Biochem. J. (1972) 130, 1063–1080
Printed in Great Britain

Choline Metabolism in the Cerebral Cortex of Guinea Pigs

STABLE-BOUND ACETYLCHOLINE

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(*Received* 24 *August* 1972)

1. The turnover of synaptosomal (vesicular-cytoplasmic) and stable-bound (vesicular) acetylcholine isolated from cortical tissue was investigated after the administration, under local anaesthesia, of [*N-Me*-^{3}H]choline into the lateral ventricles of guinea pigs. 2. Radioactive acetylcholine and choline present in acid extracts of subcellular fractions were separated by a combination of liquid and column ion-exchange and thin-layer chromatography. 3. The specific radioactivity and pattern of labelling of acetylcholine present in a fraction of monodisperse synaptic vesicles was found to be essentially the same as that of synaptosomal acetylcholine. 4. The specific radioactivity of stable-bound acetylcholine present in partially disrupted synaptosomes (fraction *H*) at short times (10–20 min) after the injection of [*N-Me*-^{3}H]choline was very variable and inversely related to the yield of acetylcholine in that fraction. 5. Evidence was found for the existence of two small, but highly labelled pools of acetylcholine, one which could be isolated in fraction *H* and the other which was lost when synaptosomes, after isolation by gradient centrifugation, were left at 0°C or pelleted. 6. It is concluded that the results are best explained by metabolic differences among the nerve-ending compartments (thought to be vesicles) which contain stable-bound acetylcholine. Computer simulation of our experiments supports this possibility and suggests that the highly labelled pool in fraction *H* is present in vesicles close to the external membrane.

Investigations of the subcellular distribution of endogenous brain acetylcholine have given rise to the hypothesis (Whittaker, 1965) that three forms of acetylcholine exist in brain tissue: free, present in cell body cytoplasm; osmotically resistant (stable-bound), present in synaptic vesicles; and osmotically labile (labile-bound), present in nerve-ending cytoplasm (Whittaker *et al.*, 1964). These pools along with their presumed subcellular localization are summarized in Table 1.

The three-pool hypothesis was tested by Chakrin & Whittaker (1969) in an investigation of the subcellular distribution of [*N-Me*-^{3}H]acetylcholine ([^{3}H]-acetylcholine) synthesized by brain tissue *in vivo* in guinea pigs anaesthetized with sodium pentobarbital. The specific radioactivities of the various forms of acetylcholine isolated (see Scheme 1) from tissue excised 1 h after intracerebral administration of [^{3}H]choline were significantly different. Labile-bound (synaptosomal cytoplasmic) had the highest, stable-bound (monodisperse synaptic vesicular) acetylcholine the next highest and free (cell body cytoplasmic) acetylcholine had the lowest specific radioactivity. It is most unlikely that these results would have obtained from the redistribution of a single pool during fractionation.

Chakrin *et al.* (1972) and L. W. Chakrin, J. F. Mitchell & V. P. Whittaker (unpublished work, cited by Whittaker, 1969) attempted to determine the subcellular site (usually assumed to be synaptic vesicles) from which acetylcholine is released upon stimulation. They compared the specific radioactivity of acetylcholine released from the exposed occipital cortex of anaesthetized rabbits upon stimulation of the lateral geniculate body with those of the synaptosomal cytoplasmic and vesicular pools isolated at the end of the collection period. They found that the specific radioactivity of released acetylcholine was much closer to that calculated for the synaptosomal cytoplasmic pool than to that of the monodisperse synaptic vesicles (fraction *D*), a result not obviously compatible with the vesicle hypothesis and strongly suggesting that the released transmitter is derived from a pool other than the stable-bound vesicular pool as represented by fraction *D*.

Evaluation of these experiments is complicated because the animals used were anaesthetized with barbiturates and eserine was used to stabilize released

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Table 1. *Compartments of brain acetylcholine*

For nomenclature of fractions see Scheme 1.

Type of acetylcholine	Fraction	Presumed location
(1) Free acetylcholine	S_3 from eserinized homogenate	Cytoplasm from cell bodies or disrupted synaptosomes from cholinergic neurons
(2) Bound acetylcholine	P_2 or B from non-eserinized homogenate	Synaptosomes
(*a*) 'labile'	O from eserinized P_2 or B	Synaptosomal cytoplasm
(*b*) 'stable'	D from non-eserinized P_2 or B	Synaptic vesicles
	H from same	Synaptic vesicles in partially disrupted synaptosomes

acetylcholine. Barbiturates, on the one hand, have been shown to decrease the turnover of brain acetylcholine (Schuberth *et al.*, 1969), increase free and labile-bound acetylcholine (Beani *et al.*, 1969) and decrease the mean quantum content in spinal monosynaptic pathways (Weakly, 1969). Eserine, on the other hand, inhibits the synaptosomal choline transport system (Marchbanks, 1969; Diamond & Kennedy, 1969) and also the uptake of acetylcholine by brain slices (Schuberth & Sundwall, 1967; Liang & Quastel, 1969) and synaptosomes (Marchbanks, 1969).

We therefore decided to investigate choline metabolism at nerve endings *in vivo* further, using an experimental approach that would circumvent these objections. Conscious rather than anaesthetized guinea pigs were used and the subsequent isolation of subcellular fractions was conducted in the absence of eserine. [*N-Me*-^{3}H]Choline ([^{3}H]choline) was administered intraventricularly into guinea pigs under local anaesthesia. In this paper we present the results for acetylcholine; the turnover of phosphorylcholine and choline-containing phospholipids in the various subcellular fractions was also followed and is the subject of the accompanying paper (Dowdall *et al.*, 1972).

Summaries of this work have been published (Barker *et al.*, 1970, 1972; Whittaker, 1971).

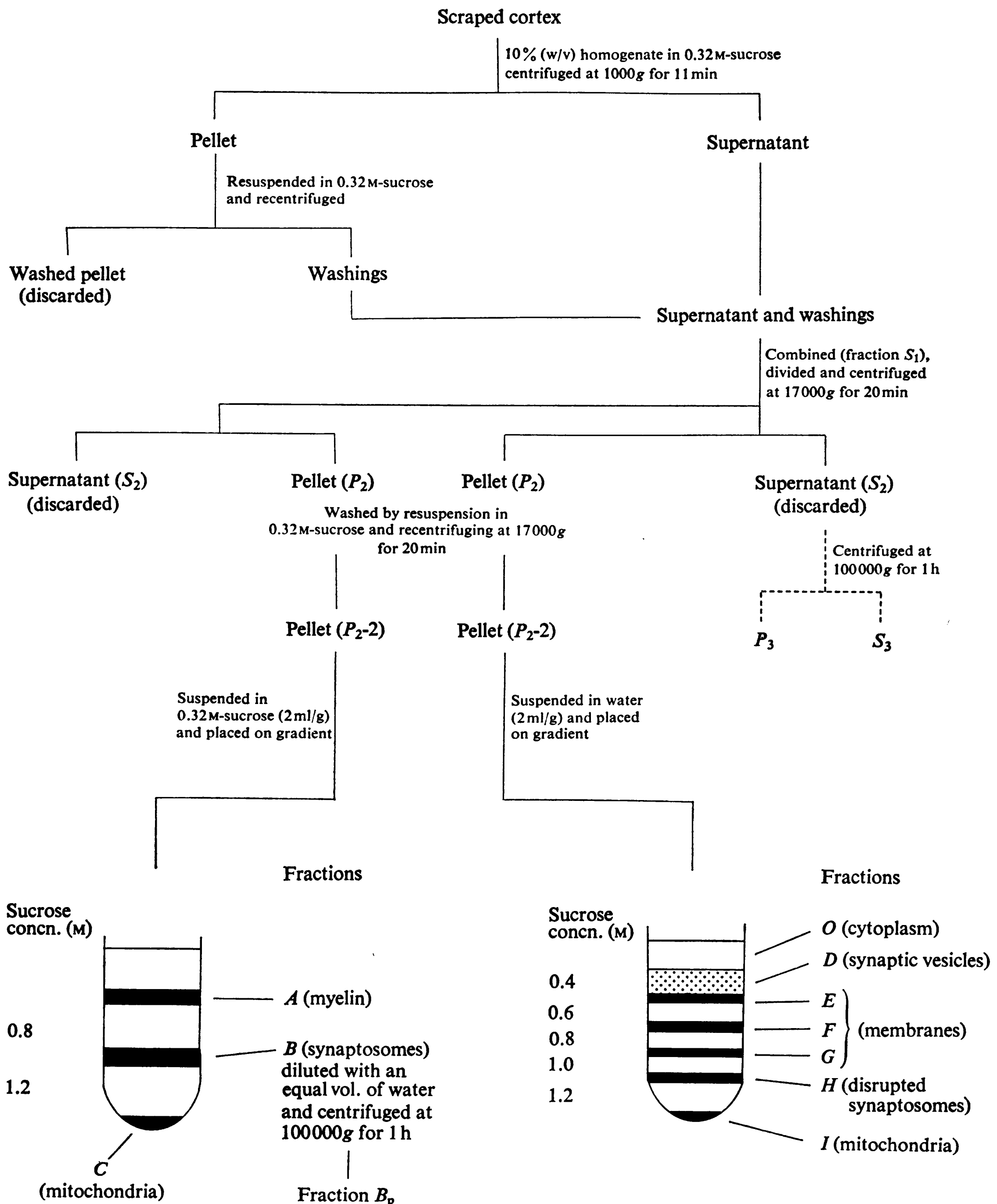
Methods

Labelling and separation of acetylcholine pools

Surgical preparation. Male guinea pigs (350–400 g) were placed in an animal jar and anaesthetized with diethyl ether or 2,2-dichloro-1,1-difluoroethyl methyl ether (methoxyflurane; Penthrane, Abbott Laboratories, Chicago, Ill., U.S.A.). After loss of consciousness, the scalp was shaved and the animal transferred to a small-animal stereotaxic frame (David Kopf Instruments, Tujunga, Calif. 91042, U.S.A.; cat. no. 400). Anaesthesia was maintained with diethyl ether or methoxyflurane by means of an open-drop procedure. A mid-line incision was made on the scalp and the calvarium exposed. The stereotaxic coordinates (8.6 mm anterior and 2 mm lateral; Luparello, 1967) which lie over each lateral ventricle were located. At these points, a small hole was made in the calvarium with a size 0 dental drill. Care was taken not to pierce the meninges. The holes were filled with bone wax and the surfaces of the wax marked with black ink to permit future identification of the site. Then the scalp incision was closed with a wound clip, and the animals allowed to recover.

Intraventricular injection. The animals were used 4–7 days after the surgical procedure. The region of the initial scalp incision was anaesthetized by local cooling and the wound re-opened. The exposed region was immediately bathed with a 2% (w/v) solution of procaine hydrochloride (Ambot Solution; Cutter Laboratories, U.S.A.). When regional anaesthesia was complete, the filled-in holes were located. From 30 to 45 min later, 10 μCi of [*N-Me*-^{3}H]choline chloride (Amersham–Searle, Chicago, Ill., U.S.A.; specific radioactivity 150–215 mCi/mmol) in 50 μl of aq. 0.9% (w/v) NaCl was injected into each ventricle by inserting the needle of a 50 μl Hamilton syringe into each hole to a depth of 4 mm and then injecting the contents of the syringe into the ventricle. A polyethylene guard on the needle prevented injections being made at depths greater than 4 mm. At various times after the injection, the animals were killed; the forebrains were removed, and the cortices were separated, scraped free of white matter, weighed and homogenized (Gray & Whittaker, 1962) in enough ice-cold 0.32 M-sucrose to give a 10% (w/v) homogenate.

Isolation of subcellular fractions. Synaptosomes and subfractions of an osmotically shocked crude synaptosome preparation were isolated simultaneously (Scheme 1) in a cold laboratory at 0–4°C by a



Scheme 1. *Isolation of synaptosomes and synaptic vesicles*

The broken lines identify fraction S_3, referred to in Table 1 but not used in the present work.

modification of the procedures of Gray & Whittaker (1962), Whittaker *et al.* (1964) and Chakrin & Whittaker (1969) as follows.

The first pellet (Scheme 1) obtained by centrifugation of the homogenate at 1000*g* (Sorvall RC-2 centrifuge, $r_{av.}$ 7.8cm) for 11 min (*g* values are averages) was washed only once; the washing was added to the initial supernatant to give fraction S_1. The volume of the combined supernatants was measured and then the supernatant was divided approximately 4:1. A crude synaptosomal pellet, P_2, and supernatant, S_2, were obtained from each portion by centrifuging at 17000*g* for 20min. The resulting pellets were washed once by resuspension in 0.32M-sucrose and re-sedimentation as described above to give fraction P_2-2. The pellet obtained from the major portion of S_1 was used for the isolation of synaptosomal subfractions. It was osmotically disrupted by suspending it in double-distilled water (2ml/g of tissue) and layering it over a density gradient consisting of equal volumes of 0.4, 0.6, 0.8, 1.0 and 1.2M-sucrose. The pellet derived from the minor portion of fraction S_1 was used for the isolation of synaptosomes. It was resuspended in 2ml of 0.32M-sucrose and layered over a discontinuous density gradient consisting of equal volumes of 0.8 and 1.2M-sucrose. The density-gradient separations of the disrupted and undisrupted preparations were done simultaneously in a SW 27 rotor of a Beckman L2-65B preparative ultracentrifuge by centrifugation at 57500*g* ($r_{av.}$ 11.7cm) for 2h. The resulting fractions (*O–I*) were removed by aspiration. Particular attention was paid to fractions *D* (synaptic vesicles) and *H* (disrupted synaptosomes that contain some synaptic vesicles and intraterminal mitochondria, but have largely lost their soluble cytoplasm). The purified synaptosome fraction (*B*) was diluted with an equal volume of cold water and pelleted by centrifugation in a no. 65 rotor at 100000*g* ($r_{av.}$ 5.7cm) for 60min. The pellet (B_p) was separated from its supernatant (B_s) and resuspended in 1ml of 0.02M-NaH_2PO_4; the pH was adjusted to 4.0. Other fractions were left suspended in sucrose solutions.

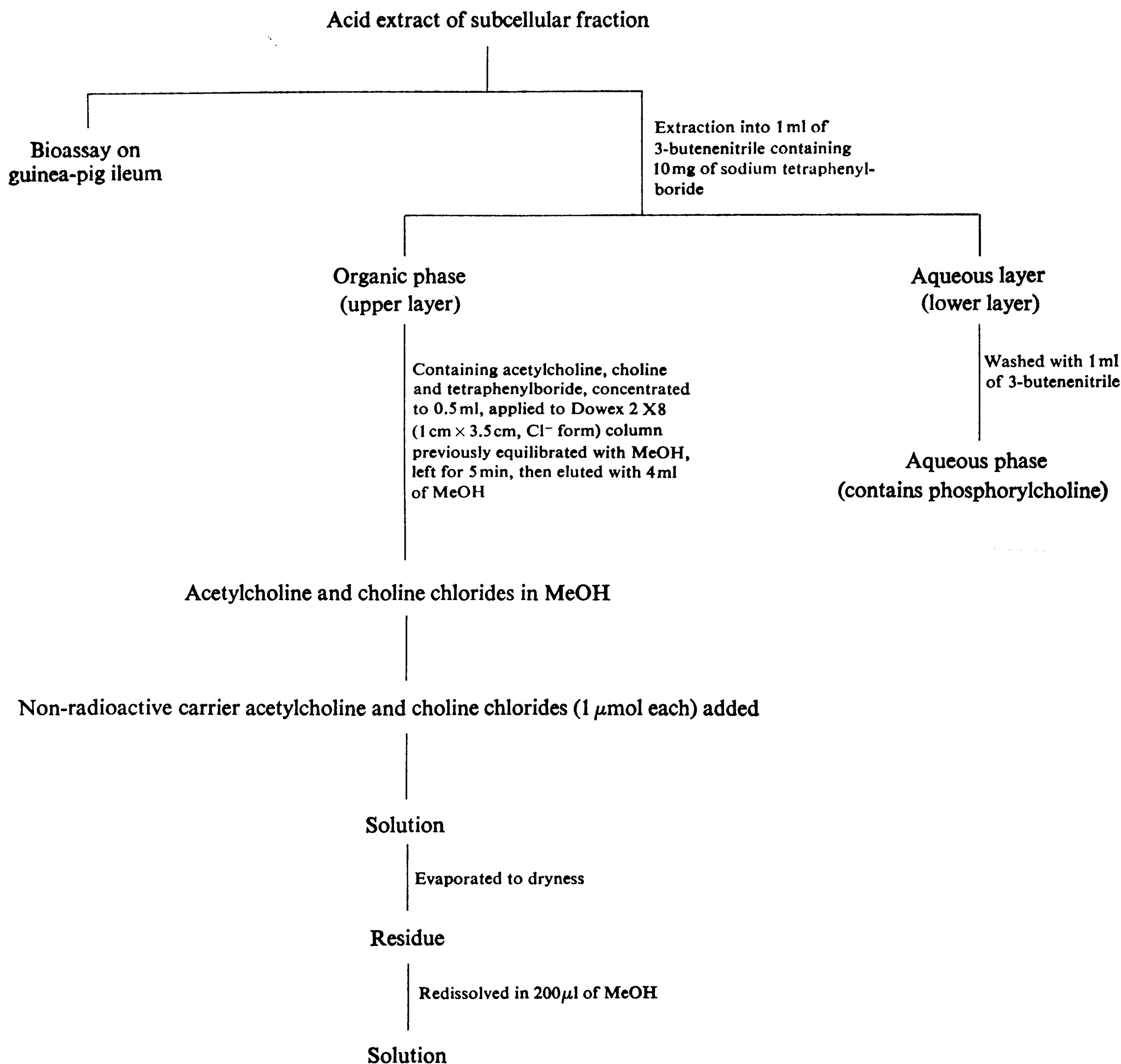
Application of graded osmotic shock. Samples of fraction P_2 prepared (Scheme 1) from guinea pigs killed 10min after injection with [^{3}H]choline (20μCi) were suspended in ice-cold 0.16M- or 0.032M-sucrose or water (3ml/g of tissue) containing eserine sulphate (100μg/ml) to conserve any acetylcholine liberated by these treatments. After 15min at 0–4°C the sucrose concentrations of the suspensions were adjusted where necessary to 0.16M and the released cytoplasm separated from the disrupted synaptosomes and other particulate material by centrifuging at 150000*g* for 1h or 60000*g* for 2h.

Extraction of bound acetylcholine. Bound acetylcholine was extracted from subcellular fractions by adjusting the pH of the suspensions to 4.0 and heating at 100°C for 10min (Hebb & Whittaker, 1958). After this the samples were centrifuged in a Sorvall centrifuge at 2000*g* for 20min to precipitate insoluble material. The pellets were washed once by resuspension in 1.0ml of 0.02M-NaH_2PO_4 and re-centrifugation as described above.

Analytical methods

Bioassay of acetylcholine and determination of specific radioactivity. The specific radioactivity of acetylcholine present in the various fractions examined was determined on acidic extracts of the fractions. Acetylcholine present in the extracts was assayed on the superfused guinea-pig ileum by using small samples and authentic acetylcholine perchlorate as a standard (for details see Whittaker & Barker, 1971). The following criteria were used to establish that the biological activity was due to acetylcholine. The activity was destroyed by treating a neutralized (pH7) sample with bovine erythrocyte acetylcholinesterase or by heating at pH10–12 for 10min; also, it was abolished or decreased proportionally to the activity of the standard acetylcholine solution by treating the ileum with atropine sulphate (0.1–0.2mg/l of Tyrode solution).

To separate radioactive acetylcholine, t.l.c. was found to be more sensitive and convenient than the column chromatographic method of Chakrin & Whittaker (1969). The acetylcholine and choline present in the major portion of the extract were transferred (Scheme 2) from the aqueous phase to 3-butenenitrile by extraction (Fonnum, 1969) with this solvent (1ml) to which sodium tetraphenylboride (10mg/ml) had been added. The two-phase system was lightly shaken by hand for 3min; the phases were then separated by centrifugation at 2000*g* for 15min in a MSE Mistral 6L refrigerated centrifuge. The upper, organic, phase was removed by aspiration and transferred to a small vial. The lower phase was washed with 1ml of 3-butenenitrile and the phases again separated as described above. The upper phase from the wash was added to the initial upper phase. The 3-butenenitrile containing acetylcholine and choline tetraphenylborides was concentrated to about 0.5ml by heating in a sand bath (60–70°C) while blowing a gentle stream of air over the surface of the liquid. To exchange the tetraphenylboride for chloride, the concentrate was applied to an anion-exchange column (Dowex 2X8, chloride form, column dimensions 1cm × 3.5cm) and eluted with a total of 4ml of methanol. The first 2ml were used to rinse out the vial which had contained the 3-butenenitrile phase. Unlabelled carrier acetylcholine and choline chloride (1μmol each in 200μl of methanol) were added to the eluate. The eluate was evaporated to dryness and stored overnight at −20°C. Next day, the dried material was dissolved in 200μl of methanol.



Two 10μl samples were used for t.l.c. Samples were streaked on plate in narrow bands (2mm) and chromatographed on cellulose (Eastman Chromagrams) in butan-1-ol–methanol–acetic acid–water (8:2:1:3, by vol.). After development of the chromatogram, the bands were detected with I_2 vapour, outlined in pencil and, after allowing the I_2 to evaporate, scraped into a Pasteur pipette and eluted with 2×1ml of MeOH–1M-HCl (19:1, by vol.) into a scintillation vial containing 1 mg of choline chloride. Scintillation medium was added directly to this and after cold adaption the samples were counted for radioactivity.

Scheme 2. *Determination of acetylcholine and separation of radioactive acetylcholine from other radioactive products of choline metabolism*

The radioactive compounds present in 10μl of the solution were separated by t.l.c. on cellulose layers (Eastman Chromagram Sheet 6064) in butan-1-ol–methanol–acetic acid–water (8:2:1:3, by vol.). The choline and acetylcholine bands were stained by exposure to iodine vapour and outlined in pencil. After the iodine had evaporated, the bands containing acetylcholine and choline were scraped off the sheet and the scrapings transferred to a Pasteur pipette which had been plugged with glass wool. Acetylcholine or choline were then eluted from the cellulose scrapings into a scintillation vial with 2×1ml of

methanol–1M-HCl (19:1, v/v); 1mg of choline chloride (in 0.2ml of methanol) and 10ml of scintillation solution [toluene–Triton X-100, 7:3 v/v, containing 2,5-diphenyloxazole (5.4g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.108g/l)] were then added. The radioactivity was measured by liquid scintillation counting in a Packard Tri-Carb 3380 scintillation counter with an Absolute Activity Analyser. The efficiency of counting was about 25%. Duplicate chromatographic separations and determinations of radioactivity were performed on each extract. The specific radioactivity of the acetylcholine was calculated (as d.p.m./pmol) from the results of the radioactivity determinations and bioassays.

Lactate dehydrogenase activity. This was measured spectrophotometrically (Whittaker *et al.*, 1964). Mitochondrial oxidation of $NADH_2$ was inhibited by KCN (1mM). The endogenous oxidation of $NADH_2$ by the preparation was determined for 30s; the substrate, sodium pyruvate, was then added and measurements continued for a further 1–2min. Results are expressed as ΔE_{340}/min at 25°C and are corrected for the small amount of activity present in the absence of added sodium pyruvate.

Protein. This was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Potassium. This was determined on a Perkin Elmer model 290B atomic absorption spectrophotometer. Neither sucrose, sodium nor protein in concentrations present in the samples at the dilutions used interfered with the determination of potassium.

Statistical expression of results. Unless otherwise stated, values are means±S.E.M. (three or more experiments) or range (two experiments) followed by the number of experiments in parentheses. Values of probability (P) were obtained from two-tailed t tests.

Results

Extraction and chromatography of acetylcholine and choline

Authentic [N-Me-^{3}H]choline and [N-Me-^{3}H]-acetylcholine were added to acid extracts of a preparation of fraction P_2 derived from 750mg of tissue. The extracts were carried through the procedure described in Scheme 2. The recoveries after the final ion-exchange step were 97.5±0.5% (3) for acetylcholine and 95% (1) for choline. Labelled choline was similarly extracted from 0.02M-NaH_2PO_4. The average recovery was 95±0.5% (6). The average recovery of total radioactivity after t.l.c. was 94±3% (13). Overall recoveries were thus about 87%.

Control experiments were performed to assess the contribution of labelled choline to radioactivity present in the acetylcholine band. Labelled choline was co-chromatographed with 50nmol each of unlabelled acetylcholine and choline. No radioactivity was found in the acetylcholine band when 35–2300d.p.m. of labelled choline was present. Cochromatography with approximately 5μCi of labelled choline (approx. 24nmol) showed an overlap of 0.15%. Treatment of neutralized acid extracts of tissue containing labelled acetylcholine with acetylcholinesterase or alkaline hydrolysis of the final methanolic extract (1M-NH_3 in methanol, 30min at room temperature) resulted in a total disappearance of radioactivity in the acetylcholine band and a proportional increase in radioactivity chromatographing with choline. Thus under the conditions employed for the chromatographic separation of acetylcholine from choline, the acetylcholine band is not significantly contaminated with other radioactive material.

Fate of choline at the nerve ending

The radioactivity present in nerve endings at various times after the administration of [^{3}H]choline was distributed mainly among choline, acetylcholine, phosphorylcholine, phosphatidylcholine and sphingomyelin. Radioactive betaine was not detected. This is not unexpected, since choline oxidase is not present in guinea pigs (Mann & Quastel, 1937; Bernheim & Bernheim, 1933). The metabolic fate of choline at the nerve ending is summarized in Fig. 2 of the accompanying paper (Dowdall *et al.*, 1972), from which it will be seen that acetylcholine is the first compound to be formed from choline.

Turnover of bound acetylcholine at the nerve ending

The time-course of the labelling of bound acetylcholine in fraction B_p (synaptosomes) and of stable-bound acetylcholine in fractions D (monodisperse synaptic vesicles) and H (partially disrupted synaptosomes) is shown in Figs. 1*a*, 1*b* and 1*c* respectively. Maximum labelling of all fractions occurs between 5 and 10min; the labelling of acetylcholine in monodisperse synaptic vesicles is quite similar to that in the intact nerve ending as represented by fraction B_p. However, the labelling in fraction H is on the average at 10 and 20min after injection greater but also much more variable than that observed in monodisperse synaptic vesicles or synaptosomes.

The amount of endogenous acetylcholine in fraction H varied by a factor of about 6 in animals killed 10min after injection and by a factor of about 8 in those killed 20min after injection, whereas the yield of [^{3}H]acetylcholine varied by factors of 3 and 1.5 respectively in such animals. Plots of the specific radioactivity against the amount of endogenous acetylcholine in fractions H, D and B_p (Figs. 2*a*, 2*b* and 2*c*) suggest that at 10 and 20min in fraction H and possibly at 10min in fraction D and at 5min in fraction B_p) a reciprocal relationship may exist be-

tween these two quantities. Least-squares analysis of the reciprocal of the specific radioactivity and the amount of endogenous acetylcholine in these fractions (Figs. 3*a*, 3*b*, 3*c* and 3*d*) confirms that such a relationship exists at 10 and 20min in fraction *H* [$r = 0.96$, $P<0.01$ (10min); $r = 0.93$, $P<0.001$ (20min)] but not in fraction *D* at 10min ($r = 0.80$, $P>0.05$) or B_p at 5min ($r = 0.48$, $P>0.4$). A reciprocal relationship of this kind can be explained by assuming that there are at least two pools of acetylcholine in fraction *H*, a relatively constant small pool of rapidly labelled acetylcholine and a larger, variable pool of more slowly labelled transmitter, similar to that found in fraction *D*. The reciprocal relationship will be clearly seen only at relatively short time-intervals (5–20min) after injection of [^{3}H]choline, when the difference in the labelling of the two pools is close to maximum. At these time-intervals, variations from experiment to experiment of the proportion of the less highly labelled pool present in fraction *H* will have a large effect on the specific radioactivity of its acetylcholine, thus accounting for the large variance in

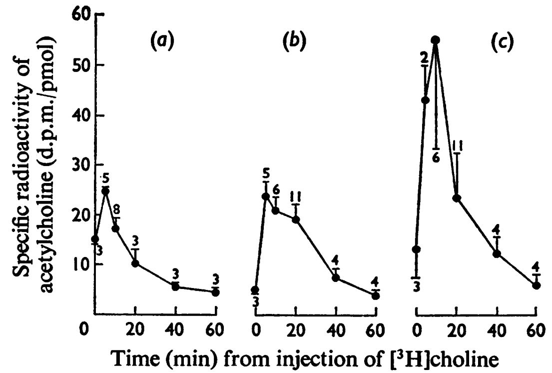


Fig. 1. *Time-course of labelling of brain acetylcholine pools*

(*a*) Synaptosomes (fraction B_p); (*b*) vesicles (fraction *D*); (*c*) partially disrupted synaptosomes (fraction *H*). The points and bars are the means ± S.E.M. (or range for mean of two) of the numbers of experiments given for each point.

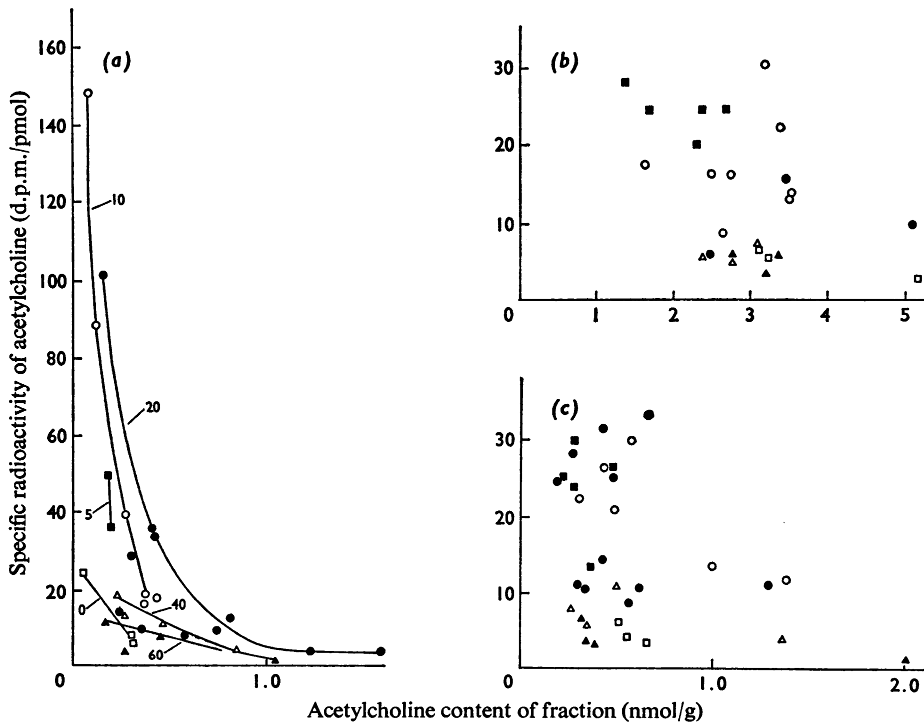


Fig. 2. *Relationship between endogenous acetylcholine and specific radioactivity of acetylcholine in fractions B_p, D and H*

Symbols refer to animals killed 0 (□), 5 (■), 10 (○), 20 (●), 40 (△) and 60 (▲) min after injection of [^{3}H]choline. (*a*) Fraction *H*; (*b*) fraction B_p; (*c*) fraction *D*. Note that the abscissae for fractions *D* and *H* are on a scale 2.5 times that for fraction B_p and that the ordinates for fractions *D* and B_p are on a scale twice that for fraction *H*.

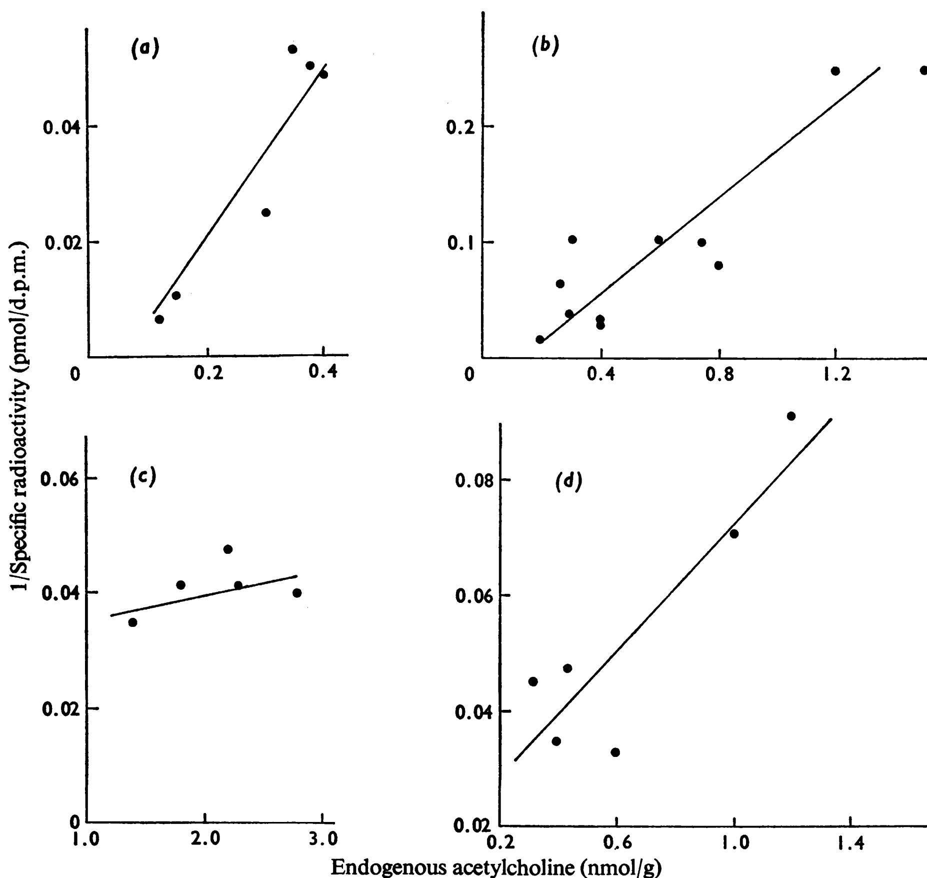


Fig. 3. *Least-squares analysis of reciprocal relationship between endogenous acetylcholine and specific radioactivity of subcellular fractions*

(*a*) Fraction *H* at 10min, (*b*) at 20min; (*c*) fraction B_p at 5min; (*d*) fraction *D* at 10min. Only in (*a*) and (*b*) are the slopes of the regression lines through the points significantly different from zero (for further details see text).

Table 2. *Specific radioactivity of bound acetylcholine at various stages of isolation*

For nomenclature of fractions and definition of enzyme action see Scheme 1 and the text. Results are for a typical experiment in which fractions were isolated from animals killed 10min after injection of [^{3}H]choline. Values relate to the amount of fractions derived from 1g of tissue. Dashes indicate that the quantity concerned was not measured.

				Acetylcholine		
					Specific radioactivity (d.p.m./pmol)	
Fraction	Protein (mg/g)	Lactate dehydrogenase (ΔE_{340}/min per g)	Potassium (μg-atom/g)	Endogenous (nmol/g)	In fraction	Lost from fraction
Homogenate	89.0	76.8	71.5	12.60	14.4	—
S_1	59.5	72.5	61.3	8.40	15.5	12.3
P_2	28.9	21.3	17.1	7.63	14.3	27.3
P_2-2	24.8	16.8	9.1	6.15	14.2	14.7
B	15.5	9.5	5.6	3.65	16.8	10.4
B_p	—	—	—	3.50	12.8	110.5
B_s	0.5	0	1.3	0	—	—

Table 3. *Loss of a highly labelled pool of acetylcholine on separating and sedimenting synaptosomes*

Details are as in Table 2.

Fraction	Acetylcholine of fraction: Concentration (nmol/g)	Acetylcholine of fraction: Sp. radioactivity (d.p.m./pmol)	Mean specific radioactivity of lost acetylcholine (d.p.m./pmol)
P_2	6.8±0.8 (3)	17.8±3.1 (3)	—
B	3.4±0.2 (4)	18.0±5.1 (5)	17.5
B_p	3.1±0.2 (6)	15.0±1.82 (6)	44.3

Table 4. *Effect of sedimentation and of exposure to hyperosmotic sucrose on the specific radioactivity of synaptosomal acetylcholine*

Specific radioactivities of lost acetylcholine are calculated from the differences in endogenous and radioactive acetylcholine between the control values for fraction B taken immediately after isolation (upper line in each experiment) and the values for a sample of fraction B that had been sedimented (fraction B_p, expts. 1–4, 5*a*), exposed to hyperosmotic sucrose for 1 h (fraction $B_{1\,h}$, expts. 5*b*–5*d*, 6*a*,*b*) or exposed to hyperosmotic sucrose for 1 h and then pelleted [fraction $B_{(1\,h)p}$, expt. 6*c*]. Values given in parentheses are the specific radioactivity of injected choline: the calculated values would have been infinite since the endogenous acetylcholine concentrations were below the limit of detection. For further details see the text and Scheme 1. Fractions were prepared from animals killed 10min after intraventricular injection of [^{3}H]choline. Units of acetylcholine: endogenous, nmol/vol. of fraction derived from 1 g of tissue; radioactive, d.p.m./vol. of fraction derived from 1 mg of tissue.

Expt. no.	Fraction	Synaptosomal acetylcholine: Endogenous (nmol/g)	Synaptosomal acetylcholine: Radioactive (d.p.m./mg)	Synaptosomal acetylcholine: Sp. radioactivity* (d.p.m./pmol)	Lost acetylcholine: Endogenous (nmol/g)	Lost acetylcholine: Radioactive (d.p.m./mg)	Lost acetylcholine: Sp. radioactivity (d.p.m./pmol)
1	B	3.15	98.38	31.3	0.02	—†	—
	B_p	3.13	98.09	31.3			
2	B	3.65	60.88	16.8	0.15	15.88	100
	B_p	3.50	45.00	12.8			
3	B	2.91	39.15	13.6	0.26	16.20	62
	B_p	2.65	22.95	8.6			
4	B	2.09	30.92	14.8	0.25	7.48	30
	B_p	1.84	23.44	12.8			
5*a*	B	3.14	98.38	31.2	0.04	14.68	300
	B_p	3.10	92.70	26.2			
5*b*	B	3.14	98.38	31.2	0.05	10.59	212
	$B_{1\,h}$	3.09	87.79	28.4			
5*c*	B	3.14	98.38	31.2	—	10.13	(475)
	$B_{1\,h}$	3.14	88.25	28.1			
5*d*	B	3.14	98.38	31.2	—	8.78	(475)
	$B_{1\,h}$	3.14	89.60	28.4			
6*a*	B	1.73	53.36	30.6	0.10	7.97	79
	$B_{1\,h}$	1.63	46.36	27.7			
6*b*	B	1.73	53.36	30.6	0.10	9.22	92
	$B_{1\,h}$	1.63	44.12	26.9			
6*c*	B	1.73	53.36	30.6	0.10	24.46	244
	$B_{(1\,h)p}$	1.63	28.90	17.7			

* The mean difference in the specific radioactivity of B and B_p is 3.1; B and B_p are significantly different (paired t test, $P<0.02$). The corresponding difference between B and $B_{1\,h}$ is 3.06 and is also significant in a paired t test ($P<0.002$).

† Difference within error of counting.

Table 5. *Release of cytoplasmic markers and acetylcholine from synaptosomes by graded osmotic shock*

For more than one determination, values are means±range (two determinations) or ±S.E.M. (more than two). The number of determinations, when more than two, is given in parentheses. For methods see the text.

Component released	Units	Concn. in fraction P_2 (units/g)	Recovery (%)	% released in cytoplasm after suspension in medium indicated: 0.16M-Sucrose	0.032M-Sucrose	Water
Protein	mg	22.4±1.0 (6)*	—	11±0	28±1	30±2
Potassium	μg-atom	15.3±0.8 (6)	105±6 (6)	28±6	77±1	78±2
Lactate dehydrogenase	ΔE_{340}/min	42.7±0.9 (6)	93±4 (6)	4±0	60±4	67±0
Acetylcholine						
Endogenous	nmol	8.7±0.8 (4)	88±23 (4)	13±1	—	24±9
Radioactive	10^{-3}×d.p.m.	63 ±2	93±5	17	—	58
Specific radioactivity	d.p.m./pmol	6.3±0.2†	—	7.8†	—	13.3†

* Estimated from sum of fractions.
† d.p.m./pmol, not per g or %.

the peak values in Fig. 2*a*. The identity of the two pools is considered in the Discussion section and a theoretical treatment of a two-pool model is given in the Appendix.

Investigations of the highly labelled acetylcholine

The possible existence of a very small, highly labelled pool of acetylcholine in fraction *H* led us to compare the specific radioactivity of bound acetylcholine at various stages of the isolation procedure. This comparison (Tables 2 and 3) suggests the existence of a small highly labelled pool of acetylcholine which escaped notice in experiments involving only the measurement of endogenous acetylcholine. This pool is apparently largely retained (Table 2) up to the point when synaptosomes (fraction *B*) are sedimented (fraction B_p) (Table 2, line 6), since the calculated specific radioactivity of the acetylcholine lost at each stage (column 7) is close to that of the parent fraction. Then, when fraction B_p is formed, considerable amounts of radioactive acetylcholine are lost, although the decline in the endogenous acetylcholine is quite small. This implies that the loss occurs preferentially from a highly labelled pool of acetylcholine. This point is further substantiated in Table 3. Subsequent experiments (Table 4) showed that this observation is reproducible and that the loss could occur on standing for 1h at 0°C with [$B_{(1\,h)p}$] or without ($B_{1\,h}$) pelleting (Table 4, expts. 5 and 6). Treatment of fraction *B* with 1.14 to 57nmol/ml of hemicholinium-3 for 1h at 0°C did not significantly affect the loss of [^{3}H]acetylcholine that occurred, suggesting that the choline carrier was not involved.

Examination of the supernatant fraction (B_s) obtained when synaptosomes were pelleted (Table 2) showed that no detectable lactate dehydrogenase was released; however, 20±3% (2) of the synaptosomal potassium was released. The recovery of radioactive acetylcholine and choline in fractions B_p and B_s was 95±4% (5) of fraction *B*. Only [^{3}H]-choline was detected in fraction B_s; any acetylcholine released would have been destroyed by the acetylcholinesterase present in the preparation.

Several attempts were made to dislodge the highly labelled pool at the P_2-2 stage (Scheme 1). The following treatments were tried, but were unsuccessful: (1) exposure to hyperosmotic (0.8M) sucrose; (2) treatment with 20mM-KCl–280mM-sucrose; (3) treatment with 50μM-eserine–0.32M-sucrose; (4) keeping in 0.32M-sucrose; (5) gel filtration of P_2-2 in 0.32M-sucrose through Bio-Gel P-10 that had been equilibrated with 0.32M-sucrose. Treatments (1)–(4) were all for 1h at 0°C followed by dilution with an equal volume of water [treatment (1) only] and centrifuging at 100000g (Beckman no. 40 rotor, $r_{av.}$ 5.9cm) for 30min.

The effect of graded osmotic shock on fraction P_2 was also studied. Table 5 shows that the release of potassium and lactate dehydrogenase from synaptosomes was proportional to the severity of the osmotic shock and that potassium leaked out more completely than lactate dehydrogenase, as would be expected from their relative molecular sizes. Table 5 also shows that the specific radioactivity of the acetylcholine that leaked out as a result of mild osmotic shock (exposure to 0.16M-sucrose) and was therefore most likely to consist of cytoplasmic acetylcholine uncontaminated by vesicular acetylcholine, had a specific radioactivity close to that of the parent fraction. The specific radioactivity of the acetylcholine released by suspension in water had a somewhat

higher value, suggesting that under these more disruptive conditions a small amount of acetylcholine from the highly labelled pool might have been released along with the cytoplasmic acetylcholine.

Discussion

The two-pool model for stable-bound acetylcholine

The most interesting results in the present work are those suggesting the existence of two small highly labelled pools of acetylcholine, one of which (pool 1*a*) appears to be lost when synaptosomes are pelleted or are left, and the other of which (pool 1*b*) is apparently present in fraction *H*. It was hoped to find out whether these pools are identical by noting whether a fraction *H* prepared from a fraction P_2 from which pool 1*a* had been discharged still contained its highly radioactive acetylcholine. This approach was unsuccessful since all attempts to remove pool 1*a* from fraction P_2 failed. It was also felt that observations on a fraction *H* prepared from fraction B_p would be somewhat inconclusive since synaptosomes separated on a sucrose density gradient are known to be more resistant to osmotic disruption than those of fraction P_2. However, the similarity in the specific radioactivities of the two putative pools suggests that they are two manifestations of a single phenomenon.

It seems unlikely that either pool 1*a* or pool 1*b* is cytoplasmic in origin. Fraction *H*, as in previous work (Whittaker *et al.*, 1964), had an extremely low content of cytoplasmic markers [only 4.8±S.D. 1.6 (7) % of the potassium and 17.6±S.D. 9.1 (7) % of the lactate dehydrogenase of the parent fraction P_2; see the accompanying paper (Dowdall *et al.*, 1972) for further details]. Cytoplasmic acetylcholine would be expected to behave more like potassium than lactate dehydrogenase, the values for which include non-specifically adsorbed enzyme (Fonnum, 1967). Experiments in which graded osmotic shock was applied to fraction P_2 showed that the labile-bound (presumably cytoplasmic) acetylcholine released along with potassium when fraction P_2 was suspended in either 0.16M-sucrose or water had a specific radioactivity similar to that of the parent fraction and that the most readily diffusible acetylcholine (i.e. that released by suspension in 0.16M-sucrose) had the lower specific radioactivity. This again suggests that the rapidly labelled pool(s) is (are) not cytoplasmic in origin but comprise(s) a fraction of the stable-bound acetylcholine of the synaptosome, metabolically more active than the remainder (pool 2). Fraction *H* is known to consist (Whittaker *et al.*, 1964) of incompletely disrupted synaptosomes that have largely lost their soluble cytoplasmic constituents but contain synaptic vesicles and a few intraterminal mitochondria within ruptured external membranes. It has previously been assumed that the stable-bound acetylcholine of this fraction was accounted for by its content of synaptic vesicles. Conceivably, a pulse of incoming [^{3}H]choline does not mix instantaneously with endogenous choline but forms a cytoplasmic gradient whose specific radioactivity determines that of the acetylcholine formed from it locally. This acetylcholine may be taken up more avidly by synaptic vesicles that are close to the external membrane and therefore more likely to have recently discharged their quantum of transmitter than by more centrally located vesicles that are fully charged and metabolically less active. Both factors would contribute to a gradient in the specific radioactivity of vesicular acetylcholine. If now the highly labelled peripheral vesicles are preferentially carried down into fraction *H* along with external synaptosome membranes, together with various amounts of more centrally located vesicles, leaving the remainder of the centrally located vesicles in fraction *D*, then the specific radioactivity of the acetylcholine in fraction *H* would be expected to show, at the appropriate time interval, just that elevated value (relative to fraction *D*) and that reciprocal relationship to endogenous acetylcholine content that is, in fact, observed. This may be the basis of the now well-documented finding, in a number of systems, cholinergic [superior cervical ganglion (Collier, 1969); diaphragm (Potter, 1970); cerebral cortex (Chakrin *et al.*, 1972, and unpublished work cited by Whittaker, 1969)], adrenergic (Kopin *et al.*, 1968) and dopaminergic (Besson *et al.*, 1969), that the most recently synthesized transmitter is that preferentially released, and for the observations of Hubbard & Kwanbunbumpen (1968) and Jones & Kwanbunbumpen (1970) that there is a stimulation-dependent decrease in a small vesicle population localized near the external membrane at the neuromuscular junction.

To test our hypothesis and to determine the effect of the various variables on the predicted radioactivity of fractions *D* and *H* as a function of time, a rather general theoretical model was developed and its behaviour simulated on a computer. This model provides for variations (*a*) in the rate of uptake of [^{3}H]choline by different synaptosomes, (*b*) in the rate of diffusion of [^{3}H]choline through the cytoplasm, (*c*) in the rate at which radioactivity is incorporated into vesicular acetylcholine and (*d*) in the proportion of peripheral and more centrally placed vesicles recovered in fractions *D* and *H*. The simplifying assumption has been made that there are only two types of vesicle accounting respectively for pools 1 and 2, 'peripheral' (P-type) and 'central' (C-type) differing in the rate at which they take up acetylcholine; however, in reality there might well be a gradient of metabolic activity from the membrane to the centre of the terminal. We are grateful to Dr. J. R. Gradijan and Dr. P.-E. E. Bergner for developing this model. The results are summarized in the

Appendix (Gradijan & Bergner, 1972*b*) and are presented more fully elsewhere (Gradijan & Bergner, 1972*a*).

Briefly, it was found that to simulate the observed time-course of incorporation of [^{3}H]choline into the stable-bound acetylcholine of fractions *D* and *H* and to account for the different variances of these respective time-courses with this model it is necessary to assume: (*a*) that the terminals take up [^{3}H]choline at different rates; (*b*) that incoming [^{3}H]choline does not mix instantaneously with the endogenous cytoplasmic pool; (*c*) that the partition of metabolically more active peripheral and metabolically less active central synaptic vesicles into fractions *H* and *D* varies from experiment to experiment in a random manner. An analogue of this last feature would be difficult (though perhaps not impossible) to incorporate in a model in which the rapidly labelled pool of acetylcholine is assigned to patches on the external membrane rather than to peripheral vesicles.

The model is purely qualitative; no attempt was made to work out the actual proportion of vesicles in the two pools or to fit the curves to the experimental data. However, depending on the value assumed for the mean specific radioactivity of the rapidly labelled pool it is estimated that between 2 and 30% of the vesicles belong to it (for specific radioactivities of pool between 400 and 40d.p.m./pmol).

The source of the highly labelled pool 1*a* lost during the preparation of fractions *B* and B_p may well be the peripheral vesicles since it is well known that hyperosmotic sucrose augments the frequency of quantal release at the neuromuscular junction (Furshpan, 1956). We are unable to say why the highly labelled pool 1*a* is more readily displaced from fraction *B* than from fraction P_2-2, while the reverse appears to be true for cytoplasmic acetylcholine. However, the preparation of fraction *B*, involving as it does intense centrifugal forces and movement through a hyperosmotic sucrose density gradient, may represent a greater total osmotic and mechanical stress than was applied to fraction P_2-2 during the attempts to dislodge the highly labelled pool from this fraction. Conceivably, the proportion of acetylcholine in the various compartments of the surviving synaptosomes may have changed relative to fraction P_2-2 and the acetylcholine lost from pool 1*a* during the final step may be a larger proportion of the total lost at this stage and thus be partially unmasked.

In conclusion, it is clear that the compartments of the nerve terminal, whether defined morphologically (cytoplasm, vesicles) or by the properties of endogenous acetylcholine (labile, stable-bound), cannot be regarded as constituting metabolically homogeneous pools. The vesicles, especially, are in all probability metabolically heterogeneous and their ability to take up recently synthesized (and therefore more radioactive) acetylcholine is determined by their history and their location within the terminal. Microheterogeneity within the vesicles may also exist (Marchbanks & Israël, 1971; Richter & Marchbanks, 1971).

Comparison with previous work

The present findings differ from those of Chakrin & Whittaker (1969) who showed that 1h after intracortical injection of [^{3}H]choline, the specific radioactivity of the acetylcholine in fraction P_2 was about 1.5 times higher than that of fraction *D* and in the limited number of comparisons made, the specific radioactivities of the acetylcholine in fractions *D* and *H* were about the same. They concluded that the acetylcholine of the synaptosome cytoplasm must be about twice as radioactive as that of the vesicles. Somewhat similar labelling patterns were obtained by Richter & Marchbanks (1971) in subcellular fractions isolated from cerebral cortical tissue blocks incubated with [^{3}H]choline before homogenization.

The specific radioactivities obtained by Chakrin & Whittaker (1969) were comparable to ours at 1h; thus the difference in the routes of administration used in the two series of experiments is probably immaterial. The significant difference is probably the use of barbiturate anaesthesia in the earlier series: barbiturates, by decreasing the turnover of brain acetylcholine, increasing labile-bound (synaptosomal cytoplasmic) acetylcholine and decreasing transmitter release from presynaptic terminals (for references see introduction), would have the effect of decreasing transmitter release from, and hence turnover of, transmitter in the peripheral vesicles preferentially sedimented into fraction *H*; they would also promote the formation of cytoplasmic acetylcholine in the terminal and thus increase the labelling of this pool. The labelling pattern in incubated slices and isolated synaptosomes would tend to resemble that of the anaesthetized brain due to the absence of synaptic activity in these systems. Clearly a study of the complete time-course of labelling in anaesthetized animals will be required to resolve these differences.

We express our appreciation to Mr. W. Bear and Mrs. S. Brassel for skilled technical assistance. L. A. B. was a Postdoctoral Research Fellow of the U.S. Public Health Service (1968–70).

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APPENDIX

A Mathematical Model of the Acetylcholine Compartments in Synaptosomes

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(*Received* 24 *August* 1972)

To examine the time-course of labelling of the various compartments of bound acetylcholine in synaptosomes, the linear model shown diagrammatically in Scheme 1 was investigated. Radioactive choline, in concentration x_0, is taken up into the synaptosome cytoplasm from the extracellular fluid at a rate defined by the rate constant l. It penetrates to the centre of the synaptosome, mixing with the endogenous pool *en route*, through a series of concentric spherical zones here represented as a linear series of n compartments running from the periphery to the centre of the synaptosome. The rate constant defining the rate of diffusion of choline from one cytoplasmic compartment to the next is k_2. Meanwhile, choline (whether as such or as acetylcholine is immaterial for the purpose of the present analysis) is assumed to be taken up by vesicles in contact with the n compartments at a rate defined by the constant k_1. Finally, radioactivity is being lost from the vesicles at a rate defined by the constant k_3. The concentrations of radioactivity at any given time t, in the vesicles and in the regions of cytoplasm with which they are in contact are denoted by x_1 to x_n and x'_1 to x'_n respectively.

It is further assumed that vesicles can be divided into two classes: metabolically more active ('P-type') vesicles (1 to j) close to the periphery of the synaptosome, of the type mainly recovered in fraction H, and metabolically less active ('C-type') vesicles ($j+1$ to n) nearer to the centre of the synaptosome and of the type mainly recovered in fraction D. The concentrations of radioactivity x_1 to x_n present in these vesicles are defined by rate constants (for P-type vesicles) k_1^{P} and k_3^{P} and for C-type vesicles k_1^{C} and k_3^{C}, the assumption being made that $k_1^{\mathrm{P}} > k_1^{\mathrm{C}}$ and $k_3^{\mathrm{P}} > k_3^{\mathrm{C}}$.

Finally, as a further refinement of the model, it is assumed that the partitioning of vesicles between fraction H (vesicles 1 to y) and fraction D (vesicles $y+1$ to n) may vary from one experiment to another, so that y is not necessarily equal to j.