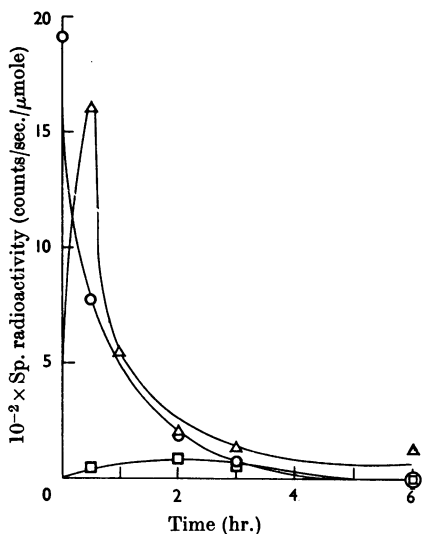

Fig. 5. Specific radioactivities of acid-soluble choline components in the brain at various times after the injection of phosphoryl[Me-^{14}C]choline. △, Choline; ○, phosphorylcholine; □, CDP-choline.

injection of labelled choline, phosphorylcholine and orthophosphate. It was found that when [^{32}P]-orthophosphate was injected into the brain of the rat the rate 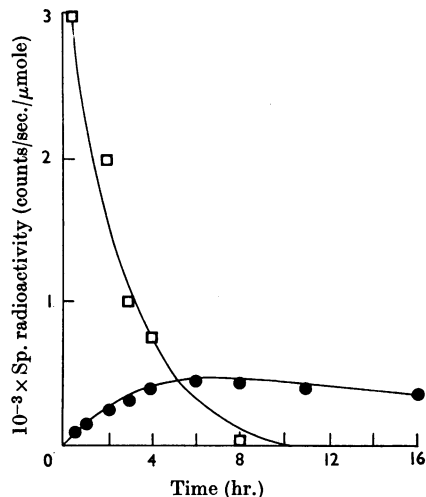of transfer of phosphorylcholine to phosphatidylcholine, as measured by the ratio of the two specific radioactivities, was very similar to that found after the injection of [Me-^{14}C]choline (Table 2). Further, it was found that, for the few experiments where phosphoryl[Me-^{14}C]choline was injected, the ratio of the specific radioactivity of phosphatidylcholine to that of phosphorylcholine was also similar to that obtained with [^{32}P]ortho-phosphate and [Me-^{14}C]choline (Table 2). Such a close correlation would suggest an obligatory phosphorylation of the choline before its incorporation into phosphatidylcholine.

DISCUSSION

When [Me-^{14}C]choline or phosphoryl[Me-^{14}C]-choline was injected into brain by the intracerebral route, there was initially a very rapid loss of the radioactivity. At 0·5 hr. after injection, only 40% of the injected dose was recovered in the brain (Figs. 1 and 4). This amount was, however, maintained and after 24 hr. over 30% of the injected label was still recovered (Fig. 1). This result was very different from that obtained with [^{14}C]ethanol-amine (Ansell & Spanner, 1967), when after an exchange period of 1 hr. 88% of the injected dose was recovered but after 24 hr. this value had dropped to 47% of that injected. This rapid initial loss of choline and phosphorylcholine is difficult to explain. Overloading the brain pool was not apparently the reason for the rapid loss. If the amount of choline injected was 0·017 μmole instead of 0·03 μmole (5·6% of the brain pool instead of 10%), the

Table 2. *Ratio of the specific radioactivity (counts/sec./μmole) of phosphatidylcholine to that of phosphoryl-choline of brain after the injection of [Me-^{14}C]choline, phosphoryl[Me-^{14}C]choline or [^{32}P]orthophosphate*

Numbers of determinations made are given in parentheses.

Time after injection (hr.)	Substance injected ...	Phosphatidylcholine/phosphorylcholine sp. radioactivity ratio		
		[Me-^{14}C]Choline	Phosphoryl[Me-^{14}C]choline	[^{32}P]Orthophosphate
1		0·013 (3)	0·011	0·010
2		0·034	0·040	0·032
3		0·080 (2)	0·075	0·071
4		0·170 (2)	—	0·097
6		0·239	—	0·185
8		0·307	—	0·203

percentage of radioactivity recovered was identical. Results from experiments with both amounts are included in Fig. 1.

The rapid appearance of labelled phosphoryl-choline after the injection of labelled choline indicated a rapid phosphorylation of this base, presumably by the enzyme choline kinase (EC 2.7.1.32). The rate of this phosphorylation (0·076 μmole/g. of brain/hr.) was low compared with the rate (2 μmoles/g. of grey matter/hr.) found *in vitro* at neutral pH by McCaman (1962). The rate found *in vivo* for the phosphorylation of choline was about 50% of that found for the phosphorylation of ethanolamine (Ansell & Spanner, 1967). On the other hand, the concentration of free choline in the brain (0·17 μmole/g. of brain) was only about one-tenth of that of ethanolamine (1·35 μmoles/g. of brain).

From the relationship between the specific radioactivity–time curves in Fig. 2, it appears that choline is the precursor of phosphorylcholine and phosphorylcholine the precursor of CDP-choline. However, if the criteria of Zilversmit *et al.* (1943) are applied, the curve for the precursor would have to be initially higher than that of the product, cross the latter at its peak and then fall below it. These criteria are not entirely fulfilled for choline–phosphorylcholine or phosphorylcholine–CDP-choline. It is, of course, unusual for the ideal criteria of Zilversmit *et al.* (1943) to be fulfilled in a biological system (Reiner, 1953), though they appear to be for the relationship between CDP-choline and phosphatidylcholine in Fig. 6. It is possible that there are intermediate steps, for example an active transport of phosphorylcholine to the site of CDP-choline synthesis, that might account for the discrepancies in Fig. 2. Nevertheless the results indicate strongly that the major pathway for the incorporation of choline into phosphatidylcholine in brain is the cytidine pathway. Further evidence is given in Table 2. This pathway has been demonstrated for brain tissue

in vitro on a number of occasions (see, e.g., Rossiter, 1964).

A comparison between Fig. 2 and Fig. 5 indicates further the closeness of the relationship between choline and phosphorylcholine in brain. Just as there was a rapid formation of labelled phosphoryl-choline from labelled choline, the reverse was also true. Within 2 hr. an equilibrium was established between the two compounds with the specific radioactivity of the choline slightly higher than that of the phosphorylcholine in both instances.

The specific radioactivity–time curves for CDP-choline and phosphatidylcholine in brain (Fig. 6) indicate a relationship between these two compounds that is different from that found in liver by Bjørnstad & Bremer (1966). They showed that, when [1,2-^{14}C$_2$]choline was injected intraperi-toneally, the phosphorylcholine, CDP-choline and phosphatidylcholine of liver were all labelled but that the specific radioactivity of the CDP-choline was very low compared with that of the phosphorylcholine up to 80 min. It was more comparable with that of phosphatidylcholine, suggesting that in this instance the specific radio-activity of the CDP-choline was kept low by equilibrium with preformed phosphatidylcholine. This was confirmed by experiments with [Me-^{3}H]-methionine, which, when injected intraperitoneally, yielded labelled phosphatidylcholine of much greater specific radioactivity than phosphoryl-choline but of similar specific radioactivity to CDP-choline. In the results presented in Fig. 6 no such equilibration of CDP-choline with phos-phatidylcholine in brain could be demonstrated.

The results suggest that the cytidine pathway is very important in the synthesis of phosphatidyl-choline in the brain, and the question of the source of the choline therefore arises. Ansell & Spanner (1967) observed very little conversion of radioactive ethanolamine into choline in the brain of the rat, in the free state, or as phosphorylethanolamine or as lipid-bound ethanolamine. Bremer & Greenberg

(1961) could not demonstrate significant methylation of the endogenous ethanolamine phospholipids present in brain microsomes by L-S-adenosyl-[Me-^{14}C]methionine. Marshall et al. (1965) also failed to demonstrate the methylation of [^{32}P]-phosphatidyldimethylaminoethanol or [^{32}P]phosphatidylmonomethylaminoethanol by S-adenosyl-[Me-^{14}C]methionine in brain dispersions. On injection of [Me-^{3}H]methionine into rats, the labelling of the phosphatidylcholine in extrahepatic tissues was slow compared with the labelling in the liver (Bjørnstad & Bremer, 1966); similar observations were made on lung tissue by Spitzer, Morrison & Norman (1968). The labelling in brain was not investigated.

Such observations suggest that choline is supplied to the brain from the liver. There is no doubt that injected labelled choline is incorporated into lipids of all organs. Groth et al. (1958) showed that [Me-^{14}C]choline and [1,2-^{14}C$_2$]choline were taken up into brain phosphatidylcholine, though the methyl-labelled choline was a more effective precursor than the hydroxyethyl-labelled choline. Bjørnstad & Bremer (1966) showed, moreover, that the uptake of labelled choline into extrahepatic tissues was decreased when the amount of free circulating unlabelled choline was artificially raised. However, the labelling of the lipids in extrahepatic tissues after the injection of [Me-^{3}H]methionine was not decreased by choline injection. From these experiments Bjørnstad & Bremer (1966) concluded that, if choline was being made in the liver by the methylation pathway and transported to other tissues for utilization via the cytidine pathway, it was not transported as free choline.

The route by which choline enters the choline plasmalogen of brain is less clear. At least two routes are possible and there may well be more. The cytidine pathway with a plasmalogenic diglyceride (2-acyl-1-alk-1′-enylglycerol) as substrate was demonstrated in vitro by McMurray & Rossiter (1962) and by E. F. Marshall (unpublished work). One difficulty in accepting this route is that no plasmalogenic diglyceride has, as yet, been detected in brain. The formation of the plasmalogen from the diacyl phospholipid was also postulated for the ethanolamine analogue by Bickerstaffe & Mead (1967). The results described in this paper and shown in Fig. 3 would indicate that the choline plasmalogen could be formed from the diacyl phospholipid, but experiments of a different type would be necessary to prove it.

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