The Subcellular Distribution of [*N*-Me-³H]Acetylcholine Synthesized by Brain *in vivo*

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1. Three forms of acetylcholine occur in subcellular fractions of brain tissue: free acetylcholine, present in the high-speed supernatant from eserinizd sucrose homogenates; stable bound acetylcholine, present in synaptic vesicles; and labile bound acetylcholine, present in the cytoplasm of synaptosomes (detached pre-synaptic nerve terminals). 2. The relationship between these forms has been investigated by isolating the subcellular fractions from the cortical tissue of cats and guinea pigs excised 1 hr. after infiltration of [*N*-Me-³H]choline into the cortex *in vivo*. 3. Since choline is a ubiquitous metabolite, means were devised for isolating the radioactive acetylcholine on columns of the weak acid ion-exchange resin IRF-97; control experiments with samples of extracts treated with acetylcholinesterase showed that the radioactivity attributed to acetylcholine migrated to the choline peak after cholinesterase treatment. 4. The specific radioactivities of the various forms of acetylcholine were different: labile bound (synaptosomal cytoplasmic) acetylcholine had the highest, stable bound (vesicular) acetylcholine the next highest, and the high-speed-supernatant form the lowest. 5. It is concluded that the various forms of acetylcholine could not have arisen during fractionation from a single pre-existing pool of acetylcholine.

When brain tissue is comminuted in physiological saline media, most of the tissue acetylcholine remains bound to particulate material; the particle-bound ester is unaffected by cholinesterases and is pharmacologically inactive unless released by treatments destructive to lipoprotein membrane structures (for a review of the older literature see Feldberg, 1945).

Hebb & Whittaker (1958) showed that the same is true of brain tissue homogenized in iso-osmotic aqueous sucrose solutions; even when an anti-cholinesterase is added to stabilize any free acetylcholine present, only about 20–25% of the total acetylcholine in the tissue can be detected pharmacologically; the greater part remains particle-bound and pharmacologically inactive until released (e.g. by heating at pH 4.0 and 100° for 10 min.). On subcellular fractionation, the free acetylcholine of the homogenate, as expected, is recovered in the high-speed-supernatant (cell-sap) fraction (*S*₃ in the terminology of Gray & Whittaker, 1962); the bound acetylcholine, on the other hand, is found mainly in the crude mitochondrial fraction (*P*₂) and, on further separation of this fraction in a sucrose density gradient, is recovered (Whittaker, 1959, 1960; Gray & Whittaker, 1960, 1962) in a

particulate fraction denser than myelin and lighter than mitochondria, the other two main constituents of this fraction. This intermediate (*B*) fraction consists largely of detached presynaptic nerve terminals (synaptosomes); the bound acetylcholine in it evidently represents the store of acetylcholine in the immediately presynaptic portions of cholinergic neurones that have become detached during homogenization and have been isolated, along with other presynaptic nerve terminals, by the fractionation procedure.

Further work (Whittaker, 1959; Johnson & Whittaker, 1963; Whittaker, Michaelson & Kirkland, 1964; Marchbanks, 1968) has shown that bound (i.e. synaptosomal) acetylcholine exists in two forms. When synaptosomes are suspended in hypo-osmotic media, the synaptosomes burst and soluble constituents of the synaptosomal cytoplasm diffuse out. Part of the bound acetylcholine (the 'labile bound' fraction described by Whittaker, 1959) diffuses out, and, in the absence of an anti-cholinesterase, is destroyed by the cholinesterases in the preparation. This form behaves in all respects like any soluble cytoplasmic constituent: thus it can be augmented by placing synaptosomes in acetylcholine solution (Whittaker, 1959), and is therefore readily labelled when the suspension medium contains radioactive acetylcholine

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Table 1. *Types of brain acetylcholine*

The nomenclature of the fractions is that of Gray & Whittaker (1962) and Whittaker *et al.* (1964).

Type of acetylcholine	Subcellular fraction	Presumed location
Free acetylcholine	S_3 from eserinizd homogenate	Cell sap from cell bodies broken during homogenization
Bound acetylcholine	P_2 or B from non-eserinized homogenate	Synaptosomes
(a) 'Labile'	O from eserinizd P_2 or B	Synaptosomal cytoplasm
(b) 'Stable'	D from non-eserinized P_2 or B ; H from same	Synaptic vesicles in monodisperse form (D) or in clumps in incompletely disrupted synaptosomes (H)

(Marchbanks, 1968). The form of bound acetylcholine surviving hypo-osmotic shock (the 'stable bound' form described by Whittaker, 1959) has been shown (Whittaker *et al.* 1964) to be localized in synaptic vesicles: when the disrupted synaptosomes are fractionated on a density gradient, the stable bound form is recovered mainly in a fraction (D) consisting of almost pure monodispersed synaptic vesicles, and in a second fraction (H) that contains partially disrupted synaptosomes also rich in synaptic vesicles. The stable bound (vesicular) form of bound acetylcholine does not exchange significantly with radioactively labelled acetylcholine *in vitro* under a variety of experimental conditions (Marchbanks, 1968) and radioactively labelled choline does not serve as a precursor *in vitro*. The subcellular location of the three kinds of cerebral acetylcholine (free, labile bound and stable bound) is summarized in Table 1.

The work reported in this paper was done in an effort to determine whether these three fractions correspond to pre-existing pools of acetylcholine in brain tissue, or were formed by the redistribution of acetylcholine from a single pool during homogenization and fractionation. Acetylcholine might, *in vivo*, be exclusively localized in synaptic vesicles and the labile bound synaptosomal cytoplasmic and high-speed-supernatant (cell-sap) fractions might have resulted from the breakdown of synaptic vesicles and synaptosomes during fractionation. Alternatively, though less plausibly in view of the control experiments performed by Whittaker (1959), all the acetylcholine in brain tissue might have been in the 'free' state and might have become associated with particulate material by a process of entrainment of soluble materials within lipid micelles of various sizes formed during homogenization.

Since radioactive choline injected into cerebral cortical tissue *in vivo* is incorporated into brain acetylcholine (Chakrin & Shideman, 1968; Chakrin, Shideman & Marrazzi, 1968), it was decided to investigate the relationship between the various forms of acetylcholine by determining their specific radioactivity at a suitable time after infiltration of

[3H]choline. If the various forms of acetylcholine in subcellular fractions were derived from a single pre-formed pool of acetylcholine, a constant specific radioactivity in all fractions would be expected.

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METHODS

Isolation of labelled subcellular fractions

Intracortical injection of radioactive choline *in vivo*. Adult cats (2–3 kg.) of either sex were anaesthetized with sodium 5-ethyl-5-(1-methylbutyl)-barbiturate (pentobarbital sodium) (35 mg./kg. intraperitoneally) and surgically prepared as described by Chakrin & Shideman (1968). The surface of the cortex in the region of the suprasylvian and ectosylvian gyri was exposed; a bridge of bone was retained over the longitudinal cerebral fissure to retard bleeding. The exposed cortex was kept warm and moist with aq. 0.9% NaCl. Guinea pigs (500–700 g.) were similarly prepared, except that no central ridge of bone was preserved.

[N -Me- 3H]Choline chloride (The Radiochemical Centre, Amersham, Bucks., specific radioactivity 150–200 mc/m-mole) was injected into the cortical tissue in four or five portions by means of a 50 μ l. Hamilton syringe; cats received 40 μ l. in 200 μ l. of aq. 0.9% NaCl and guinea pigs half this amount. Then 1 hr. later cortical tissue (1–2 g.) was excised from the injection area, weighed and homogenized in enough ice-cold aq. 0.32 M-sucrose to give a final tissue concentration of 10% (w/v). With guinea pigs, the tissue was freed from white matter as completely as possible by gentle scraping with a blunt scalpel, but with cat tissue the convolutions of the tissue made this impracticable.

Isolation of subcellular fractions. The specific radioactivity of synaptosomal and vesicular acetylcholine was determined on extracts of fractions containing intact synaptosomes or synaptic vesicles obtained by slight modifications of methods used as a routine in this laboratory.

The procedure finally adopted is shown in Scheme 1. In the experiments with cats, the pellet containing synaptosomes was washed by suspension in 0.32 M-sucrose and centrifuging at 12000 g for 30 min. (g values are averages) before being submitted to hypo-osmotic disruption, and the

Isolation of radioactive acetylcholine. Since choline is a precursor of many tissue constituents, it was necessary to isolate labelled acetylcholine from the extracts of the subcellular fractions in order to determine the radioactivity associated with endogenous acetylcholine. The procedure finally adopted is shown in Scheme 2. Fractions (P_2 or D of Scheme 1, or S_3 equivalent to 1–2 g. of tissue) were heated at 100° and pH 4.0 for 10 min. to extract bound acetylcholine (Hebb & Whittaker, 1958): the amount extracted was determined on a small sample of the extract by the leech micro-assay procedure of Szerb (1961) as modified by Whittaker *et al.* (1964), except that a vertical organ bath

Scheme 2. Separation of radioactive acetylcholine from other radioactive products of choline metabolism.

of 0.2 ml. capacity replaced the horizontal one of 0.05 ml. capacity previously used. To check that the response of the muscle was due to acetylcholine, the latter was destroyed by treatment for 30 min. at 37° with ox erythrocyte acetylcholinesterase (Sigma Chemical Co., St Louis, Mo., U.S.A.; 3–10 μ g., equivalent to 0.2–1.0 unit of enzyme activity/0.5 ml. sample) or by heating at pH 10–12 for 10 min., and the preparation was reassayed. After acetylcholinesterase treatment samples were adjusted to pH 4.0 and heated at 100° for 10 min. to destroy the enzyme, which otherwise interfered with the assay.

The main portion of the extract (2–6 ml.) was usually divided into two: one part was neutralized to pH 8.0 with solid NaHCO₃ and treated for 30 min. at 37° with acetylcholinesterase (2–10 units) to destroy endogenous acetylcholine; this part was adjusted to pH 4.0 and heated at 100° for 10 min. to destroy the enzyme; then to both parts non-radioactive carrier acetylcholine (5 nmoles) was added; and finally each part was separately subjected to chromatography on a column (12 cm. $\times$ 1 cm.) of the weak acid ion-exchange resin IRF-97 (formerly named XE-97) (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) that had been previously prepared by cycling it through the H⁺ and Na⁺ forms and buffering it to pH 4.3 with 0.02 M-NaH₂PO₄ (Gardiner & Whittaker, 1954; Whittaker, 1963). The addition of carrier acetylcholine was found to be essential

to eliminate adsorption of the very small amounts of radioactive acetylcholine on to glassware and consequent irregularities in peaks. Extracts containing eserine were not subjected to cholinesterase treatment.

In general, with extracts that had not been pretreated with acetylcholinesterase, three peaks of radioactivity were observed (Scheme 2) in the column effluents after elution with 0.02 M-NaH₂PO₄: an initial peak that emerged in the void volume of the column and probably consisted of choline that had become incorporated into phospholipid; a second peak that, as shown by analyses carried out by the method of Appleton, La Du, Levi, Steele & Brodie (1953), contained much free choline, but probably phosphorylcholine and other acidic derivatives were also present; and a third peak that coincided in retention volume with unlabelled carrier acetylcholine as determined by bioassay. Further prolonged elution of the columns with 0.02 M-NaH₂PO₄ or displacement of cations with 0.1 M-HCl did not give any further peaks of radioactivity. In extracts treated with acetylcholinesterase, no radioactive material could be detected in association with unlabelled carrier acetylcholine, but the peak corresponding in retention volume to free choline was correspondingly augmented. It was concluded, therefore, that the radioactivity in the third peak was due to labelled endogenous acetylcholine. The specific radioactivity of the endogenous acetylcholine of the fraction was then the ratio

of the total d.p.m. in the third peak to the amount (in pmoles) of endogenous acetylcholine in an equivalent volume of the extract. In control experiments with authentic acetylcholine the recovery of the ester was about 85%; values for specific radioactivities are given uncorrected for chromatographic losses.

Estimation of radioactivity. The radioactivity of extracts and chromatographic effluents was measured at 4–5° in a Nuclear-Chicago model 725 liquid-scintillation counter. To each sample (0.1–1.0 ml.) was added 10 ml. of dioxan-phosphor scintillation solution (formula no. 3 of Hayes, 1963), and the samples were precooled. The counting efficiency was 12–14%.

Gel filtration of fractions. The effect of hypo-osmotic conditions on the radioactivity associated with fractions containing synaptic vesicles was determined by the gel-filtration method of Marchbanks (1967). Columns containing 3 g. of Sephadex G-50 (bead form; Pharmacia AB, Uppsala, Sweden) were made iso-osmotic with synaptic vesicles by equilibrating the gel with 0.4 M-sucrose. Other columns were equilibrated with glass-distilled water. Vesicle preparations (3 ml.) were added to the top of the column and allowed to drain through. The columns were then eluted with the solution with which they had been equilibrated, and fractions (1 ml.) of eluate were collected for protein and radioactivity determinations. In some experiments, the fractions emerging in the void volume of the column and containing the macromolecular constituents were pooled for further analysis.

Characterization of radioactive acetylcholine as the aurichloride. Unlabelled carrier acetylcholine (usually 200–250 mg.) was added to solutions suspected to contain radioactive acetylcholine, and excess of aq. AuCl_3 was added. The resultant precipitate was recrystallized from water repeatedly until constant specific radioactivity (d.p.m./mg. of aurichloride) was attained. Since choline aurichloride is considerably more soluble than acetylcholine aurichloride in cold water (Dudley, 1929), any radioactivity due to the precursor is gradually removed (Goldberg, 1966; Wallach, Goldberg & Shideman, 1967).

Determination of protein. The protein content of subcellular fractions and effluents from Sephadex columns was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) as the standard.

Electron microscopy. For electron-microscopic examination, subcellular fractions were fixed in suspension by the addition of 5 vol. of 2.5% (w/v) glutaraldehyde in 0.1 M Sørensen's phosphate buffer, pH 7.4, collected by centrifuging, stained with 1% (w/v) OsO_4 in 0.1 M-phosphate buffer, dehydrated in ethanol and embedded in Epikote (Epon) resin (Luft, 1956). Silver-grey sections were stained with lead citrate before being viewed in the A.E.I. EM 6B electron microscope.

RESULTS

Experiments with cats

Since earlier experiments on the incorporation into acetylcholine of radioactive choline injected into cortical tissue *in vivo* had been done with cats (Chakrin & Shideman, 1968; Chakrin *et al.* 1968), preliminary studies were carried out on this species.

When [*N-Me- ^3H*]choline chloride was homogenized with cat cerebral tissue at 0–4° most of the radioactivity was found in the high-speed supernatant and the small amount of particle-bound ^3H was not associated with any particular fraction. In contrast, when radioactive choline was injected into the exposed cortex of anaesthetized animals and, 1 hr. later, tissue was removed from the injection sites, homogenized and fractionated, considerable amounts of radioactivity were found to be associated with particulate material. The crude mitochondrial fraction (P_2) had the most, and when this was subfractionated by means of a discontinuous gradient (Gray & Whittaker, 1962), the purified mitochondrial fraction (*C*) again had the most, the synaptosome fraction (*B*) somewhat less, and the myelin fraction (*A*) the least radioactivity.

When the synaptosome fraction was subfractionated by osmotic rupture and separation on a discontinuous gradient by essentially the same procedure as shown in Scheme 1, seven subfractions were obtained as in previous work with guinea pigs. About one-third of the radioactivity was released and passed into fraction *O* (Table 2, column 2). This evidently represented soluble radioactive compounds present in the synaptosome cytoplasm. Again, among the particulate fractions the one with most radioactivity was the mitochondrial fraction (*I*), and, as shown by the distribution of protein in the fraction (Table 2, column 3), this also had the highest specific radioactivity on a protein basis.

When, however, that part of the radioactivity attributable to acetylcholine was measured by using the chromatographic technique outlined in Scheme 2, a bimodal distribution (Table 2, column 4) was disclosed; the most radioactive fraction was now the vesicle fraction (*D*) and the next most radioactive particulate fraction was the one containing partially disrupted synaptosomes with entrapped synaptic vesicles (fraction *H*). The distribution was quite similar to that of endogenous acetylcholine (Table 2, column 5), except that there was considerably more radioactivity in fraction *O* than could be expected from its endogenous acetylcholine content, suggesting the presence of a fraction of acetylcholine with a specific radioactivity (in d.p.m./pmole) almost an order of magnitude greater than the mean of the particulate fractions. However, it should be pointed out that the radioactivity associated with the acetylcholine peak in the chromatogram of fraction *O* represented only < 5% of the total radioactivity in this fraction and may well have included some material that had 'tailed' from the choline peak. In the experiments with cats, unlabelled carrier acetylcholine was not added before chromatography and the separation

Table 2. *Distribution of total radioactivity, radioactive acetylcholine and endogenous acetylcholine in subfractions of synaptosomes from cat and guinea-pig cortex*

For identification of the fractions see Whittaker *et al.* (1964), Table 1 and Scheme 1. In columns 4–7, the values in parentheses are the percentages of the total recovered radioactivity or endogenous acetylcholine found in each fraction; the other values in these columns are the amounts of each fraction derived from 1 g. of cortex, and are the results of single representative experiments. Columns 2 and 3 give the means of eight experiments $\pm$ S.E.M.

Fraction	Cat				Guinea pig	
	Distribution (% of total recovered)		Acetylcholine		Acetylcholine	
	Total radioactivity	Protein	Radioactive (d.p.m.)	Endogenous (pmoles)	Radioactive (d.p.m.)	Endogenous (pmoles)
O	33.5 $\pm$ 6.3	14.3 $\pm$ 3.6	5230* (30)	7 (5)	312 (13)	60 (14)
D	7.3 $\pm$ 2.4	8.3 $\pm$ 1.8	6770 (38)	75 (51)	2110 (54)	210 (46)
E	6.6 $\pm$ 1.2	12.8 $\pm$ 5.3	1780 (10)	18 (12)	432 (11)	32 (7)
F	10.5 $\pm$ 2.1	17.2 $\pm$ 4.5	220 (1)	14 (10)	29 (1)	15 (3)
G	11.1 $\pm$ 3.7	12.4 $\pm$ 2.4	1060 (6)	13 (8)	108 (3)	21 (5)
H	13.5 $\pm$ 4.1	18.2 $\pm$ 4.6	2430 (14)	18 (12)	860 (22)	105 (23)
I	17.3 $\pm$ 3.3	16.4 $\pm$ 3.3	230 (1)	3 (2)	78 (2)	10 (2)

* <5% of the total radioactivity recovered from the column.

Fig. 1. Distribution of radioactive material in effluents after gel filtration of untreated fractions O (a) and D (b) prepared from cat cortex excised 1 hr. after infiltration with radioactive choline *in vivo*. The black profiles show the distribution of protein and indicate the void-volume effluents; the continuous lines show the radioactivity.

of radioactive acetylcholine from radioactive choline was therefore less efficient than in later experiments with guinea pigs, where acetylcholine of high apparent specific radioactivity was not found in fraction O derived from synaptosomes suspended in non-esterinized water.

Table 3. *Radioactivity of aurichlorides of material derived from synaptic vesicles of cat cortex after recrystallization in the presence of carrier acetylcholine*

Preparations of synaptic vesicles (fraction D, Scheme 1) were submitted to gel filtration on columns of gel equilibrated with aqueous sucrose solution iso-osmotic with the vesicle preparation or with water. To the void-volume effluent was added carrier acetylcholine and excess of AuCl₃, and the precipitate that formed was recrystallized from water to constant specific radioactivity as described in the Methods section. The results given below are the mean specific radioactivities $\pm$ S.E.M. of the aurichlorides in three experiments; [*N-Me-³H*]choline was carried through the same procedure as a control.

Condition	Sp. radioactivity of aurichloride (d.p.m./mg.)
Iso-osmotic column	25.2 $\pm$ 6.1*
Hypo-osmotic column	12.8 $\pm$ 4.2*
[<i>N-Me-³H</i>]Choline (5 μ C having approx. 1.11×10^7 d.p.m.)	8.3 $\pm$ 3.4

* Significantly different (paired *t* test) (0.05 > *P* > 0.01)

Evidence that the radioactivity in fraction D was vesicular was obtained from gel-filtration experiments, since it is known (Marchbanks, 1968) that endogenous vesicular acetylcholine remains bound and emerges in the void-volume effluent of the gel column along with vesicles when the latter are passed through gels that have previously been equilibrated with sucrose solutions iso-osmotic with the vesicle suspension medium. Fig. 1 compares the distribution of total radioactivity in

effluents from iso-osmotic Sephadex columns after filtration of fraction *O* (Fig. 1a) and fraction *D* (Fig. 1b). Most of the radioactivity in fraction *O* emerged in the non-excluded fraction, confirming its association with small diffusible molecules, whereas that in fraction *D* was almost all recovered in the void volume.

When fraction *D* was passed through hypo-osmotic Sephadex, there was a marked decrease in the amount of radioactivity emerging in the void volume, and the greater part of the radioactivity was now recovered in the non-excluded fraction of the eluate. The results were similar to those shown in Fig. 3 (discussed below), which represents an experiment with a guinea-pig preparation. On addition of unlabelled carrier acetylcholine to the void-volume effluent, precipitation of the bases as aurichlorides and recrystallization from water, the specific radioactivity (Table 3) of the material isolated from the void-volume effluent derived from the hypo-osmotic column rapidly fell to a value close to that of a choline control, whereas the material isolated from the void-volume effluent derived from the iso-osmotic column retained the value it initially had in the presence of carrier acetylcholine.

The demonstration that vesicular acetylcholine had become labelled was of particular interest in that, in experiments with synaptosomes and synaptic vesicles *in vitro*, Marchbanks (1968) had been unable to demonstrate significant labelling of vesicular acetylcholine under a variety of conditions, although cytoplasmic acetylcholine was readily labelled either by exchange with radioactive acetylcholine in the suspension medium or (Marchbanks, 1969) from radioactive choline as a precursor. However, electron-micrographic examination of the vesicle fraction from cat cortex revealed that it was considerably more contaminated with larger membrane fragments than the same fraction from guinea-pig cortex; the content of endogenous acetylcholine/g. of original tissue was also much lower, and both shortcomings were probably due to the relatively high content of myelin in the original tissue. Since the highly convoluted structure of the cat cortex precluded the ready removal of myelin by dissection before homogenization it was decided to carry out further work with the guinea pig.

Experiments with guinea pigs

Labelling of vesicular acetylcholine. With the guinea pig, as in the cat, appreciable amounts of

Fig. 2. Chromatographic separation on IRF-97 of radioactive material in extracts of fraction *O* (a) and fraction *D* (b) prepared as shown in Schemes 1 and 2 from guinea-pig cortex previously infiltrated with radioactive choline *in vivo*. The continuous lines show the radioactivity, and the black profiles the bioassay of carrier acetylcholine. ACh, Acetylcholine.

radioactive acetylcholine were found to be associated with subfractions of disrupted synaptosomes (Table 2, column 6); the distribution of this radioactive acetylcholine once again paralleled that of endogenous acetylcholine (Table 2, column 7) and had the bimodal distribution expected of a vesicular constituent. With the use of unlabelled carrier acetylcholine in the chromatographic separation of radioactive acetylcholine from radioactive choline, the analytical results were more reliable and the amount of radioactivity associated with the acetylcholine peak in chromatograms of extracts of fraction *O* was always very small.

Fig. 2 shows the contrast between chromatograms of extracts of fractions *O* and *D* obtained as shown in Scheme 2. Although there was considerable radioactivity in the extract from fraction *O* (Fig. 2*a*) there was no peak associated with the unlabelled carrier acetylcholine. In the synaptic-vesicle fraction there was less total radioactivity, but an appreciable amount was associated with unlabelled carrier acetylcholine (Fig. 2*b*).

To make sure that none of the radioactivity in the acetylcholine peak derived from the vesicle fraction was attributable to soluble radioactive choline products diffusing from fraction *O*, use was made of gel filtration to eliminate non-vesicular small molecules from fraction *D*. The technique is illustrated in Fig. 3. When samples of vesicles were passed through a column of Sephadex equilibrated with sucrose iso-osmotic with the vesicle preparation (thick line), much of the radioactivity passed through the gel in the void-volume effluent, emerg-

ing with protein and other macromolecular constituents, including vesicles. On filtration through a gel equilibrated with water (broken line), the amount of radioactivity emerging in the void

Fig. 3. Distribution of radioactivity in effluents after gel filtration of untreated fraction *D* prepared from guinea-pig cortex previously infiltrated with radioactive choline *in vivo*. The thick continuous line shows the radioactivity in the effluent from a column equilibrated beforehand with 0.4 M-sucrose iso-osmotic with the vesicle preparation; the broken line shows the radioactivity in the effluent from a column equilibrated beforehand with water and thus strongly hypo-osmotic to the vesicle preparation; the thin continuous line shows the mean distribution of protein in the effluents.

Fig. 4. Chromatogram on IRF-97 of radioactive materials present in the void-volume effluent after iso-osmotic gel filtration of an untreated fraction *D* from guinea-pig cortex previously infiltrated with radioactive choline *in vivo*. Before chromatography, the radioactive materials were released from macromolecular constituents of the void-volume effluent by heating the latter at pH 4 and 100° for 10 min. and subsequently proceeding as shown in Scheme 2. The continuous line shows the distribution of radioactivity before, and the broken line shows the radioactivity after treatment of the extracts with cholinesterase (ChE). The black profiles show the distribution, measured by bioassay, of carrier acetylcholine (ACh).

volume was greatly decreased, and most of it now appeared in the non-excluded volume. This showed that most of the radioactivity in the void volume was associated with a substance (or substances) released by hypo-osmotic stress and therefore presumably vesicle-bound. Fig. 4 (continuous line) shows that this substance was mainly acetylcholine. This chromatogram is that of an extract of the void-volume effluent from a vesicle fraction passed through iso-osmotic gel before acid extraction and resin chromatography. Most of the radioactivity was associated with acetylcholine, though there was a small peak of choline (probably produced by hydrolysis of acetylcholine during manipulation) and some 'lipid choline' which may have represented [^3H]choline incorporated into the vesicle wall. This last material may account for the osmotically insensitive ^3H emerging in the void volume of the hypo-osmotic column in Fig. 3. After cholinesterase treatment (dotted line) the radioactivity of the acetylcholine peak was transferred, as one would expect, to the choline peak, but the lipid choline was unaffected.

Comparison of specific radioactivities of vesicular, synaptosomal and supernatant acetylcholine. The results thus far demonstrated that vesicular acetylcholine became labelled 1 hr. after injection of radioactive choline into the surviving cortex. It was now desired to compare the specific radioactivity of vesicular acetylcholine with that of the other two forms of acetylcholine demonstrable in brain homogenates.

Attempts were made to find conditions under which the specific radioactivities of labile bound (synaptosomal cytoplasmic) and stable bound (vesicular) acetylcholine could be determined in the same preparation. Synaptosomes were ruptured by suspension in water containing $10\mu\text{M}$ -eserine to stabilize labile bound acetylcholine after release and then submitted either to gel filtration or to separation on a gradient like that shown in Scheme 2 but made of sucrose solutions containing $10\mu\text{M}$ -eserine. It was assumed that liberated labile bound acetylcholine would enter the gel and be recovered later in the non-excluded phase, whereas stable bound acetylcholine would pass through in the void volume. For satisfactory results, it was essential that complete rupture of synaptosomes should take place, since any surviving intact synaptosomes would pass through the gel along with synaptic vesicles, and their contained labile bound acetylcholine would contaminate the vesicular acetylcholine. Potassium was used as a cytoplasmic marker. It was not found possible for various technical reasons to devise conditions of rupture that permitted the release of all the synaptosomal potassium (as shown by the absence of potassium from the void-volume effluent) while

at the same time avoiding the release of some vesicular acetylcholine and thus contamination of released labile bound acetylcholine by released stable bound acetylcholine. In the density-gradient experiments, the distribution of free acetylcholine down the gradient did not exactly parallel that of the soluble cytoplasmic markers potassium and lactate dehydrogenase; there were appreciable amounts of free acetylcholine in the denser fractions but negligible amounts of potassium or lactate dehydrogenase, though the fraction with the most free acetylcholine was still fraction *O* (cf. Whittaker & Sheridan, 1965). This result was interpreted to mean that acetylcholine had leaked from synaptic vesicles during their passage down the gradient and that the distribution of free acetylcholine was a composite of the distributions of labile bound acetylcholine released during the initial disruption and of stable bound acetylcholine that had leaked during separation. It was therefore decided to compare the specific radioactivity of vesicular acetylcholine (which, as shown above, can be prepared quite free of synaptosomal cytoplasmic acetylcholine by omitting an anticholinesterase during the initial disruption and separating the vesicles in a density gradient) with that of total synaptosomal acetylcholine.

Table 4 (rows 1-5) compares the specific radioactivity of vesicular acetylcholine with that of total synaptosomal acetylcholine, by the techniques summarized in Schemes 1 and 2, when tissue was removed 1 hr. after injection of radioactive

Table 4. *Specific radioactivities of the various types of acetylcholine in subcellular fractions from guinea-pig cerebral cortex*

Each row relates to a separate experiment. For the nomenclature of fractions see Table 1 and Scheme 1. In column 2 the values in parentheses are the specific radioactivities as a percentage of those of synaptosomal acetylcholine (column 1).

Sp. radioactivity of acetylcholine (d.p.m./pmole)		
Synaptosomes (P_2)	Vesicles (D)	High-speed supernatant (S_3)†
7.2*	4.6 (63)	—
9.4*	5.1 (54)	—
6.3*	4.4 (69)	—
6.2	5.1 (83)	—
6.4	3.9 (61)	—
—	—	2.6
—	—	2.5
—	—	2.4
Means $\pm$ S.D. 7.1 ± 1.8 ‡ 4.6 ± 0.3 (66 $\pm$ 5)‡ 2.5 ± 0.1		

* Eserinized water suspension.

† Prepared from eserinized homogenate.

‡ Significantly different (paired *t* test) ($P < 0.01$).

choline. Although vesicular acetylcholine was labelled, its specific radioactivity was only about two-thirds of that of total synaptosomal acetylcholine. Since there were about equal amounts of synaptosomal cytoplasmic and vesicular acetylcholine in synaptosomes (Whittaker, 1959), the cytoplasmic pool must have had a specific radioactivity of about twice that of the vesicular pool.

In a separate series of experiments (Table 4, rows 6-8) made with tissue that had been homogenized in eserized 0.32M-sucrose, the specific radioactivity of the supernatant fraction was even lower than that of the vesicular fraction, about one-third of that of the synaptosomal acetylcholine.

The time-course of labelling of the various forms of acetylcholine has not been studied in detail, but preliminary observations on tissue removed $\frac{1}{2}$ hr. after injection of radioactive choline showed that the vesicular acetylcholine had a lower specific activity relative to the synaptosomal cytoplasmic fraction than that isolated 1 hr. after injection.

DISCUSSION

In discussing the significance of the results, a distinction must be made between pools of acetylcholine (defined kinetically) and morphological compartments of acetylcholine, which may represent statistical aggregates of separate pools. The different specific radioactivities of the various forms of acetylcholine in the subcellular fractions show that these forms could not have arisen during fractionation from the redistribution of acetylcholine from a single pre-existing pool. Taken in conjunction with the results with isolated synaptosomes (Marchbanks, 1968) in which the synaptosomal cytoplasmic acetylcholine was found to exchange readily with radioactive acetylcholine under conditions that gave undetectable exchange of vesicular acetylcholine, they indicate the existence of a pool of synaptosomal cytoplasmic acetylcholine distinct from vesicular acetylcholine, and further suggest that the high-speed-supernatant acetylcholine does not arise from synaptosomes damaged during homogenization. The origin of this fraction of acetylcholine may well be neuronal cell bodies, since there is much evidence that transmitters and the enzymes that synthesize them are distributed throughout the length of neurones, though in general in lower concentrations than at the nerve terminals.

It has often been thought that acetylcholine and other transmitters are confined *in vivo* to a single morphological compartment, the synaptic vesicle. This view is only consistent with the present results if several additional and partly contradictory assumptions are made. What is here described as cytoplasmic acetylcholine could have arisen from

the preferential breakdown of a population of vesicles metabolically more active and also less stable than the main population: it is, indeed, not inconceivable that there is *in vivo* a population of empty, labile, vesicles that are more rapidly labelled than another population of full, stable, vesicles. However, as far as the cell-sap (fraction S_3) form of acetylcholine is concerned, a different set of assumptions must be made. Fraction S_3 acetylcholine must arise either from a population of sluggishly labelled vesicles from the perikaryon that are unstable in iso-osmotic sucrose and so less, not more, stable than the putative population of rapidly labelled vesicles in the terminals, or it must arise from a population of less rapidly labelled and less stable synaptosomes than the surviving synaptosomes. The concept of three morphologically distinct compartments involves fewer assumptions.

One objection to the notion of cytoplasmic acetylcholine is that it is not clear how such a form of acetylcholine could coexist in neurone somata and terminals with the acetylcholinesterase known to be distributed throughout the length of cholinergic neurones (reviewed by Silver, 1967). However, histochemical studies in the electron microscope (Lewis & Shute, 1966; Brzin, Tennison & Duffy, 1966) show that the products of acetylcholinesterase hydrolysis are confined in the cell bodies of cholinergic neurones to the lumen of the endoplasmic reticulum, and in their axons and nerve endings, to the external membrane. If the active centres of the enzyme were inaccessible to substrate applied to the inside of these membranes, i.e. if the enzyme were functionally, at all points, on the outside of the cell, free acetylcholine would be unhydrolysed so long as it remained intracellular. Some support for this model is provided by the observation that when the synaptosomal cytoplasmic acetylcholine concentration is artificially raised by placing synaptosomes in acetylcholine solutions and then transferring them to an acetylcholine-free medium by centrifuging or gel filtration, the synaptosomal acetylcholine declines much more slowly than would be expected from the rate of hydrolysis of similar concentrations of exogenously applied acetylcholine.

The slower labelling of vesicular acetylcholine relative to synaptosomal cytoplasmic acetylcholine implies either that acetylcholine is synthesized from choline in the cytoplasm and is then transferred to the vesicles by a rate-limiting mechanism that has so far not been duplicated *in vitro* (Marchbanks, 1968), or that synthesis occurs simultaneously in both compartments but at a lower overall rate in vesicles. The former model is consistent with the observation (Fonnum, 1967) that choline acetyltransferase, the enzyme that catalyses the synthesis

of acetylcholine from acetyl-CoA and choline, is localized exclusively in the synaptosome cytoplasm. Evidence to the contrary in some species (McCaman, Arnaiz & de Robertis, 1965; Tuček, 1967) is explained by the non-specific adsorption of the enzyme to membranes in solutions of low ionic strength; at the presumed pH and ionic strength of the synaptosome cytoplasm, choline acetyltransferase is soluble in all species examined (Fonnum, 1967, 1968). No alternative biosynthetic pathway for acetylcholine has been described.

While this work was in progress, Potter (1968) reported the labelling of vesicular acetylcholine after the administration of radioactive choline *in vivo* to rats. However, Potter (1968) did not measure the specific radioactivity of his vesicular acetylcholine and apparently overlooked the fact that under his experimental conditions the most highly labelled fraction of acetylcholine (the synaptosomal cytoplasmic fraction) is destroyed.

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