Choline Metabolism in the Cerebral Cortex of Guinea Pigs

PHOSPHORYLCHOLINE AND LIPID CHOLINE

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1. The labelling of phosphorylcholine and choline-containing phospholipids in the subcellular fractions of guinea-pig cerebral cortex after the intraventricular injection of [*N*-Me-³H]choline into conscious animals has been studied. Special emphasis was placed upon the synaptosome fraction and early time-periods after administration. 2. The labelling of phosphorylcholine was rapid compared with that of phospholipid and was confined to two distinct subcellular fractions: the soluble cytoplasmic fraction and the synaptosome fraction. Most of the labelled phosphorylcholine of the synaptosome fraction was readily released by osmotic rupture indicating location in the nerve-ending cytoplasm. The two pools of phosphorylcholine had similar specific radioactivities at all observed times. 3. ³H-labelled phospholipid was found in all membranous fractions. The labelling was confined to choline-containing phospholipids, notably phosphatidylcholine. 4. The labelling of the different membranous fractions was similar. 5. The half-life of the choline-containing phospholipids in the synaptic vesicle fraction was very much greater than the acetylcholine in this fraction. 6. Evidence is presented that synthesis *de novo* of phosphatidylcholine at nerve terminals occurs *in vivo*.

The organic base choline is a key component of nervous tissue. It is found in a covalently bound form in the neurotransmitter, acetylcholine, and in certain phospholipids that are major constituents of all neural membranes. Further, free choline is an obligatory precursor of these components. In spite of these facts there has been a paucity of knowledge concerning its metabolism in brain. Various facets of this problem, however, have quite recently received some attention.

Even though the brain does not apparently have the capacity to synthesize choline *de novo* (see Ansell & Spanner, 1970), its uptake, following administration, is exceptionally avid both *in vivo* (Ansell & Spanner, 1968; Schuberth *et al.*, 1969, 1970) and *in vitro* (Schuberth *et al.*, 1966). The limited information available on the immediate fate of the choline which is taken up *in vivo* indicates a cellular cytoplasmic location with significantly high amounts in the synaptosome fractions (Schuberth *et al.*, 1969; Gomez *et al.*, 1970). This rapid uptake of choline also occurs *in vitro* with isolated synapto-

somes (Marchbanks, 1968; Diamond & Kennedy, 1969).

The majority of reports on the metabolism of labelled choline administered to brain tissue have been limited either to a study of its incorporation into acetylcholine or into the phospholipids and to a lesser extent the intermediates (phosphorylcholine and CDP-choline) in the lipid pathway. Thus Ansell & Spanner (1968) demonstrated the incorporation of [*Me*-¹⁴C]choline into choline-containing phospholipids and their precursors in whole rat brain. The rapid labelling of phosphatidylcholine after administration of [*Me*-¹⁴C]choline into rat brain has been demonstrated for microsomal material and synaptosomes (Abdel-Latif *et al.*, 1970). In a more comprehensive study phosphatidylcholine was labelled from labelled choline and the half-lives of the lipid in various membrane fractions of rat brain were measured (Lapetina *et al.*, 1970). The incorporation of choline into phosphatidylcholine also occurs *in vitro* both in slices and in isolated synaptosomes (Lunt & Lapetina, 1970*a,b*).

The labelling of acetylcholine from labelled choline was demonstrated *in vivo* in cat cerebral cortex (Chakrin & Shideman, 1968; Chakrin *et al.*, 1968) and in mouse brain (Schuberth *et al.*, 1969) and is confined essentially to the synaptosome fraction in both species (Chakrin & Whittaker, 1969; Schuberth

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et al., 1970). The ability of isolated synaptosomes to incorporate labelled choline into acetylcholine has also been demonstrated (Marchbanks, 1968; Diamond & Kennedy, 1969).

From these studies it seems that two principal metabolic routes for choline in brain are the formation of acetylcholine and choline-containing phospholipids. The question therefore arises as to the spatial and temporal relationships between these divergent metabolic routes in neurones *in situ*. However, simultaneous investigation of the translocation of choline through both of these pathways has received little attention. Even where this has been studied the results and their interpretation are somewhat limited (Diamond & Kennedy, 1969; Gomez *et al.*, 1970).

In a study, *in vivo*, on the subcellular distribution of [*N*-Me-³H]acetylcholine synthesized from intracerebrally injected [*N*-Me-³H]choline into guinea-pig brain, an unknown ³H-labelled component was detected in the acid extracts of the synaptosomal fraction and subfractions. This component was tentatively identified as 'lipid choline' and constituted a considerable proportion of the total acid-soluble radioactivity 1 h after the administration of [³H]choline (Chakrin & Whittaker, 1969). Preliminary investigations into the nature of this unknown component showed that it was almost entirely [³H]phosphorylcholine together with traces of glycerylphosphorylcholine. However, [³H]lipid choline, mostly in the form of phosphatidylcholine, was also found in the acid-insoluble portion of various synaptosome fractions (M. J. Dowdall & L. A. Barker, unpublished work).

Since phosphorylcholine is an intermediate in the Kennedy pathway (Kennedy & Weis, 1956) for the biosynthesis of phosphatidylcholine in brain, as in other tissues, these findings suggested that some local biosynthesis of this important phosphoglyceride might be occurring at the terminal region. This is of particular interest with respect to the metabolic independence of the presynaptic terminal region since in the case of proteins the terminal is somewhat dependent on synthesis in the perikaryon followed by transport down the axon by axoplasmic flow (for review of axoplasmic transport, see Barondes, 1969).

The relatively rapid appearance of ³H-labelled choline-containing phospholipid after intracerebral injection of [³H]choline also suggested the possibility of a simultaneous study on the turnover rates of acetylcholine and membrane phospholipid in the terminals and their sub-structures in the same experiments. It was felt that such a simultaneous study would eliminate many of the variables which often complicate the interpretation of independent researches (e.g. administration of the precursor and subcellular fractionation techniques).

By using this approach it was hoped that more insight might be gained into the metabolism of choline

at the nerve terminal and in particular might shed some light on the mechanisms for the storage and release of acetylcholine.

Methods

Surgical preparation of the animals, administration of [*N*-Me-³H]choline ([³H]choline), subcellular fractionation of the brain cortices and the measurement of protein and marker components were done as described in the accompanying paper (Barker *et al.*, 1972).

Analytical methods

Isolation and determination of phosphorylcholine. Phosphorylcholine was extracted from subcellular fractions by the addition of ice-cold 40% (v/v) trichloroacetic acid (1 vol.: 3 vol. of fraction). Insoluble material was allowed to precipitate for at least 15 min at 4°C and was subsequently separated from the extract by centrifugation at 3000g for 20 min in an MSE Mistral 6L refrigerated centrifuge. The supernatant was transferred to an extraction tube and the insoluble material was washed by resuspension in a small vol. of ice-cold water (usually 1–2 ml) followed by recentrifugation. The washings and initial supernatant were combined and the precipitate was retained for lipid analysis.

The acid extracts were freed from trichloroacetic acid by successive extractions with diethyl ether (three equal vols.). After each extraction the ether phase was removed by aspiration. The final aqueous phase was then concentrated and freed of diethyl ether by evaporation *in vacuo* at a temperature not exceeding 50°C. The concentrated syrup containing acid-soluble components and contaminating sucrose was adjusted to 3–4 ml with water before ion-exchange treatment.

Phosphorylcholine was separated from sucrose by passage down a small column of Dowex 50 (H⁺ form, 4 cm × 1 cm). After sample application the column was eluted with water. The initial 15 ml of eluent from the column contained the sucrose and was discarded. A further fraction (45 ml) containing the phosphorylcholine was collected and evaporated to dryness *in vacuo*; the resultant residue was redissolved in 5 ml of methanol–water (19:1, v/v) and portions of this extract were used for the determination of radioactivity (0.5 ml) and t.l.c. (3.5 ml). T.l.c. on cellulose layers (Brinkmann-microcrystalline) was used to isolate the phosphorylcholine from the other phosphate-containing species in the fraction. The 3.5 ml portion of the extract was concentrated to facilitate sample application to the cellulose layer as a thin band 1.5 cm long. Separation was achieved by development in phenol–water–0.18 M-NH₃ (80:20:0.3, w/v/v). The developed plate was left to dry in a well-ventilated fume cupboard overnight, then the phosphorylcholine was made visible by spraying the plate with

acid molybdate (Hanes & Isherwood, 1949). The area corresponding to phosphorylcholine (as determined by the position of authentic phosphorylcholine developed on the same plate) had an R_F value close to 0.9. This area was scraped off and used for the determination of phosphate.

Isolation and determination of phospholipids. Lipids were extracted from the material insoluble in trichloroacetic acid essentially by the method of Folch *et al.* (1957). The insoluble pellet of material remaining after extraction of the subcellular fractions with trichloroacetic acid was resuspended in 5 ml of chloroform-methanol (2:1, v/v) and left to extract overnight. The insoluble material was collected by centrifugation and washed with small vols. of chloroform-methanol. Where necessary the combined extracts were adjusted to 10 ml by the addition of chloroform-methanol and then shaken with 2 ml of 0.9% NaCl solution. After phase separation the upper phase was removed and discarded and the lower phase washed once by shaking with 4 ml of fresh upper phase (made by using 0.9% NaCl). The washed lower phase was evaporated to dryness *in vacuo* and redissolved in 5 ml of chloroform. Any chloroform-insoluble material was removed by passage of the extract through a Pasteur pipette plugged with glass wool. Samples of the extract were then taken for radioactivity measurements (0.5 ml), the determination of phospholipid phosphate (1.0 ml) and t.l.c. (2 ml).

Separation of the phospholipids in the lipid extract was achieved by t.l.c. on 'basic' silica-gel plates (Skipiski *et al.*, 1964). A portion of the washed lipid extract was dried down under air and redissolved in a small vol. of chloroform. Each sample was loaded in a band (1–3 cm) by using a Hamilton syringe. Authentic phospholipid standards and mixtures of these were developed with each plate. After development the plates were then dried and developed by using I_2 vapour. The bands were carefully outlined by lightly scoring the surface of the layer with a fine needle. Iodine was allowed to evaporate from the layer by gentle warming of the plate.

The areas of the plate required for analysis were then scraped off and transferred to a Pasteur pipette plugged with glass wool. The silica gel was eluted with methanol (2 × 2 ml) and the collected eluent adjusted to 5 ml by the addition of more methanol. Portions of the eluted samples were used for the determination of phosphate and radioactivity.

General methods

Determination of radioactivity. Radioactivity was measured by liquid-scintillation counting in a Packard Tri-Carb 3380 fitted with an Absolute Activity Analyser. All samples were counted in 10 ml of scintillation medium [toluene-Triton X-100, 7:3 v/v,

containing 2,5-diphenyloxazole (5.4 g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.108 g/l)]. Lipid samples were dried down in the scintillation vial before addition of scintillation medium. In the case of total radioactivity measurements of subcellular fractions, the sample (0.1–0.2 ml) was usually solubilized in 1 ml of 'Soluene' (Packard) before addition of scintillation medium. All samples were counted at least twice and where possible to 10^4 counts on each occasion.

Determination of phosphate. Areas of cellulose chromatograms and dried lipid samples were ashed and estimated for phosphate by the method of Berenblum & Chain (1938) with minor modifications (Lyon *et al.*, 1965).

Cholesterol. This was determined by reaction with ferric chloride by using a micromodification of the method of Crawford (1958). Dried samples of total lipid extract containing 2–10 μ g of cholesterol were assayed together with cholesterol standards. After colour development the E_{540} of the reaction mixture was determined by using micro-cuvettes (capacity 0.5 ml). The assay was linear in the 0–10 μ g range with an $E_{540}^{1\text{cm}}$ of 0.6 for 10 μ g of cholesterol.

Statistical presentation of results. Unless otherwise stated values are means $\pm$ S.D. (three or more experiments) or range (two experiments) followed by the number of experiments in parentheses.

Results

Uptake of [^3H]choline

The amounts of ^3H retained by the cortex after intraventricular injection of [^3H]choline are shown in Fig. 1. Initial experiments ($\circ$) were done with an injection depth of 3 mm. However, subsequent experiments ($\bullet$) indicated that more radioactivity was retained by the cortex when the injection depth was 4 mm and this was therefore adopted as standard procedure. After an initial rapid uptake of [^3H]choline the values remained fairly constant for the first hour and a high proportion of this radioactivity was retained for much longer time-periods. The maximum uptake represented about 9% of the injected dose or 6–9 nmol/g of cortex, assuming that no dilution of the labelled choline occurred before uptake. In one experiment at 1 h after injection of [^3H]choline the combined value for radioactivity recovered from the caudate nucleus, hippocampus and midbrain was only 60% of that present in the cortex. Thus only a small portion of the injected choline was retained by the brain as a whole.

Fig. 1 also shows the proportions of radioactivity recovered from the cellular cytoplasmic fraction (S_2) compared with both the crude mitochondrial fraction (P_2) and the purified synaptosome fraction (B) at various times after injection; the bulk of the radioactivity was recovered from fraction S_2 at the time-

Fig. 1. Total radioactivity recovered from homogenates and subcellular fractions of guinea-pig brain after injection of $[^3\text{H}]$ choline

(a) Total radioactivity recovered from homogenate at different times after intraventricular injection of $[^3\text{H}]$ choline. (b), Total radioactivity of fractions S_2 , P_2 and B at different times after injection as a percentage of homogenate value. Single or mean values are plotted cumulatively. Depth of injection: $\circ$, 3 mm; $\bullet$, 4 mm. Bars are the range (two experiments) or s.d. (three or more experiments) of values for fractions S_2 , P_2 and B ; the numbers above or below the bars refer to the number of experiments. The 90 min value for fraction P_2 was obtained by interpolation. Note the change of scale in the abscissae after 110 min and in the ordinate of the lower diagram above 55%. For description of controls (i) and (ii) see the text. The various sections of blocks (i) and (ii) are related to the corresponding time-course studies by broken lines.

periods studied. However, significantly high amounts of ^3H were recovered from fraction P_2 and these could be attributed mainly to the presence of synaptosomes, particularly for time-periods up to 20 min. During the first hour after injection the proportion of total ^3H recovered in the synaptosome fraction did not vary appreciably, indicating that the uptake by these particles was extremely rapid and apparently complete after a few minutes. To check the potential contribution of uptake between the time of death and the time of isolation of each fraction two control experiments were undertaken. In one experiment (ii, Fig. 1b) the injection was made into the brain immediately after death and in the other, (i), $[^3\text{H}]$ choline was added to an homogenate prepared from the cortices of untreated animals. The results showed that the

observed uptake was predominantly occurring *in situ* and confirmed that it was extremely rapid, since even when a severed head was injected the net uptake of radioactivity was almost 50% of that observed in conscious animals killed immediately (0–2 min) after injection.

Choline metabolism at nerve terminals

The metabolic fate of choline at nerve terminals is depicted in Fig. 2. After injection the $[^3\text{H}]$ choline taken up is rapidly converted into acetylcholine, phosphorylcholine and lipid choline. The proportion of $[^3\text{H}]$ choline entering the acetylcholine and phosphorylcholine pools was about equal. Comparison of the relative rates of entry of $[^3\text{H}]$ choline

Fig. 2. Metabolic fate of choline in nerve terminals in vivo

Synaptosomes (fraction *B*) were isolated at various times after injection of [^3H]choline. Radioactive phosphorylcholine (Δ) and lipid choline ($\blacktriangle$) were measured as described in the Methods section; radioactive choline ($\circ$) and acetylcholine ($\bullet$) were isolated and measured as described by Barker *et al.* (1972). Bars indicate standard deviations or ranges of mean values represented by points. Values in parentheses represent the number of experiments for that particular time-period. For description of controls (*i* and *ii*) see the text.

into and exit from the pools of acetylcholine and phosphorylcholine indicate a faster turnover for the former. After an initial lag period of about 10 min there was considerable labelling of lipid choline which increased markedly with time. After 90 min over 70% of the recovered radioactivity was present in this form.

A detailed account of the turnover of acetylcholine in various compartments of the synaptosomal fraction is given in the accompanying paper (Barker *et al.*, 1972).

Significance of synaptosomal [^3H]phosphorylcholine

Since the formation of phosphorylcholine represents the initial step in the biosynthesis of choline-containing phospholipids via the Kennedy pathway it was of interest to compare the labelling of phosphorylcholine in the synaptosome fraction with the labelling at other subcellular sites. Preliminary experiments on the subcellular distribution of endogenous phosphorylcholine indicated that detectable amounts could be recovered from only two primary subcellular sites, the cellular cytoplasm (S_2 or S_3) and the synaptosomes (P_2 or B). This indicated that the phosphorylcholine in the synaptosomes was present because of the occluded cytoplasm present in this

fraction. More direct evidence for the cytoplasmic location of the synaptosomal phosphorylcholine is that phosphorylcholine could only be detected in fractions *O* (synaptosomal cytoplasm) and *H* (partially disrupted synaptosomes) derived from osmotically lysed synaptosomes. The amounts of phosphorylcholine recovered in the various subcellular fractions are given in Table 1. Also included in Table 1 for comparison are the amounts of cytoplasmic markers, lactate dehydrogenase and K^+ . Although the recovered phosphorylcholine was distributed equally between fractions *O* and *H* this was not true for the cytoplasmic markers, which were recovered mainly in fraction *O*. Thus the 'apparent' bimodal distribution of phosphorylcholine between fractions *O* and *H* was not primarily due to inefficient osmotic lysis of the synaptosomes in fraction P_2 .

The results indicate that the cellular cytoplasmic pool of phosphorylcholine was twice that of the synaptosomes. The combined amount of phosphorylcholine in these two primary fractions was $0.42 \mu\text{mol/g}$ (mean results), which agrees reasonably well with the values of Pumphrey (1969) for whole slices of guinea-pig cortex ($0.4 \mu\text{mol/g}$) and of Ansell & Spanner (1968) for whole rat brain ($0.38 \mu\text{mol/g}$).

After osmotic lysis of the crude synaptosome fraction phosphorylcholine was recovered equally from fractions *O* and *H*. The combined recovery was,

Table 1. *Amounts of phosphorylcholine and cytoplasmic markers recovered from subcellular fractions of guinea-pig brain cortex*

Standard phosphorylcholine (0.01–1 μ mol) in sucrose solutions, taken through the analytical procedure, gave recoveries greater than 90%. The amounts of phosphorylcholine recovered from the tissue fractions at different time-intervals after injection showed no significant differences and therefore all results have been pooled for the calculation of mean values. Both lactate dehydrogenase activity and K^+ amounts in fraction *H* were somewhat greater than reported previously for this fraction (3.1 and 0% of P_2 values respectively; Whittaker *et al.*, 1964). When expressed as a percentage of P_2 values the *H* values in the present experiments were 17.6 ± 9.1 (7)% and 4.8 ± 1.6 (7)% for lactate dehydrogenase and K^+ respectively. This difference was due to the preparation of *H* from fraction P_2W rather than W_s . Thus when *H* was prepared from both W_s and W_p derived from the same P_2W fraction then the amounts of lactate dehydrogenase and K^+ in the fraction derived from W_p were four times that in the fraction from W_s .

Subcellular fractions and components	Phosphorylcholine (nmol/g)	K^+ (μ g-atom/g)	Lactate dehydrogenase (E_{340} /min per g)
<i>S</i> ₂ Cellular cytoplasm, microsomes	268 $\pm$ 92 (22)	63.4 $\pm$ 9.2 (5)	66.6 $\pm$ 13.8 (5)
<i>B</i> Synaptosomes	155 $\pm$ 70 (13)	5.3 $\pm$ 1.1 (5)	12.1 $\pm$ 3.6 (5)
<i>O</i> Synaptosomal cytoplasm	33 $\pm$ 10 (23)	5.8 $\pm$ 1.8 (4)	7.2 $\pm$ 0.9 (4)
<i>H</i> Disrupted synaptosomes	28 $\pm$ 12 (21)	0.6 $\pm$ 0.2 (7)	2.9 $\pm$ 1.5 (7)

Table 2. *Amounts of [³H]phosphorylcholine recovered from subcellular fractions of guinea-pig brain cortex at various times after injection of [³H]choline*

Values are $10^{-3} \times$ d.p.m./g.

Subcellular fraction	Time after injection of [³ H]choline (min)					
	0	10	20	40	60	90
<i>S</i> ₂	33.7 $\pm$ 22.0 (3)	168.0 $\pm$ 46.9 (3)	303.0 $\pm$ 169.8 (8)	313.0 $\pm$ 75.6 (5)	318.6 $\pm$ 125.5 (5)	341.0
<i>A</i>	0.3 $\pm$ 0.2 (2)	2.9 $\pm$ 0.9 (2)	5.7 $\pm$ 4.3 (2)	11.4	10.9	5.6
<i>B</i>	7.2 $\pm$ 6.4 (3)	23.4 $\pm$ 10.2 (3)	36.0 $\pm$ 13.4 (5)	35.3 $\pm$ 14.1 (3)	42.7 $\pm$ 14.8 (3)	44.9
<i>C</i>	0.2 $\pm$ 0.0 (2)	5.5 $\pm$ 2.2 (2)	6.9 $\pm$ 0.6 (2)	8.4	17.4	11.1
<i>O</i>	3.4 $\pm$ 2.4 (3)	13.6 $\pm$ 5.0 (3)	32.5 $\pm$ 15.4 (8)	27.0 $\pm$ 4.9 (5)	30.4 $\pm$ 8.5 (5)	58.2
<i>D</i>	—	—	—	14.9	8.2	6.1
<i>E</i>	0.1 $\pm$ 0.2 (3)	1.1 $\pm$ 0.7 (3)	1.9 $\pm$ 2.1 (3)	3.6	3.7	4.1
<i>F</i>	0.1 $\pm$ 0.0 (3)	1.2 $\pm$ 1.1 (3)	2.3 $\pm$ 2.2 (3)	6.9	3.2	4.4
<i>G</i>	0.5 $\pm$ 0.5 (3)	2.2 $\pm$ 0.1 (2)	2.1 $\pm$ 0.2 (2)	8.1	6.2	8.0
<i>H</i>	0.6 $\pm$ 0.3 (3)	4.9 $\pm$ 2.0 (3)	7.6 $\pm$ 4.7 (8)	9.7 $\pm$ 4.3 (6)	6.3 $\pm$ 3.7 (6)	8.1
<i>I</i>	0.2 $\pm$ 0.1 (3)	1.2 $\pm$ 1.6 (3)	2.7 $\pm$ 2.6 (3)	5.8	2.5	2.7

however, only 40% of the amounts in the synaptosome fraction, indicating that considerable degradation of phosphorylcholine may have occurred during the density-gradient centrifugation.

The amounts of [³H]phosphorylcholine recovered from subcellular fractions at various times after injection of [³H]choline are given in Table 2. The much greater sensitivity of this radiochemical measurement as compared with direct chemical measurement enabled determinations to be made in all fractions. The largest incorporation was in fraction *S*₂, being nearly 10 times greater than the synaptosome fraction that had the next highest incorporation. Comparison of the amounts of [³H]-phosphorylcholine in the three P_2 subfractions shows

quite clearly that it was almost exclusively located in the synaptosome fraction. Further, subfractionation of osmotically shocked synaptosomes revealed a sharp cytoplasmic localization of the labelled phosphorylcholine in fraction *O* with a second, much smaller, peak of labelled phosphorylcholine in fraction *H*.

To ensure that the radioactivity measured was due to [³H]phosphorylcholine and not [³H]choline or other ³H-labelled compounds several control experiments were done.

The cation-exchange treatment for the separation of phosphorylcholine from both sucrose and choline was checked for efficiency of removal of choline. The phosphorylcholine fraction prepared from 3 ml of

Table 3. *Distribution of radioactivity in the phosphorylcholine fractions*

Portions of the phosphorylcholine fractions derived from fractions S_2 and B isolated at 20 and 60 min after injection of [^3H]choline were subjected to t.l.c. on cellulose layers (plastic-backed; Eastman Kodak). The developing solvent was ethanol–18M-NH₃–water (6:3:1, by vol.). After development the sheets were dried and the position of the components determined by reference to standards developed on the same sheets. Radioactivity was determined in each area after elution of the cellulose with 0.1M-HCl directly into scintillation vials containing appropriate carriers. The particular experiment on which this analysis was done showed peak labelling of the phosphorylcholine at 20 min. Values in parentheses are percentage of total recovered radioactivity (lines 1–4) or percentage recoveries (line 5).

Identity of component	$10^{-3} \times \text{Radioactivity (d.p.m.)}$			
	S_2		B	
	20 min	60 min	20 min	60 min
Phosphorylcholine	50.94 (92.9)	26.74 (95.7)	1.35 (88.8)	0.39 (88.6)
Unknown (sphingosylphosphorylcholine?)	3.80 (6.9)	1.16 (4.2)	0.11 (7.2)	0.03 (6.8)
Glycerylphosphorylcholine	0.09 (0.2)	0.04 (0.1)	0.05 (3.3)	0.01 (2.3)
Choline	0.02 (<0.1)	0 (0)	0.01 (0.7)	0.01 (2.3)
Total recovered	54.85 (129.8)	27.94 (127.0)	1.52 (124.5)	0.44 (78.5)

Fig. 3. *Time-course of the labelling of phosphorylcholine in the subcellular fractions of guinea-pig brain cortex after injection of [^3H]choline*

For details see the text. Fractions: ●, S_2 ; ○, B ; ▲, H ; △, O . Points and bars are means $\pm$ s.d. or ranges of the numbers of determinations indicated by the adjacent values.

1.2M-sucrose containing 0.23 μmol of [^3H]choline (85.7×10^3 d.p.m.) contained only 0.17% of the added radioactivity. By using this value together with the data in Tables 1 and 2 it is evident that even at 10 min after injection of [^3H]choline, which was before the peak labelling of phosphorylcholine, the proportion of radioactivity in the phosphorylcholine fraction

due to the presence of contaminating [^3H]choline was very small.

As a further test of radiochemical purity the phosphorylcholine fractions derived from both fractions S_2 and B were subjected to chromatographic analysis (Table 3). This showed that in both fractions at 20 min and 60 min, 90% of the radioactivity in the extracts was recovered from the expected position for phosphorylcholine and that neither choline nor glycerylphosphorylcholine were serious radiochemical contaminants. An unknown component (possibly sphingosylphosphorylcholine) which travelled slightly ahead of phosphorylcholine in the chromatographic system appeared to be the major radiochemical impurity, contributing up to 7% of the recovered radioactivity.

In Fig. 3 the specific radioactivities of the phosphorylcholine fractions prepared from different subcellular sites are plotted as a function of time. These data show that the apparent turnover of the total synaptosomal pool (B) was slow when compared with the cellular cytoplasmic pool (S_2). However, the cytoplasmic fraction (O) prepared from osmotically shocked synaptosomes contained phosphorylcholine with a specific radioactivity–time profile similar to that of fraction S_2 . By contrast, the specific radioactivity of phosphorylcholine in fraction H more closely resembled that seen in the undisrupted synaptosomes. Since the [^3H]phosphorylcholine content of fraction O was always greater than the combined content of fractions A and C but, with the exception of one result at 90 min, always less than that present in fraction B (Table 2), it must have been derived principally from the synaptosomes.

A possible explanation for these observations is that the unlabelled phosphorylcholine pool was produced by the breakdown of choline-containing compounds such as phosphatidylcholine, sphingomyelin and glycerylphosphorylcholine, which were of much lower specific radioactivity than the phosphorylcholine as isolated in fraction *O*. Brain concentrations of the phospholipid breakdown product glycerylphosphorylcholine are about the same as phosphorylcholine (Ansell & Spanner, 1970) and thus could account for an appreciable augmentation of the phosphorylcholine pool. However, the known breakdown products of this compound, either by the action of known enzymes or in acidic solution, are glycerophosphate and free choline. Both phosphatidylcholine and sphingomyelin give rise to phosphorylcholine when subjected to treatment with phospholipase C (EC 3.1.4.3), which has been found in brain (Druzhinina & Kritsman, 1952; Roitman & Gatt, 1963). It is particularly noteworthy that a phospholipase C has been detected in the crude mitochondrial fraction of brain (Ansell *et al.*, 1971). Both fractions *B* and *H* contained appreciable quantities of phospholipid (Table 4) of which phosphatidylcholine and sphingomyelin constituted some 50%. By using these values it can be calculated that in both fractions as little as a 3% breakdown of these phospholipids by the action of phospholipase C would account for the observed pool sizes of phosphorylcholine.

Labelling of 'lipid choline'

As shown in Fig. 2 the labelling of lipid in the synaptosome fraction was both rapid and extensive. To determine whether or not this rapid incorporation was confined to the synaptosomes it was necessary to compare it with the labelling of the lipid fraction derived from other subcellular sites. It was also of considerable interest to determine the relative labelling of the lipid in the different sub-synaptic membranes. The incorporation patterns are shown in Fig. 4 where specific radioactivity-time profiles for the various fractions are compared. Most of the results are for short time-periods (0–90 min) although for comparison results of longer time-periods (16–400 h) have been included.

Since the lipid fraction consists of a number of different phospholipids, three of which contain choline (phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) the molecular location of the ^3H was examined after separation of the phospholipids by t.l.c. (Fig. 5). This showed that the ^3H was located almost exclusively in phosphatidylcholine during the 60 min after injection. There was also slight labelling of the two other choline-containing phospholipids, sphingomyelin and lysophosphatidylcholine. As an additional check on the location

of the ^3H label the lipids derived from fraction *P*₂ (prepared 60 min after administration of [^3H]choline) were subjected to the hydrolytic procedure of Dawson (1960). This showed that 94% of the radioactivity became water-soluble after mild alkaline hydrolysis and that upon chromatography, on cellulose layers with the phenol solvent system of Dawson (1960), 94% of this radioactivity was recovered as glycerylphosphorylcholine. Thus at least 88% of the ^3H in the phospholipids was located in the choline moiety of phosphatidylcholine and its lyso-derivative.

Phosphatidylcholine is the most abundant phospholipid in all brain membrane systems and in most cases represents close to 40% of the total phospholipid in the various synaptic membranes (Whittaker, 1966). Therefore comparisons of the specific radioactivities of the total phospholipid fractions are, to a first approximation, representative of the specific radioactivities of phosphatidylcholine.

The pattern of incorporation (Fig. 4) of [^3H]choline into the phospholipid of the synaptosomes (fraction *B*) was different from that exhibited by the microsomal (*S*₂) or mitochondrial (*C*) fraction. Thus although a slight lag period was seen in the labelling patterns of all three fractions the incorporation into the synaptosome fraction (*B*) was slower than that into the microsomal or mitochondrial fractions. In addition the initial phase of incorporation into the synaptosome fraction was largely complete after 60 min, while the incorporation into both microsomal and mitochondrial fractions was still proceeding. The labelling in the small myelin (*A*) fraction was similar to that of the synaptosome fraction.

After osmotic lysis of the crude synaptosomal fraction, ^3H -labelled phospholipid was recovered from all membranous subfractions (Fig. 4). It should be noted that some of the fractions (*E*, *F* and *I*) contained membranes of mixed origin and were therefore not representative of single subcellular sites. However, interestingly, the rates of labelling in fractions *D* and *G*, assumed to represent reasonably pure preparations of synaptic vesicles and external synaptosomal membranes respectively, were similar to each other and to the intact synaptosome fraction. The phospholipid and protein yields of the various subcellular fractions derived from osmotically lysed synaptosomes are given in Table 4, where comparison is also made with the limited amount of published information on these or comparable fractions.

The synaptic vesicle

The yield of phospholipid in the synaptic vesicle fraction [1.11 ± 0.36 (24) μmol of phospholipid phosphate/g of tissue; see Table 4, line 6] was slightly higher than that previously reported for guinea-pig brain cortex (0.88 μmol of phospholipid phosphate/g of tissue) by using the same isolation procedure

Table 4. Protein and phospholipid contents of brain subcellular fractions

(a) Present experiments. (b) Results of Lapetina *et al.* (1968) with rat brain cortex, recalculated from mean values of phospholipid phosphate and protein. (c) Results of Eichberg *et al.* (1964) for guinea pig, recalculated on the assumption that the distribution of 'non-diffusible solids' is the same as for protein.

Subcellular fraction	Major components of fractions		Yield/g of cortex of		Phospholipid:protein ratio ($\mu\text{mol}/\text{mg}$)		
	a and c	b	Protein (mg) a	Phospholipid phosphate (μmol) a	a	b	c
<i>S</i> ₂			33.0 $\pm$ 5.5 (24)	5.59 $\pm$ 1.17 (18)	—	—	—
<i>A</i>	<i>M</i> ₁ 0.8	Myelin, microsomal material	5.2 $\pm$ 1.7 (7)	3.57 $\pm$ 1.63 (7)	0.69	0.86	0.81
<i>B</i>	—	Myelin	14.1 $\pm$ 3.4 (16)	8.29 $\pm$ 1.45 (14)	0.59	—	0.72
<i>C</i>	—	Synaptosomes	4.2 $\pm$ 0.8 (7)	2.07 $\pm$ 0.74 (8)	0.50	—	0.52
<i>O</i>	—	Somatic mitochondria	4.2 $\pm$ 1.3 (25)	0.02 $\pm$ 0.02 (6)	<0.005	—	—
<i>D</i>	<i>M</i> ₂ A	Intraterminal cytoplasm	2.5 $\pm$ 0.7 (26)	1.11 $\pm$ 0.36 (24)	0.44	0.76	0.53
<i>E</i>	<i>M</i> ₂ B	Synaptic vesicles	2.1 $\pm$ 0.9 (22)	1.82 $\pm$ 0.69 (18)	0.86	0.90	—
		Microsomal material, myelin and 'Membranes'					
<i>F</i>	<i>M</i> ₂ B	Microsomal material, myelin and small membrane fragments	3.0 $\pm$ 0.8 (22)	2.76 $\pm$ 0.77 (19)	0.92	0.90	—
	<i>M</i> ₁ 0.8	Larger membrane fragments and myelin				0.86	
<i>G</i>	<i>M</i> ₁ 0.9	'Membranes' and myelin	3.3 $\pm$ 0.9 (23)	1.62 $\pm$ 0.96 (19)	0.50	0.86	—
	<i>M</i> ₁ 1.0	External synaptosome membranes and synaptosome 'ghosts'				0.65	
<i>H</i>	<i>M</i> ₁ 1.2	Partially disrupted synaptosomes	5.7 $\pm$ 1.3 (26)	2.38 $\pm$ 0.97 (25)	0.42	0.51	—
		Nerve-ending membranes					
<i>I</i>	<i>M</i> ₁ P	Mitochondria (somatic and intraterminal)	6.6 $\pm$ 1.2 (16)	2.15 $\pm$ 0.86 (19)	0.33	0.35	—
		Mitochondria and nerve-ending membranes

Fig. 4. Specific radioactivities of total phospholipids in the subcellular fractions of guinea-pig brain cortex at various times after injection of $[^3\text{H}]\text{choline}$

For description of fractions see Table 4. Black areas refer to values at relatively long periods after injection of $[^3\text{H}]\text{choline}$ (greater than 1.5 h), white areas to values at relatively short time-periods (up to 1.5 h). Where more than one determination was done the mean values are given together with their standard deviations (vertical lines) and the number of determinations. —, Not determined.

(Eichberg *et al.*, 1964) and slightly less than that reported from rat brain cortex (1.30 ± 0.21) by Lapetina *et al.* (1968). It was thought that perhaps the increased yield was due to membrane fragments as found in fraction E (microsomal material and small myelin). As an index of purity of the synaptic vesicle fraction the molar ratio of phospholipid:cholesterol was determined on the lipid extracts from fraction D in three experiments. The mean yield of phospholipid in these experiments was $1.13 \pm$

$0.13 \mu\text{mol/g}$ and the mean phospholipid:cholesterol molar ratio was $1:0.39 \pm 0.03$, which is slightly lower than the value reported for fraction D ($1:0.4-0.45$) by Eichberg *et al.* (1964). Thus contamination of the vesicle fraction with membranes richer in cholesterol than synaptic vesicles, such as myelin, appears to have been negligible.

Comparison of the relative rates of labelling of phosphatidylcholine in the synaptosomes and the synaptic vesicles (Table 5) reveals that during the first

Fig. 5. Distribution of radioactivity after t.l.c. of lipid extracts from brain subcellular fractions

(a) For fraction P_2 the phospholipids were separated as described in the Methods section. After development the plate was dried and the silica layer scraped off at 1 cm intervals and put into scintillation vials. After addition of scintillation fluid the samples were shaken well and counted for radioactivity. The diagrams above the radioactivity profiles show the location of authentic phospholipids developed at the same time. Abbreviations: LPTC, lysophosphatidylcholine; SM, sphingomyelin; PTC, phosphatidylcholine; PTS and PTE, phosphatidylserine and -ethanolamine; PA, phosphatidic acid; CL, cardiolipin. (b) The total phospholipid fraction from fraction S_2 was subjected to t.l.c. on a sheet of silica-impregnated glass fibre in the solvent system chloroform-methanol-acetic acid (100:9:1, by vol.). After development the sheet was dried and cut into strips at 0.5 cm intervals. The strips were placed into vials and after addition of scintillation fluid the radioactivity in each strip was determined.

hour after injection the synaptic vesicle phosphatidylcholine was behaving as an 'average pool' of phosphatidylcholine in the synaptosomes. Table 5 also shows that lysophosphatidylcholine and sphingomyelin also become labelled during the hour after injection. The measurement of the specific radioactivity of the lysophosphatidylcholine is likely to be somewhat inaccurate because of the low amounts of endogenous phosphate recovered from the chromatography plate. However, since it was of similar specific radioactivity to phosphatidylcholine at 20 and 60 min it is possible that it arose from the breakdown of phosphatidylcholine during the isolation procedure. The labelling of sphingomyelin was considerably slower than that of phosphatidylcholine.

Other membrane fractions

The membrane fractions E , F and G all showed similar patterns of incorporation. Interestingly, in

fraction G the lag period, seen in all the other subcellular fractions, was very slight, and at 0 and 10 min the specific radioactivity of the phospholipid in fraction G was higher than that of intact synaptosomes (B) and the synaptic vesicle fraction D . After 20 min, however, this situation was reversed.

Mitochondrial fractions

The fractionation procedure used in the present study precluded direct measurement of the labelling of phospholipid in the intraterminal mitochondria. However, it was thought that some indication of this labelling would be possible by comparison of the yields and radioactivities of the phospholipids in the fractions representing somatic (C) and somatic plus intraterminal (I) mitochondria. The specific radioactivity of the intraterminal mitochondria should thus be equal to the difference in radioactive phospholipid (I minus C) divided by the difference in yield of

Table 5. Comparison of the rates of labelling of choline-containing phospholipids in synaptosomes and synaptic vesicles

The values represent either single results or the means of two or more (numbers in parentheses) determinations. The synaptosomes and synaptic vesicles were from the same experiments.

Time after injection of [³ H]choline (min)	10 ⁻³ × Specific radioactivities (d.p.m./μmol of phosphate) in			
	Synaptosomes (B)		Synaptic vesicles (D)	
	Phosphatidylcholine	Phosphatidylcholine	Lysophosphatidylcholine	Sphingomyelin
0-2	0.08 (2)	0.40	0	0
10	3.92 (2)	7.66	0.19	0.05
20	8.00 (4)	9.79 (3)	14.30 (2)	1.95 (2)
40	22.67	30.30 (2)	16.64 (2)	5.58 (2)
60	34.00 (2)	32.10 (2)	32.0	5.50 (2)

phospholipid. In practice this calculation was only occasionally possible since the yields of both endogenous and labelled phospholipid were often less in fraction *I* than in fraction *C*. This was particularly puzzling since osmotic lysis led to an increase (57%) in the protein content of the mixed mitochondrial fraction (Table 4). The mean increase in mitochondrial phospholipid was, however, only about 4%. It seems very likely, therefore, that the hypo-osmotic conditions employed for the osmotic lysis of synaptosomes caused considerable damage to the mitochondrial membranes with the preferential loss of phospholipid.

Because of these observations precise assessment of the rate of labelling of the intraterminal mitochondria was not possible.

Discussion

The primary aim of the investigation was to follow the metabolism of choline in nerve terminals *in vivo* and to compare it with choline metabolism at other subcellular sites in the brain.

The present study shows that after intraventricular injection into conscious animals a proportion of the [³H]choline is immediately taken up and retained by the nerve terminals. A similar sequestration of injected choline was observed in mouse brain synaptosomes *in vivo* by Schuberth *et al.* (1970) after its administration via the tail vein. Although a much larger proportion of the injected choline presumably enters the cell body cytoplasm the rapidity of the uptake by the nerve terminals suggests that it is a local event. It seems likely that this uptake *in situ* is mediated by the choline transport system that exists in isolated synaptosomes (Marchbanks, 1968; Diamond & Kennedy, 1969).

The incoming choline is rapidly acetylated and the newly formed acetylcholine enters various sub-

synaptic compartments (see the accompanying paper; Barker *et al.*, 1972). Simultaneously the choline is rapidly converted into phosphorylcholine, probably in the cytoplasmic compartment of the synaptosomes. Although this pool of phosphorylcholine has a similar specific radioactivity to that recovered from the cell body cytoplasm there is no evidence which suggests that there is a translocation of labelled phosphorylcholine from one pool to the other. Therefore the labelled phosphorylcholine found in the synaptosomes is almost certainly synthesized there. These findings are in agreement with previous observations on rat brain cortex *in vitro* where a synaptosomal pool of phosphorylcholine became rapidly labelled with ³²P when slices were incubated with [³²P]P_i (Dowdall, 1968). There are also other reports of the formation of phosphorylcholine from choline by synaptosomes *in vitro* (Diamond & Kennedy, 1969) and *in vivo* (Gomez *et al.*, 1970). Thus it seems that synaptosomes synthesize their own phosphorylcholine.

The present study shows that considerable labelling of phosphatidylcholine in the synaptosomes also occurs after injection of [³H]choline. The question therefore arises: does this labelled phospholipid arise from local synthesis, or by axonal flow from other subcellular sites such as the endoplasmic reticulum? The labelling of the phospholipids in the microsomal (*S*₂) and mitochondrial (*C*) fractions was faster than in the synaptosomes during the first hour after injection. It is unlikely that a significant proportion of the labelled phospholipid in the synaptosomes could have arrived during this initial period via fast axoplasmic flow, since the total amount of ³H in the synaptosomes did not change appreciably. Further, the decline in the rate of incorporation of label in the synaptosome phospholipids after 1 h is not seen in either fraction *S*₂ or fraction *C*. Conceivably at longer time-periods (1-5 days) there is an

approach to equilibration of the various subcellular pools of labelled phospholipid; at short time-intervals, however, labelling seems to be a local process.

Studies *in vitro* have shown that synaptosome fractions are capable of incorporating labelled choline into phospholipids (Lunt & Lapetina, 1970a; Miller & Dawson, 1972). The latter authors took particular care to assess the amount of incorporation due to contaminating membranous material in the synaptosome fraction and concluded that synaptosomes free from these contaminants are still capable of incorporating labelled choline into phosphatidylcholine. Both groups of workers also claim that a base exchange of free choline with lipid-bound choline is partly responsible for the observed labelling. This is of particular interest, since in whole brain *in vivo* it has been thought that the Kennedy pathway (choline $\rightarrow$ phosphorylcholine $\rightarrow$ CDP-choline $\rightarrow$ lipid-choline) is largely responsible for the incorporation of choline into choline-containing phospholipids (Ansell & Spanner, 1968). It seems unlikely that the exchange reaction is due to the state of the tissue *in vitro* since it has been shown to occur *in vivo* in liver (Treble *et al.*, 1970). In the above study it was shown that the exchange reaction accounted for 80% and the Kennedy pathway for the remainder of the incorporation into phosphatidylcholine. It therefore seems reasonable to assume that the incorporation of choline into phosphatidylcholine in brain *in vivo* may also take place simultaneously by these two separate mechanisms.

The results of the present study do not permit unequivocal discrimination between these two possibilities. However, since the specific radioactivity of the phospholipid precursor, phosphorylcholine, has been measured, some estimate of the contribution that synthesis *de novo* might make to the labelling of phosphatidylcholine is possible. It can be argued that if the labelling of phosphatidylcholine is due exclusively to synthesis *de novo* via the Kennedy pathway, then the rate of synthesis should be independent of the time after injection of choline (assuming no perturbation of precursor pool sizes) and further should correlate with the rate of labelling of the precursor. For these calculations it is assumed that CDP-choline is the immediate precursor of phosphatidylcholine and that it can only be formed from phosphorylcholine. Minimal rates of synthesis of phosphatidylcholine (r) can then be calculated approximately from the following expression: $r = \Delta TR / \bar{SA}(t_2 - t_1)$, where ΔTR is the increment of total radioactivity in the phosphatidylcholine between time t_1 and t_2 and $\bar{SA}$ is the mean specific radioactivity of phosphorylcholine between the same times.

When this calculation was applied to the results presented in this paper by using the specific radioactivity of the phosphorylcholine from fraction *O* the apparent rate of synthesis of phosphatidylcholine in

fraction *B* varied as a function of time. It was maximal (3.4 nmol/min) and the same at 20 and 40 min following injection but at shorter and longer time-periods than these it was considerably lower. Further, these minimal rates of synthesis indicate that complete replacement of the synaptosomal phosphatidylcholine should occur about every 17 h. Clearly this was not so. It is probable therefore that some labelling of the phosphatidylcholine occurs by a mechanism independent of the Kennedy pathway, such as base exchange. Quantitative evaluation of the possible relative contributions of each of these two mechanisms to the labelling of phosphatidylcholine in the synaptosome is not possible. However, it does seem that the labelling is not entirely due to a base exchange mechanism, since a lag period of about 10 min is observed in the labelling of phosphatidylcholine even though the uptake and phosphorylation of choline into the synaptosome is extremely rapid.

The labelling pattern of phospholipid in fraction *G* is highly suggestive of an exchange-type reaction in that no lag period occurs before accumulation of injected label. It is conceivable, therefore, that incorporation of choline into phosphatidylcholine in the extrasynaptosomal membrane at least occurs by a base-exchange reaction.

The present experiments show that, after intraventricular injection of [3 H]choline, the synaptic

Fig. 6. Time-course of incorporation of [3 H]choline

Incorporation of choline was into the acetylcholine (—) and phospholipid (----) of synaptic vesicles isolated by the method of Whittaker *et al.* (1964). Points and bars are means $\pm$ S.E.M. (acetylcholine) or $\pm$ S.D. (phospholipid) or range of the number of experiments indicated by the adjacent values. Note change of scale on abscissa.

vesicles become rapidly labelled in both acetylcholine (Barker *et al.*, 1972) and choline-containing lipids. A comparison of the half-lives of these two components (Fig. 6) shows that the turnover of vesicular acetylcholine is several orders of magnitude faster than the vesicular lipid in the synaptic vesicle membrane. Comparatively long half-lives of macromolecular synaptic vesicle constituents have also been reported by other workers. Thus Lapetina *et al.* (1970) found that the half-life of the choline in vesicle phosphatidylcholine was 30 days and von Hungen *et al.* (1968) report a half-life of 21 days for vesicle protein. The latter authors concluded that vesicles are not rapidly depleted and replenished as a consequence of firing of the synapse. We had hoped that a simultaneous comparison of the turnover of the membranes (as represented by choline-containing phospholipids) of the vesicle with both those of vesicular acetylcholine and other subsynaptic membranes at short time-intervals might provide some insight into the mechanisms involved in the storage and release of vesicular acetylcholine. The results show, however, that this approach is not possible since there is an apparent rapid equilibration of the labelled phospholipid between different subsynaptic sites. It may well be that this apparent equilibration of the choline-containing phospholipids is not a reflection of the movement of intact phospholipid molecules but rather represents intraterminal equilibration of their choline moieties by exchange-type processes.

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