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The Heterogeneity of Bound Acetylcholine and Synaptic Vesicles

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Synaptic vesicles containing radioactive acetylcholine have been isolated from slices of *Torpedo* electric organ incubated with radioactive choline. The recently synthesized radioactive acetylcholine is preferentially removed from the vesicles by iso-osmotic gel filtration. There is therefore a small compartment of loosely bound recently synthesized acetylcholine within the monodisperse vesicle fraction. The specific radioactivity of this compartment correlates most closely with the 'free' acetylcholine of electric organ that is lost when the tissue is homogenized. Membrane-associated vesicles did not contain any particular enrichment of this compartment. On standing at 6°C the loosely bound compartment stabilizes so that it survives iso-osmotic filtration. A study of this phenomenon revealed that it was proportional to the extent of the loss of tightly bound acetylcholine from the vesicles. Incubation with Ca^{2+}, at pH 5.5, or partial hypo-osmotic shock, caused losses of tightly bound acetylcholine and proportional increases in the stabilization of loosely bound acetylcholine of vesicles. Incubation at 20°C caused less loss of tightly bound, and less stabilization of loosely bound, acetylcholine. A theoretical treatment of these exchanges also shows that the random factors promoting loss of tightly bound acetylcholine are statistically correlated with those which cause stabilization of loosely bound acetylcholine. The reciprocal relationship between the exchanges is inconsistent with there being two distinct populations of vesicles, one containing recently synthesized, loosely bound acetylcholine and the other containing tightly bound acetylcholine. It is proposed that all the vesicles contain a core of tightly bound acetylcholine and a surface layer of loosely bound acetylcholine. The origin of the extravesicular acetylcholine and also of the acetylcholine released on stimulation is discussed in the light of these results.

When the acetylcholine of synaptic vesicles is labelled by incubation of the parent tissue with [^{3}H]choline we have observed that the radioactive and bioassayable acetylcholine are heterogeneous and behave differently when the vesicles are submitted to iso-osmotic gel filtration (Marchbanks & Israël, 1971; Richter & Marchbanks, 1971*b*). The more radioactively labelled acetylcholine is washed off the vesicles, whereas most of the bioassayable acetylcholine passes through in the void volume of the column. We have investigated this phenomenon with the particular intention of determining whether the radioactive acetylcholine is present in all the vesicles, or whether it is derived from a distinct population of highly labelled vesicles unstable to gel filtration. The answer to this problem is important in evaluating the origin and role of the compartment of acetylcholine in the nerve terminal, which does not appear to be associated with vesicles isolated by subcellular fractionation. This compartment was originally termed the 'labile bound' compartment by Whittaker *et al.* (1964), and subsequently called the 'cytoplasmic' (indicating its distribution in subcellular fractionation studies), 'free' or 'extravesicular' compartment of acetylcholine.

In a wide variety of preparations it has become clear that the acetylcholine preferentially released on stimulation is that which has been most recently synthesized. These include electrically stimulated superior cervical ganglion of the cat (Collier & MacIntosh, 1969), electrically stimulated hemidiaphragm of the rat (Potter, 1970), cerebral-cortex slices stimulated by K^+ (Richter & Marchbanks, 1971*a*; Molenaar *et al.*, 1971) and electrically stimulated electric organ of *Torpedo marmorata* (Dunant *et al.*, 1971, 1972). However, the vesicular compartment as isolated by subcellular fractionation procedures does not preferentially receive newly synthesized acetylcholine. This has been shown in cerebral-cortex synaptosomes *in vitro* (Marchbanks, 1969), cerebral-cortex slices *in vitro* (Richter & Marchbanks, 1971*b*; Collier *et al.*, 1972) and *Torpedo* electric-organ slices

in vitro (Marchbanks & Israël, 1971). In addition the enzyme synthesizing acetylcholine, choline acetyltransferase, appears to be in the cytoplasm of synaptic terminals and not bound to the vesicles (Fonnum, 1967).

These results suggest that a substantial proportion of the acetylcholine released on stimulation comes from the extravesicular compartment. A particularly striking demonstration of this is to be found in the study by Dunant *et al.* (1971), in which electrical stimulation of slices of electric organ caused a substantial decrease in the extravesicular (free) acetylcholine and very little change in the bound (vesicular) acetylcholine.

However, it remains possible that there is a population of vesicles filled with recently synthesized acetylcholine that are so unstable that they tend to disintegrate when the tissue is homogenized and are therefore not completely isolated by subcellular fractionation procedures. If this were so both extravesicular acetylcholine and released acetylcholine could be explained as originating from this labile population. In addition it has been suggested, originally by Curtis & Eccles (1960), that there is a special population of vesicles close to the pre-synaptic membrane whose function it is to provide the acetylcholine 'readily releasable' on stimulation, and which are therefore presumably filled with recently synthesized acetylcholine (see Hubbard, 1970, p. 50, for discussion).

A possible explanation of the heterogeneity that we have observed in the acetylcholine of vesicles is that it represents the residue of a population of unstable recently filled vesicles. In the present paper we put forward the reasons for excluding this possibility and offer an alternative explanation. The experiments were done with synaptic vesicles from the electric organ of *Torpedo marmorata* because this is a purely cholinergic tissue (Feldberg & Fessard, 1942) analogous in many respects to the motor end-plate of skeletal muscle.

Methods

Preparation of labelled vesicles

Torpedo marmorata were obtained live from the Station de Biologie Marine, Arcachon, France. Dorso-ventral slices of the electric organ about 1 prism thick were incubated at 20°C in the medium (5ml/g of tissue) described by Marchbanks & Israël (1971). In a few experiments the medium used by Dunant *et al.* (1971) was employed. This medium has a lower Mg^{2+} concentration and improves the physiological performance of the slices. Yields of vesicular acetylcholine are slightly higher but the properties of the radioactively labelled and bioassayable acetylcholine are the same. [^{3}H]Choline (1–2μCi/ml; specific radioactivity 6–15Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the medium and the incubation was carried on for 1–3h. The incorporation of [^{3}H]-choline into acetylcholine of the tissue rises linearly during this period (Marchbanks & Israël, 1971).

At the end of the incubation period the tissue was briefly washed with fresh medium and samples were taken for analysis; the rest was homogenized in 0.325M-NaCl – 0.15M-sucrose and centrifuged by technique 1 of Israël *et al.* (1968, 1970). The density gradient was slightly modified by the addition of a layer of 0.275M-NaCl – 0.25M-sucrose on top of the gradient to prevent diffusion of soluble constituents (see Marchbanks & Israël, 1971). Fig. 1 summarizes the steps in the procedure and the acetylcholine distribution in the various fractions. The vesicle fraction was harvested and the experiments were started immediately. Gel filtration of the vesicle fraction was normally carried out on small columns containing Sephadex G-50 exactly as described previously (Marchbanks, 1968), except that they were eluted with 0.21M-NaCl – 0.38M-sucrose, which is iso-osmotic for *Torpedo* tissue. Larger columns were used for certain experiments as described in the text.

Assay procedures

Bioassayable acetylcholine. Samples for acetylcholine assay were treated immediately with trichloroacetic acid (final concn. 5%, w/v), the precipitated protein was removed by centrifugation and the trichloroacetic acid was removed by ether extraction until the sample was at pH4.5. The samples were stored frozen in this condition until assayed on a slip of the dorsal muscle of the leech (Szerb, 1961).

Choline. Choline was assayed by acetylation with acetic anhydride according to the procedure of Bligh (1952). The acetylcholine produced was assayed as described above, correction being made for any acetylcholine being present in the sample originally. The procedure of Appleton *et al.* (1953) was found to be unsuitable because of the presence of interfering material.

Choline acetyltransferase. This enzyme was assayed by measuring the incorporation of [^{14}C]acetate into acetylcholine via an acetate-activating system (Fonnum, 1966). [^{14}C]Acetylcholine was extracted by using sodium tetraphenylboron in butyl ethyl ketone (Fonnum, 1969).

Lactate dehydrogenase. This was measured on a recording spectrophotometer by the method described by Kornberg (1955).

Radioactive acetylcholine

Choline bases from samples of volume up to 4.0ml, from which the protein precipitate had been removed

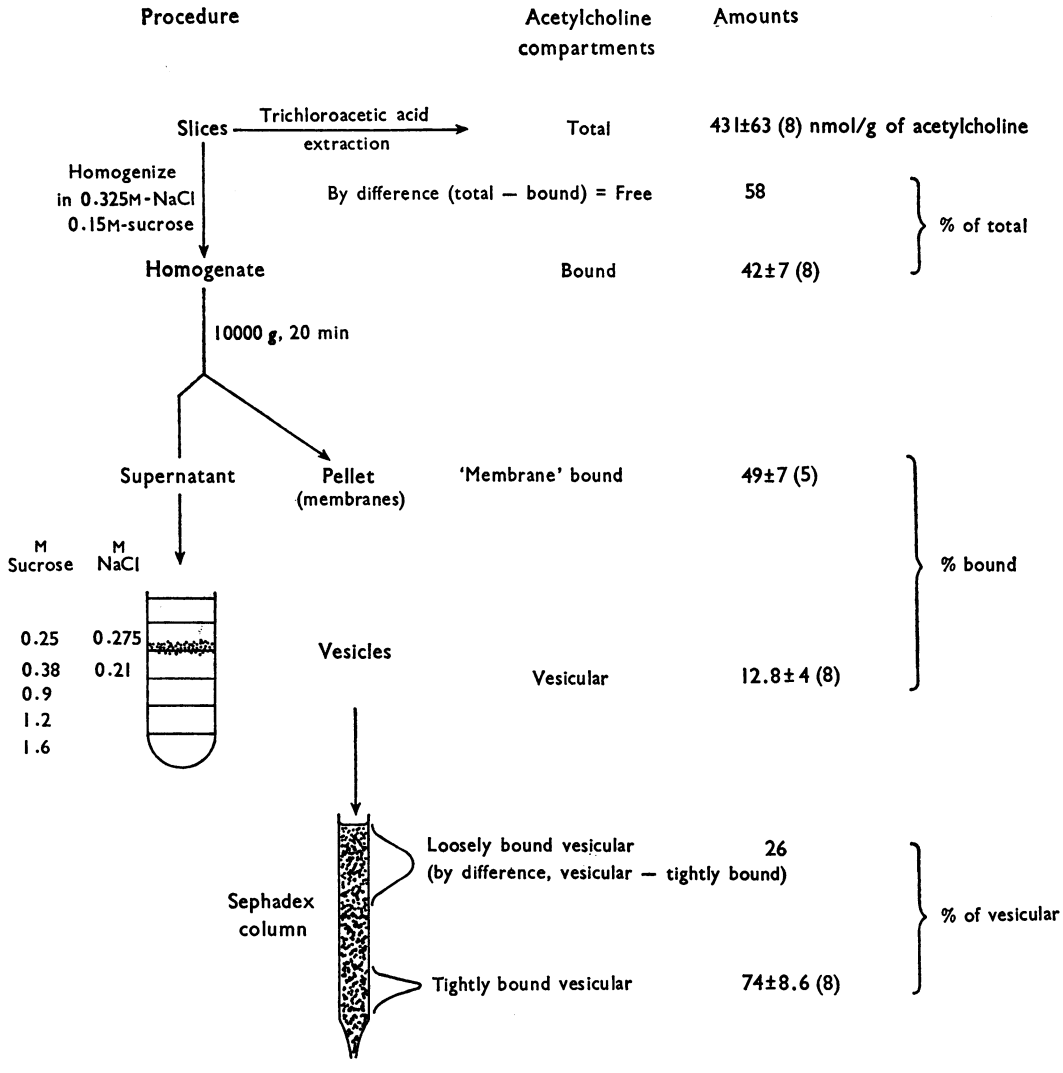

Fig. 1. *Fractionation procedure for incubated electric organ and the distribution of acetylcholine*

Values are means±S.E.M. (numbers of experiments in parentheses) of percentage acetylcholine distribution in the experiments whose results are discussed in Tables 1 and 2. Percentage of 'membrane' bound acetylcholine and vesicular acetylcholine do not add up to 100 because of losses during density-gradient centrifugation; they were also a different series of experiments.

after addition of 5% (w/v) trichloroacetic acid, were extracted by shaking vigorously for 15s with 1.0ml of vinylacetonitrile containing 20mg of sodium tetraphenylboron/ml. The vinylacetonitrile–tetraphenylboron solution was washed once with water before use. After being shaken the samples were centrifuged to separate the phases and 0.8ml of the organic solvent was removed. To this were added 1.6ml of ether and 0.8ml of 0.07M-silver acetate (recrystallized). The mixture was shaken vigorously for 15s and excess of silver precipitated by addition of 0.1ml of 1.0M-HCl. After shaking for a further 15s the samples were centrifuged lightly and the organic phase was removed. Too vigorous centrifugation at this stage was avoided because it compacted the precipitate at the interphase, making it difficult to remove. The aqueous phase containing choline bases was washed twice with 2.0ml of ether (itself washed with 1.0M-HCl). Care

was taken to remove all the ether by aspiration, otherwise traces of trichloroacetic acid revert to the aqueous phase and interfere with subsequent chromatography. Water (0.5ml) was added and the samples were centrifuged for 5min at the maximum speed of an MSE bench centrifuge. A portion (1.0ml) of the clear aqueous layer was removed by aspiration and evaporated to dryness under vacuum in a desiccator. The samples were dissolved in 25μl of methanol containing 12.5mM-acetylcholine chloride and 12.5mM-choline chloride and 10μl in duplicate was applied to a t.l.c. plate with a layer of cellulose powder (without $CaSO_4$ binder) MN-300H (Machery, Nagel and Co., 516 Düren, Germany). The plates were eluted with butan-1-ol–ethanol–acetic acid–water (8:2:1:3, by vol.) and choline bases were identified by spraying with iodoplatinate reagent [1 part of aq. 5% (w/v) chloroplatinic acid added to 20 parts of 4.5% (w/v) KI in aq. 50% (v/v) ethanol]. Choline bases appear as blue spots on a pink background, choline and acetylcholine having R_F values 0.45 and 0.56 respectively.

The extraction into the organic solvent was virtually quantitative and only slightly affected by high salt concentrations even though no carrier acetylcholine was added at this stage. High protein concentrations, however, prevented a clear phase separation. Most of the losses resulted from the manipulations with small volumes before application of the sample to the chromatogram. An internal standard of 2.5mμCi of [*N*-*Me*-^{14}C]acetylcholine was always added before extraction for the estimation of and correction for recovery. The internal standard was estimated by chromatography without extraction and its average percentage recovery on extraction was 50$\pm$1 (S.E.M.; $n = 75$). The overlap of choline radioactivity into the acetylcholine position was found to be 3%$\pm$2% (S.D.; $n = 30$) and values for acetylcholine radioactivity were corrected accordingly. Further evidence of the authenticity of chromatographically separated radioactive acetylcholine was obtained by adding to representative samples after vacuum evaporation 1.0ml of a solution of acetylcholinesterase (0.5mg/ml; Sigma Chemical Co.) adjusted to pH8.0 with 0.1M-NH_3. These samples were incubated for 120min at 37°C and then evaporated to dryness and chromatographed in the normal fashion. The radioactivity was lost from the acetylcholine position and appeared in the choline position of the chromatogram.

The radioactivities of ^{14}C and ^{3}H in the chromatogram spots of acetylcholine were estimated simultaneously with a liquid-scintillation spectrometer. The spots were scraped off into a scintillation counting vial; 0.1ml of 5% (w/v) $Na_2S_2O_3$ in aq. 50% (v/v) ethanol was added to decolorize the sample, 1.0ml of ethanolic 0.1M-NH_3 soln. was added and the cellulose dispersed into a fine powder by agitation with glass beads. Finally 18ml of a mixture of 14 parts of toluene–1,4-bis-(5-phenyloxazol-2-yl)benzene–2,5-diphenyloxazole and 5 parts of Triton X-100 containing 3.5% (w/v) Cab-O-Sil (Packard Instrument Co., Inc., Downers Grove, Ill., U.S.A.) was added. Liquid samples containing radioactivity were counted similarly except that thiosulphate and Cab-O-Sil were omitted. The counting efficiencies for all samples of ^{14}C and ^{3}H were 26% and 20% respectively with a 30% crossover of counts of carbon radioactivity into the tritium channel.

Treatment of results

The radioactivity of acetylcholine (d.p.m.) and its bioassayable activity (pmol) in a fraction were calculated per g wet wt. of original tissue. The specific radioactivity (d.p.m./pmol) of acetylcholine is the ratio of these two quantities. The amounts of acetylcholine (d.p.m. and pmol) in the free, and the loosely bound, vesicular compartments were calculated as the difference respectively between total and bound and between vesicular and stable vesicular acetylcholine. The specific radioactivity of the acetylcholine in such compartments is the ratio of the differences of d.p.m. and pmol.

The amounts of radioactive and bioassayable acetylcholine in the vesicle fraction vary from preparation to preparation (see Table 1). To compare the results of a particular treatment (Tables 3, 4 and 5) in several different preparations the amounts of acetylcholine (d.p.m. and pmol) were expressed as fractions of that in the vesicle preparation from which they originated. Such fractions are not suitable for statistical manipulation because of their non-normal distribution. Accordingly a logarithmic transformation was carried out and statistical calculations were performed on the logarithm of the original fraction (Geigy, 1970).

Results

Identity of the acetylcholine lost by gel filtration

Fig. 2 shows the results of applying a labelled vesicle preparation to an iso-osmotic Sephadex column followed by serial collection of the effluent. Whereas bioassayable acetylcholine appeared almost entirely in the void-volume peak the radioactive acetylcholine showed two well-defined peaks: one associated with the bioassayable acetylcholine and the other in the included volume of the column. The radioactive acetylcholine in the included volume was presumably associated with an amount of bioassayable acetylcholine too small to be reliably measured in this case, and therefore had a high specific radioactivity, and had been recently synthesized. Its appearance in the included volume indicates that it

was not associated with any molecule having a molecular weight of more than about 1000. It cannot have originated from free acetylcholine in the parent fraction that may have diffused into the vesicle band of the density gradient, because there were no anti-

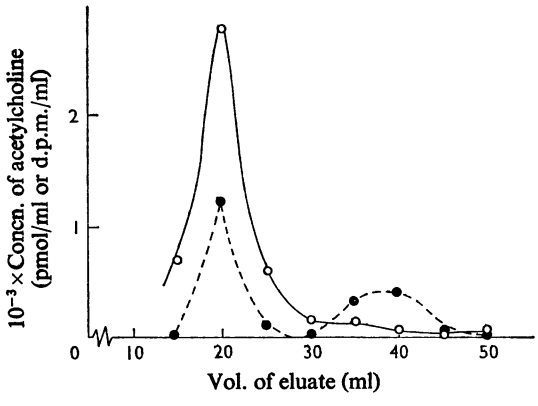

Fig. 2. *Exclusion chromatography of synaptic vesicles containing radioactively labelled acetylcholine*

A sample (4.0ml) of vesicle fraction containing 15200pmol (10900d.p.m.) of acetylcholine was applied to the top of the column. The column was 1.2cm diam. and contained 40ml wet vol. of Sephadex G-50 (bead form) in equilibration and subsequently eluted with a solution containing 0.38M-sucrose, 0.21M-NaCl, 20mM-sodium phosphate pH7.0, and 100μM-neostigmine. Fractions collected were 5ml: o, bioassayable acetylcholine; •, radioactive acetylcholine. The fraction immediately above that from which the vesicles were harvested contained 3200pmol (5200d.p.m.) of acetylcholine in the same volume.

cholinesterases present to permit its survival by preventing its hydrolysis. Further, the amounts of radioactive acetylcholine in the cushion layer above (values given in the legend to Fig. 2) would not be sufficient, even if it was free to diffuse, to account for that found in the included peak of the column. We conclude that the highly labelled acetylcholine originated from the vesicle fraction and was released from it into an unassociated form by the column treatment.

There must have been some compartmentation within the vesicle fraction to prevent equalization of the specific radioactivities of the acetylcholine. We have called the compartment whose acetylcholine remains within the included volume the 'loosely bound vesicular' and that which appears in the void volume the 'tightly bound vesicular' compartment. In subsequent experiments the loosely bound was measured as the difference between the tightly bound acetylcholine in the void volume of the column and the acetylcholine in the parent vesicle fraction.

Relationship to other compartments

We have compared the specific radioactivity of the loosely bound vesicular acetylcholine with that of the acetylcholine in other fractions. In Table 1 are shown the mean specific radioactivities of the free (i.e. total trichloroacetic acid-extractable acetylcholine minus the bound), the bound acetylcholine that survives homogenization, the parent vesicular and vesicular subcompartments. There was considerable variability, but the specific radioactivity of the loosely bound vesicular acetylcholine was higher than that of the bound. If the specific radioactivities of the fractions are correlated by a least-squares linear regression it can be seen from Table 1 that the loosely bound vesicular acetylcholine has a statistically significant

Table 1. *Specific radioactivities of compartments of acetylcholine*

Specific radioactivities (means±S.E.M.; $n = 8$) of acetylcholine compartments in electric organ incubated as described in the Methods section are shown. The regressions correlating the specific radioactivities of the compartments shown were calculated from the paired specific radioactivities in each experiment. n.s., Not significant.

Compartment ...		Free	Bound	Vesicular	Loosely bound vesicular	Tightly bound vesicular
Specific radioactivity (d.p.m./pmol)		5.9±4.5	0.36±0.1	0.53±0.16	1.54±0.67	0.11±0.047
Least-squares linear regression						
Against specific radioactivity of free acetylcholine	Slope				0.1309	−0.0040
	Correlation coefficient				0.8450	0.3737
	P (slope>0)				<0.01	n.s.
Against specific radioactivity of bound acetylcholine	Slope				0.5641	0.1658
	Correlation coefficient				0.0847	0.3499
	P (slope>0)				n.s.	n.s.

correlation (slope > 0) with the free acetylcholine only. The slope indicates that the specific radioactivity of the loosely bound vesicular acetylcholine is about one-sixth that of the free acetylcholine. The tightly bound vesicular acetylcholine does not correlate with either free or bound acetylcholine. This is not surprising, since the bound compartment is composed principally of vesicles and thus gives rise to both the vesicular subcompartments (Marchbanks & Israël, 1971). However, the slopes suggest that the tightly bound vesicular acetylcholine has more relationship with the bound than with the free acetylcholine compartments.

It has been suggested that in cerebral cortex there is a compartment of recently synthesized acetylcholine, which exists in vesicles associated with synaptic membranes (Barker *et al.*, 1970; Whittaker, 1971). In the electric organ the membrane fraction separated by centrifugation before the density-gradient fractionation does contain acetylcholine (Israël *et al.*, 1970) and can be seen to contain some partially disrupted terminals with vesicles adhering to membranes. We have therefore in separate but similar experiments examined the specific radioactivity of the acetylcholine in the membranous pellet (Table 2). The specific radioactivity of the acetylcholine of the membranous pellet approximated closely to that of the bound acetylcholine rather than to that of the free. Regression analysis shows that it correlates well with both bound and free acetylcholine, but the slope against bound acetylcholine is much closer to 1 than that against free. There is therefore no reason to suppose that the membranous-pellet acetylcholine is any different from bound acetylcholine or contains any significantly increased proportion of recently synthesized acetylcholine, as do the free and loosely bound vesicular compartments.

We have surveyed the bioassayable and radioactive acetylcholine in all the fractions of the density gradient and a typical result is shown in Table 3. Both bioassayable and radioactive acetylcholine reached a peak at 0.38 M-sucrose – 0.21 M-NaCl, which is the layer to which vesicles sediment. The correspondence of the peaks suggests that the loosely bound and tightly bound compartments are not separable by density-gradient centrifugation. There

Table 2. *Specific radioactivities of the acetylcholine in the membrane pellet fraction*

Specific radioactivities (means ± S.E.M.; $n = 5$) of acetylcholine in the membrane pellet fraction obtained after incubation of the electric organ as described in the Methods section are shown. The regressions correlating the specific radioactivities of the compartments shown were calculated from the paired specific radioactivities in each experiment.

Compartment ...	Free	Bound	Membrane pellet
Specific radioactivity (d.p.m./pmol)	1.26 ± 1.2	0.55 ± 0.47	0.41 ± 0.37
Least-squares linear regression			
Against specific radioactivity of free acetylcholine: Slope			0.3018
Correlation coefficient			0.9987
P (slope > 0)			<0.001
Against specific radioactivity of bound acetylcholine: Slope			0.804
Correlation coefficient			0.9995
P (slope > 0)			<0.001

Table 3. *Distribution of radioactive and bioassayable acetylcholine in the density gradient*

After incubation with [^{3}H]choline the free and the bound acetylcholine compartments had specific radioactivities of 1.54 and 0.171 d.p.m./pmol respectively. After removal of the membrane pellet (see the Methods section) the fraction was submitted to density-gradient centrifugation and the subfractions were assayed for bioassayable and radioactive acetylcholine.

Fraction	Bioassayable acetylcholine (nmol)	10^{-3} × Radioactive acetylcholine (d.p.m.)
Parent fraction	140.0	39.6
Top layer	0.57	1.92
Equilibrating at:		
0.1 M-Sucrose – 0.35 M-NaCl	0.21	0
0.25 M-Sucrose – 0.275 M-NaCl	22.5	7.9
0.38 M-Sucrose – 0.21 M-NaCl (vesicles)	125.0	29.2
0.6 M-Sucrose – 0.1 M-NaCl	22.5	9.42

was more radioactive acetylcholine in the top layer of the gradient than would be predicted from the bioassayable-acetylcholine concentration. However, this observation was erratic and it presumably represents a few vesicles that have not sedimented. We think that there is a disproportionate amount of radioactivity present because the vesicles have not been 'washed' by passage through the gradient (Richter & Marchbanks, 1971*b*). Whatever causes the loss of radioactive acetylcholine on gel filtration could be expected to happen to a lesser extent as these vesicles pass through the gradient. Therefore any few vesicles left at the top of the gradient would be expected to have a higher specific radioactivity. In any case it represents less than 0.5 and 3% respectively of the total bioassayable and radioactive acetylcholine.

We conclude that the loosely bound vesicular compartment is most closely related to the free compartment in terms of specific radioactivity. It derives principally from the monodisperse vesicle fraction and not 'membrane-associated vesicles'. The free compartment of acetylcholine in the electric-organ slice preferentially receives the newly synthesized acetylcholine (Marchbanks & Israël, 1971), so it follows that the loosely bound vesicular compartment also contains more recently synthesized acetylcholine, at least in comparison with the tightly bound vesicular compartment. At the same time it is clear that the loosely bound compartment is not as rich in recently synthesized acetylcholine as the free compartment.

Stabilization of the loosely bound vesicular acetylcholine

If the labelled vesicle fraction was left at 6°C before being submitted to gel filtration it was found that after about 20–30 min the radioactive acetylcholine was no longer lost but appeared in the void volume of the column effluent (Fig. 3). A similar effect was shown by the unfractionated homogenate. Bioassayable acetylcholine showed no such increase on standing.

A possible explanation for this effect is that there is enzymic synthesis of small amounts of radioactive acetylcholine within the tightly bound vesicular pool. To exclude this possibility we have examined the changes with time of the radioactive acetylcholine in a homogenate from incubated electric organ left at 6°C (Fig. 4). There were no significant changes in the concentration of either radioactive or bioassayable acetylcholine during a 200min period.

As a further check we have investigated the possibility that there might be small, hitherto undetected, amounts of choline acetyltransferase within the vesicles, which might be able to carry out this synthesis. Fig. 5 shows the results of gel filtration of a vesicle fraction on Bio-Gel 5-M (excludes particles > 5 $\times 10^6$ daltons). Virtually all the acetylcholine occurred

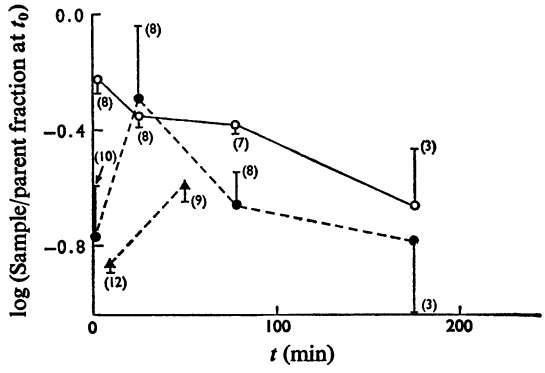

Fig. 3. *Fractional changes in the radioactive and bioassayable acetylcholine in the void volume of the column effluent after standing at 6°C*

Results are expressed as the log of the ratio of the column effluent sample value at times indicated to the parent fraction value at zero time. ●, Radioactive acetylcholine from a vesicle fraction; ○, bioassayable acetylcholine from a vesicle fraction. Results are means±S.E.M. (vertical bars) with the number of separate experiments indicated by adjacent small numbers. ▲, Radioactive acetylcholine from a homogenate of electric organ treated similarly but with results displaced by +0.8 on the vertical axis. One experiment was done with homogenate and the points represent mean±S.E.M. of the number of determinations shown by the adjacent small numbers.

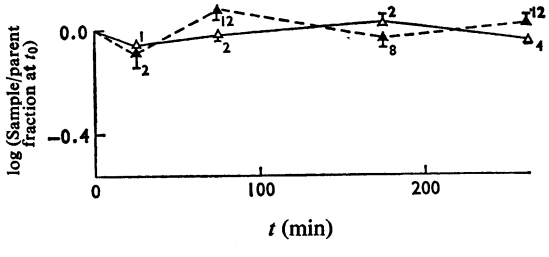

Fig. 4. *Fractional changes in radioactive and bioassayable acetylcholine in an incubated electric-organ homogenate kept at 6°C*

Results are expressed as log of the ratio of the sample value at times indicated to the parent fraction value at zero time. ▲, Radioactive acetylcholine; △, bioassayable acetylcholine. Points are means±S.E.M. (or range if less than three observations), shown by vertical bars; numbers of observations are shown by adjacent small numbers.

in the void-volume peak and was well separated from residual traces of choline acetyltransferase and lactate dehydrogenase, which were retained in the included

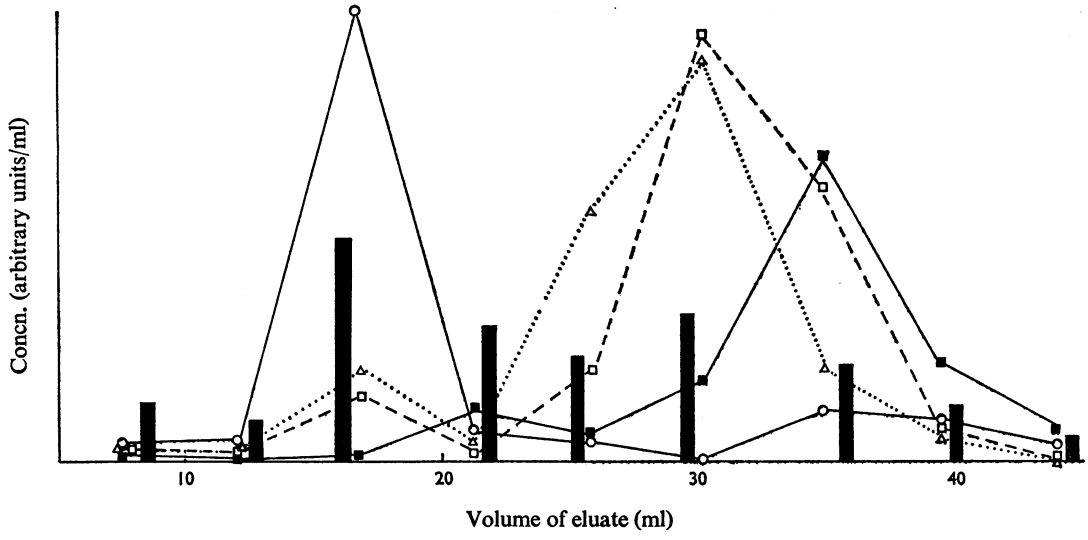

Fig. 5. *Exclusion chromatography on Bio-Gel 5-M of synaptic vesicle fraction from Torpedo electric organ*

The column was 1.2cm in diam. and contained 40ml (wet vol.) of the gel equilibrated and subsequently eluted with a solution containing 0.38 M-sucrose, 0.21 M-NaCl, 10mM-sodium phosphate, pH7.4, and1 mM-neostigmine. A portion (5ml) of a synaptic vesicle fraction was added to the top of the column and samples were collected and analysed with the results shown. The recovery of acetylcholine was 94%. The choline acetyltransferase and lactate dehydrogenase found are <3% and <5% respectively of the amounts found in the supernatant fraction from which the vesicle fraction was isolated. ○, Acetylcholine; □, choline acetyltransferase; △, lactate dehydrogenase; ■, choline. Vertical bars represent protein concentration.

volume of the column. The residual choline acetyltransferase is therefore neither within the vesicles nor bound to them, a result in accordance with those of Fonnum (1967). Fig. 5 also shows that there was no choline within the vesicles.

Since there was no synthesis of radioactive acetylcholine it must be that it became stabilized during the incubation so that it survived gel filtration in a bound form. The magnitude of this effect is masked by a decrease in the bioassayable acetylcholine appearing in the void volume as the incubation proceeds. If stabilization had not occurred the radioactivity in the void volume would be expected to be that appearing initially, decreased in proportion to the decrease in bioassayable acetylcholine. The expected log (fractional radioactivity), no stabilization being assumed, can therefore be calculated as the sum of the log (fraction radioactive acetylcholine initially) and the change of log (fractional bioassayable acetylcholine) during incubation. The expected log (fractional radioactivity) at the $t = 25$min point in Fig. 3 is therefore -0.907, whereas the actual value is $-0.286 \pm 0.258(8)$, the difference being significant at the $P < 0.05$ level. Fig. 3 shows that the variability in the radioactivity was considerably higher than that of the bioassayable acetylcholine. This is unexpected because the standard deviation of the radioactivity measurement is if anything less than that of the bioassay. The explanation of this is discussed in the last part of the Results section.

Conditions for stabilization of loosely bound vesicular acetylcholine

We have investigated stabilization under a variety of conditions. Table 4 shows the effect of eluting the columns at different osmolarities compared with elution with a solution iso-osmolar to the vesicle-suspension medium. As expected the decrease of the osmolarity of the eluting medium caused losses of bioassayable acetylcholine in the column effluent, which were extreme for the columns eluted at osmolarities <0.08. The loss of radioactive acetylcholine was proportionately much less, and with the column eluted at 0.4 M osmolarity there was an average increase in the amount of radioactive acetylcholine. At both osmolarities the amount of radioactive acetylcholine was significantly higher than that expected on a proportional basis (see the preceding section).

The effect of incubation under various conditions

Table 4. *Effect of partial osmotic shock on radioactive and bioassayable acetylcholine*

Synaptic vesicle preparations containing [^{3}H]acetylcholine were submitted to gel filtration at different column osmolarities and the bioassayable and radioactive acetylcholine were determined in the void-volume effluent. The results were expressed as fractions of that present in the original vesicle preparation and the Table shows the mean ± S.E.M. (number of samples in parentheses) of the log of such fractions. The controls (columns eluted iso-osmotically) were from the same preparations as the experimental samples, but usually several experimental samples were taken from each preparation. The differences between the log (fraction radioactive acetylcholine) expected on a proportional basis (see the text) and that found are significant at the $P<0.01$ level.

	log (Sample/parent fraction)		
	Bioassayable acetylcholine	Radioactive acetylcholine	Expected radioactive acetylcholine
Columns eluted iso-osmotically	−0.3036 ± 0.097 (6)	−0.7845 ± 0.128 (17)	—
Columns eluted at osmolarity 0.4 M	−0.5762 ± 0.150 (8)	−0.4669 ± 0.204 (18)	−1.0571
Columns eluted at osmolarity <0.08 M	−1.5552 ± 0.288 (4)	−1.683 ± 0.127 (30)	−2.0361

before gel filtration was examined, with the results shown in Table 5. Technical reasons prevented all the incubations being for the same period, so the experimental samples have been matched with samples taken from control incubations (6°C, without additions) of the same preparation at exactly the same time, although sometimes more than one experimental sample was matched against one control. The different control groups are not therefore comparable but each experimental group is exactly comparable with its control.

Preincubation at 20°C caused a small decrease (not significant) in the bioassayable acetylcholine. Previous experience with vesicles from unincubated tissue would lead us to expect greater losses of bioassayable acetylcholine (Marchbanks, 1968; Marchbanks & Israël, 1971). There was no increase in the amount of radioactive acetylcholine in the column effluent as a result of preincubation, and the amount approximates to the expected value.

Preincubation in the presence of Ca^{2+} (10 mM) caused significant losses of the bioassayable acetylcholine and an increase in the amount of radioactive acetylcholine, significantly higher than the expected value. Preincubation at pH 5.5 caused substantial losses of bioassayable acetylcholine, but only small losses of radioactive acetylcholine, so that the amount of radioactive acetylcholine is significantly higher than would be expected on a proportional basis.

The conclusion that emerges from the experiments whose results are shown in Tables 4 and 5 is that procedures that tend to decrease the amount of bioassayable acetylcholine in the column effluent tend to increase on a proportional basis the amount of radioactive acetylcholine in the column effluent. This is particularly noticeable when the effects of preincubation at pH 5.5, in the presence of Ca^{2+}, or elution at 0.4 M are compared with the effects of incubation at 20°C. Stabilization of the loosely bound vesicular compartment seems to be inversely proportional to the loss of acetylcholine from tightly bound vesicular compartment.

Calculation of the fractional exchange between vesicular compartments

The reciprocal relationship between the bioassayable acetylcholine lost from and the radioactive acetylcholine gained by tightly bound acetylcholine led us to evaluate the phenomenon in a more quantitative manner. The original vesicle fraction can be regarded as containing L pmol of loosely bound acetylcholine and T pmol of tightly bound acetylcholine, only the latter remaining bound during iso-osmotic exclusion chromatography and being determined as such. During incubation under the various conditions, we may envisage (without assuming any particular mechanism) a transfer or exchange of U pmol from the loosely bound to the tightly bound form, and V pmol from the tightly bound form. The transfer from the tightly bound form may be either into the loosely bound form or it may be lost entirely from the vesicles; these possibilities cannot be directly distinguished from the information available, but it does not affect the argument.

As a consequence of these transfers the bioassayable acetylcholine in the void volume of the column effluent (denoted A) after incubation will be:

$$T+U-V=A \text{ (pmol)} \qquad (1)$$

and the radioactive acetylcholine (denoted D) also in the void volume will be:

$$TR_T+UR_L-VR_T=D \text{ (d.p.m.)} \qquad (2)$$

where R_T and R_L are the specific radioactivities (d.p.m./pmol) of the tightly and loosely bound vesicular acetylcholine respectively. Solving eqn. (1) for

Table 5. *Effects of preincubation under various conditions on radioactive and bioassayable acetylcholine*

Synaptic vesicle preparations containing [^{3}H]acetylcholine were preincubated under the conditions shown and then submitted to iso-osmotic gel filtration and the bioassayable and radioactive acetylcholine were estimated in the void-volume effluent. The results were expressed as fractions of that present in the original vesicle preparation and the table shows the mean±S.E.M. (number of samples in parentheses) of the log of such fractions. As explained in the text the controls are not comparable with each other, but are exactly comparable with their associated experimental group. n.s.,Not significant.

	log (Sample/parent fraction)			
	Bioassayable acetylcholine	Radioactive acetylcholine	Expected radioactive acetylcholine	Significance of difference (expected radioactivity–radioactivity found)
Incubation at 20°C	−0.3886±0.081 (17)	−1.047 ±0.138 (12)	−0.9544	n.s.
Control at 6°C	−0.2718±0.06 (17)	−0.8376±0.119 (10)		
Incubation with 10mM-Ca^{2+}	−0.3532±0.030 (14)	−0.1971±0.107 (18)	−0.4612	$P<0.025$
Control without Ca^{2+}	−0.1655±0.047 (7)	−0.2735±0.155 (9)		
Incubation at pH5.5	−0.7141±0.183 (7)	−0.4416±0.176 (9)	−0.8623	$P<0.05$
Control at pH6.9	−0.2410±0.028 (4)	−0.3892±0.126 (5)		

Table 6. *Fractional exchange of acetylcholine into and out of the tightly bound acetylcholine compartment of vesicles*

The exchanges in each direction were calculated by means of the equations derived and explained in the text and were then expressed as fractions of the originating compartment size. Each value shown is the mean±S.E.M. (number of samples in parentheses) of the log of such fractions. The regression coefficients were calculated from the paired log fractional exchanges in each direction for each condition and also over all conditions combined.

	log (Fractional exchange)			
Condition	Tightly → loosely bound	Loosely → tightly bound	Regression coefficient (b)	P ($b>0$)
Incubation at 6°C for 25min (control without incubation)	−0.594±0.17 (10)	−1.11 ±0.224	0.363	<0.1
Incubation at 20°C for 20min (control at 6°C)	−0.77 ±0.205 (12)	−1.48 ±0.24	0.588	<0.01
Incubation with 10mM-Ca^{2+} (control without Ca^{2+})	−0.316±0.246 (13)	−0.248±0.303	0.585	<0.02
Incubation at pH5.5 (control at pH6.9)	−0.172±0.66 (4)	−0.242±0.68	0.972	<0.05
Column eluted at 0.4M (control column at 0.8M)	−0.76 ±0.308 (8)	−1.22 ±0.32	0.706	<0.05
Column eluted at <0.08M (control column at 0.8M)	−0.021±0.05 (8)	−0.67 ±0.277	−0.036	n.s.
All conditions combined			0.55	<0.001

V and substituting in eqn. (2), and repeating the process for U, we get:

Loosely bound → tightly bound =
$$U = (AR_T - D)/(R_T - R_L)$$
Tightly bound → loosely bound =
$$V = (AR_L - D)/(R_T - R_L) + T$$

The validity of these expressions is limited by the assumption that the exchanges will not affect the specific radioactivities of the compartments appreciably, but otherwise no assumptions have been made about the nature of the compartments or the transfer process. The specific radioactivities of the compartments R_T and R_L can be determined as described in the Methods section from the column effluent of samples incubated under matched control conditions (as shown in Table 6), by making the assumption that the total acetylcholine of the vesicle preparation has not changed appreciably (see Fig. 3). The values for amounts (pmol) transferred from each compartment are more meaningful if expressed as fractions of the originating compartment size, also calculated as described in the Methods section. In Table 6 the means of the log of the fractional exchanges in each direction are given for each condition and also the linear regression coefficient obtained when the fractional exchange in the tightly bound → loosely bound direction is plotted against that in the reverse direction.

A high exchange in the tightly bound → loosely bound direction was associated with a high exchange in the reverse direction, and vice versa. This can be seen when the conditions of incubation in the presence of Ca^{2+} and at pH 5.5 are compared with incubation at 20°C, 6°C or column elution at 0.4M osmolarity. The exception to this generalization was the column with <0.08M osmolarity, but this may fairly be regarded as an atypical condition since very little vesicular acetylcholine survives such treatment. With the exception of this anomalous condition the regression coefficients within each group are all positive, mostly with high statistical significance. The exchanges are therefore covariant, and factors, at present unknown, which positively affect the exchange in the $T \rightarrow L$ direction positively affect the exchange in the $L \rightarrow T$ direction and vice versa. Taking all conditions together this tendency is highly significant.

The positive covariance of the exchanges, equivalent to negative covariance of radioactive and bioassayable acetylcholine, will counteract to magnify their joint variabilities when their ratios are formed in the calculation of specific radioactivities. This goes some way towards explaining the dispiriting variability we have encountered in these phenomena.

Discussion

Our results show that the monodisperse synaptic vesicle fraction of electric organ contains a small labile compartment of acetylcholine, which is more related to the free or cytoplasmic than to the bound acetylcholine. It is therefore more recently synthesized than the tightly bound acetylcholine of vesicles. We have not been able to show that 'membrane-associated vesicles' or any other fraction of bound acetylcholine contains any specific enrichment of the loosely bound component. Our results do not therefore support the notion that there is a discrete population of labile recently filled vesicles associated with the membrane, as has been suggested for the cerebral cortex by Barker *et al.* (1970) and Whittaker (1971).

On the question of whether the monodisperse synaptic vesicle fraction itself contains two distinct populations, i.e. labile and stable with respect to acetylcholine storage, we argue as follows. To explain the stabilization phenomena we would have to attribute the following properties to the hypothetical labile vesicles: instability to iso-osmotic column conditions but stability to hypo-osmotic conditions, instability to incubation at 20°C but increasing stability at 6°C, and increased stability as a result of incubation with Ca^{2+} or at pH 5.5. Not only are the osmolarity conditions mutually inconsistent, but to explain the results we would have to attribute exactly opposite properties to the stable vesicles. In addition, the random factors that affect stabilization of the labile vesicles are positively correlated (Table 6) with the factors that affect losses of acetylcholine from the stable vesicles. To maintain the hypothesis of two distinct populations of vesicles we would therefore have to attribute inconsistent and exactly opposite properties to the two populations. This we find implausible.

A mechanism for stabilization involving transfer of free acetylcholine can be rejected because the acetylcholine would be instantly hydrolysed by residual cholinesterases. We cannot rigorously exclude the possibility that acetylcholine protected by being loosely bound to a small molecule (mol.wt. <1000) is transferred to the stable compartment but this seems unlikely in view of the particulate distribution of loosely bound acetylcholine. The loosely bound vesicular pool of acetylcholine must therefore reside within each vesicle.

Although it is possible that the loosely bound acetylcholine is distributed homogeneously throughout the vesicle we propose that the results are best explained by postulating that it exists on or near the surface of the vesicle. Processes that release the tightly bound acetylcholine in the core are in effect causing it to exchange with the outer layers or be lost from the vesicle. It can readily be envisaged that the exchange or movement of tightly bound acetylcholine outwards will be accompanied by a movement of loosely bound acetylcholine inwards. No special mechanism need be invoked to explain this coupling; it could, for example, arise as a result of there being only

limited binding sites in the core of the vesicle such that some of the tightly bound acetylcholine must be lost, exposing vacant sites for the loosely bound acetylcholine to move into. Alternatively the coupling may result from the action of the vesicle membranes in allowing the loosely bound acetylcholine to enter the vesicle only at the same time as it allows the tightly bound acetylcholine to pass out. This would imply that loosely bound acetylcholine is situated outside the vesicle membrane and further information is needed before this could be established. In any event either mechanism would explain the equivalence of exchange in both directions disclosed by the results of Table 6.

The loosely bound acetylcholine compartment in vesicles may not have a distinct location. Indeed we think that it is likely that the lability of the vesicular acetylcholine as we have described it simply reflects how near the vesicle surface it is situated. As one proceeds towards the core of the vesicle each concentric shell can be envisaged as containing acetylcholine that is more tightly bound. The most labile acetylcholine would then be on the surface layer of the vesicle and presumably in rapid equilibrium with the free or cytoplasmic acetylcholine. An arrangement similar to this has been proposed by Taugner & Hasselbach (1966) for the storage of catecholamine in granules from the adrenal medulla.

In a previous study (Marchbanks, 1968) it was found by using gel-filtration methods that the acetylcholine of cerebral-cortex vesicles was only very slowly exchangeable compared with the cytoplasmic acetylcholine of synaptosomes. That conclusion does not need to be modified as a consequence of the present results except to point out that the methods were measuring the tightly bound or core vesicular acetylcholine. Use of the pre-labelled vesicles in the present study has disclosed some exchange into the tightly bound acetylcholine, but quantitatively this is small. Taking the most favourable example (10mM-Ca^{2+}; Table 6) there is an increase in fractional exchange $L \rightarrow T$ of 0.57 as a result of incubation at 6°C in the presence of Ca^{2+}. At 6°C for 25min the extent of fractional exchange $L \rightarrow T$ is 0.07. Therefore the exchange into the tightly bound form during this period in the presence of Ca^{2+} is approx. 0.64 of the labile vesicular form, i.e. approx. 16% of the total vesicular acetylcholine. Losses from the tightly bound form during the same period are approx. 55% of the total vesicular acetylcholine. We are alert to the possibility that this effect of Ca^{2+} may have a physiological role, but an exact formulation must await more extensive studies.

The conditions that favour net incorporation into the tightly bound acetylcholine of vesicles remain unknown. Kuriyama *et al.* (1968) report binding of acetylcholine to vesicles, but it was virtually completely abolished by 50mM-NaCl or 50mM-KCl, so it appears to be a salt effect and is unlikely to have any physiological function.

The ease with which the loosely bound vesicular acetylcholine can be washed off the vesicle suggests that it is in very rapid equilibrium with the free acetylcholine and it may well be that the free compartment of acetylcholine is contaminated with some loosely bound vesicular acetylcholine washed off during homogenization. This equilibrium may be so rapid as to make it difficult to distinguish between free acetylcholine and the most labile outer layer of vesicular acetylcholine. It is clear, however, that the free acetylcholine cannot have arisen from the breakdown of any discrete population of unstable vesicles containing recently synthesized acetylcholine unless such vesicles never even survived the initial homogenization. In this case they are hardly accessible to biochemical investigation, and we leave the reader to decide whether vesicles of such lability would also survive the procedures for electron-microscopic examination.

In the introduction to the present paper we pointed out that the acetylcholine released on stimulation of the synapse in electric organ and other tissues was that which had been most recently synthesized. Since the most recently synthesized acetylcholine exists free, and probably on the surface of the vesicles, it is difficult to see how total exocytosis of the vesicle contents could provide much of the acetylcholine released on stimulation. Total exocytosis would release the core of the vesicle, which contains little or no recently synthesized acetylcholine. The precision of the measurements does not allow us to say that total exocytosis never occurs, but it does not contribute the majority of the acetylcholine released on stimulation.

The properties that we have described of the vesicular binding of acetylcholine fit the vesicles rather well for the role of the reserve fraction of the 'depot' acetylcholine described by Birks & MacIntosh (1961). They envisage that the larger 'reserve' fraction feeds into a smaller 'readily releasable' fraction of acetylcholine before being released. This rate-controlling process accounts for the steady acetylcholine output rate that occurs on stimulation after an initial sharp decrease. The sharp initial fall in acetylcholine output is due to the depletion of the 'readily releasable' fraction. The rapid exchangeability of the free acetylcholine with loosely bound vesicular acetylcholine and the slower exchange of the latter with tightly bound vesicular acetylcholine would clearly correspond to the model described by Birks & MacIntosh (1961).

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References

Appleton, H. D., La Du, B. N., Levy, B. B., Steele, J. M. & Brodie, B. B. (1953) *J. Biol. Chem.* **205**, 803–813

Barker, L. A., Dowdall, M. J., Essman, W. B. & Whittaker, V. P. (1970) in *Drugs and Cholinergic Mechanisms in the CNS* (Helibronn, E. & Winter, A., eds.), p. 193, Almquist and Wiksell, Stockholm

Birks, R. & MacIntosh, F. C. (1961) *Can. J. Biochem. Physiol.* **39**, 787–827

Bligh, J. (1952) *J. Physiol. (London)* **117**, 234–240

Collier, B. & MacIntosh, F. C. (1969) *Can. J. Physiol. Pharmacol.* **47**, 127–135

Collier, B., Poon, P. & Salehmoghaddam, S. (1972) *J. Neurochem.* **19**, 51–60

Curtis, D. R. & Eccles, J. C. (1960) *J. Physiol. (London)* **150**, 374–398

Dunant, Y., Gautron, J., Israël, M., Lesbats, B. & Manaranche, R. (1971) *C. R. Acad. Sci. Ser. D* **273**, 233–236

Dunant, Y., Gautron, J., Israël, M., Lesbats, B. & Manaranche, R. (1972) *J. Neurochem.* **19**, 1987–2002

Feldberg, W. & Fessard, A. (1942) *J. Physiol. (London)* **101**, 200–216

Fonnum, F. (1966) *Biochem. J.* **100**, 479–484

Fonnum, F. (1967) *Biochem. J.* **103**, 262–270

Fonnum, F. (1969) *Biochem. J.* **115**, 465–472

Geigy, J. R. (1970) *Documenta Geigy Scientific Tables* (Diem, K., & Leutner, C., eds.), 7th. edn., p. 165, Geigy S. A., Basle

Hubbard, J. I. (1970) *Progr. Biophys. Mol. Biol.* **21**, 33–124

Israël, M., Gautron, J. & Lesbats, B. (1968) *C. R. Acad. Sci. Ser. D* **266**, 273–275

Israël, M., Gautron, J. & Lesbats, B. (1970) *J. Neurochem.* **17**, 1441–1450

Kornberg, A. (1955) *Methods Enzymol.* **1**, 441–443

Kuriyama, K., Roberts, E. & Vos, J. (1968) *Brain Res.* **9**, 231–252

Marchbanks, R. M. (1968) *Biochem. J.* **106**, 87–95

Marchbanks, R. M. (1969) *Biochem. Pharmacol.* **18**, 1763–1766

Marchbanks, R. M. & Israël, M. (1971) *J. Neurochem.* **18**, 439–448

Molenaar, P. C., Nickolson, V. J. & Polak, R. L. (1971) *J. Physiol. (London)* **213**, 64*P*

Potter, L. T. (1970) *J. Physiol. (London)* **206**, 145–166

Richter, J. A. & Marchbanks, R. M. (1971*a*) *J. Neurochem.* **18**, 691–703

Richter, J. A. & Marchbanks, R. M. (1971*b*) *J. Neurochem.* **18**, 703–712

Szerb, J. (1961) *J. Physiol. (London)* **158**, 8*P*

Taugner, G. & Hasselbach, W. (1966) *Naunyn-Schmeidebergs Arch. Pharmakol. Exp. Pathol.* **255**, 266–286

Whittaker, V. P. (1971) *Ann. N.Y. Acad. Sci.* **183**, 21–32

Whittaker, V. P., Michaelson, I. A. & Kirland, R. J. A. (1964) *Biochem. J.* **90**, 293–303