The Isolation of Pure Cholinergic Synaptic Vesicles from the Electric Organs of Elasmobranch Fish of the Family Torpedinidae

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1. Zonal centrifuging permitted the separation, on the milligram scale and in a form largely free from contamination by soluble cytoplasmic protein or membrane fragments derived from other structures, of synaptic vesicles from the purely cholinergic terminals of the electric organ of *Torpedo*. Up to 100g of tissue could be processed in a single run.
2. As much as 46% of the bound acetylcholine from the original tissue preparation was recovered as a single peak of density equivalent to 0.38M-sucrose–0.21M-NaCl and with a concentration of up to 680nmol of acetylcholine/mg of protein.
3. The limiting concentration of acetylcholine in isolated vesicles when allowance had been made for non-vesicular protein appeared to be about 600nmol/mg of protein.
4. Vesicle counts by a 'bead-tagging' procedure indicated an acetylcholine content of about 360 μ mol/ml of vesicles; thus the vesicle protein content would be about 60% (w/v).
5. Calculations showed that the core of the vesicle, accounting for about 55% of the vesicle volume, could be largely filled with acetylcholine and protein.

Elasmobranch fish of the family Torpedinidae possess bilateral electric organs, on each side of the head, which receive a copious cholinergic innervation (Feldberg & Fessard, 1942) from paired nuclei (the electric lobes) in the brain-stem (Fig. 1). The electric organs are considered to be derived embryologically from muscle, with loss of the contractile elements, and consist of vertical stacks of cells (electroplaques) arranged in a honeycomb pattern. Each cell is innervated on its ventral surface by cholinergic nerve fibres, whose cell bodies are located in the electric lobes (Bennett, 1970), and which run in four large bundles between the gill arches before ramifying in the tissue. The discharge of these fibres causes a release of acetylcholine, which in turn generates a local, non-propagated depolarization of the electroplaques analogous to the end-plate potential in muscle (Grundfest, 1957). A synchronized discharge is followed by a summation of electroplaque potentials over the entire thickness of the organ and in this way shocks are generated amounting to tens or even, in a large specimen of *Torpedo nobiliana*, hundreds of volts which are probably sufficient to stun or kill small prey.

This system is thus an excellent one in which to study cholinergic transmission, since the whole cholinergic innervation is available from the cell bodies of the cholinergic neurons in the lateral line (electric) lobes (homologous *inter alia* with the hypoglossal

nuclei and readily identified by their yellow colour) through the nerve trunks (measuring 10mm or more in diameter in large specimens of *T. nobiliana*) to the terminals in the electric tissue with their associated postsynaptic membranes. In the present paper we describe how pure preparations of cholinergic synaptic vesicles may be isolated in milligram quantities for studies of lipid and protein composition from extracts of electric organ by using density-gradient centrifuging in zonal rotors. A few measurements have also been made of the total and bound acetylcholine content of the cholinergic nerves.

The first attempts to submit electric tissue to sub-cellular fractionation were made by Frontali & Toschi (1958), who were interested in the localization of acetylcholinesterase. Sheridan *et al.* (1966) showed that synaptosomes were not generated by homogenization in significant numbers and concluded that the mechanical and geometric conditions for synaptosome formation were not present in electric tissue. They noted that synaptic vesicles were liberated in large numbers, but attempts to purify these by pelleting and density-gradient separation of the re-suspended crude vesicle pellet were not completely successful. They showed, however, that if all the bound acetylcholine of the vesicle fractions were located in the vesicle core, it would account for about 4% of the wet weight of the vesicle, equivalent to a 0.4M solution of acetylcholine chloride or about 40000 molecules of acetylcholine per vesicle. Israël *et al.* (1968, 1970) introduced several modifications

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Fig. 1. Diagram of *Torpedo* showing location, innervation and structure of electric organs

(a) *Torpedo* showing position of electric organs; (b) brain, showing electric lobes and nerves to organ; (c) vertical section through a stack of electroplaque cells showing upper, non-innervated face and lower, innervated face. *Narcine* has an additional nerve on each side which innervates the small accessory electric organ caudal to the main organ.

of the original procedure which we have found helpful in the present work: they homogenized in a low-density medium (0.2M-sucrose–0.3M-NaCl); they applied a low-speed supernatant of the homogenate directly to the density gradient without initial pelleting of the vesicles; and their density gradient was made iso-osmotic with elasmobranch plasma by means of appropriate additions of NaCl throughout the portion through which the vesicles sedimented.

Brief accounts of this work have already been published (Essman & Whittaker, 1970; Barker *et al.*, 1970; Whittaker, 1971a, 1972).

Experimental

Fish

Five species of Torpedinidae were used: *Narcine brasiliensis* (Caribbean; Gulf Specimen Co., Panama, Fla., U.S.A.), *Torpedo torpedo*, *Torpedo marmorata* (Mediterranean; Stazione di Biologia Marina, Naples, Italy), *Torpedo nobiliana* (N. Atlantic; Marine Biological Laboratory, Woods Hole, Mass., U.S.A.) and *Torpedo californica* (N. Pacific; Pacific Bio-Marine Co., Venice, Calif., U.S.A.). Live fish were transported by air in sealed polythene bags containing oxygen and sea-water, and on arrival were kept at 10°C (*Torpedo*) or 20°C (*Narcine*) until killed (by pithing). Experiments in which anaesthesia was

induced by means of urethane and chloralose before killing showed no consistent effects on acetylcholine concentrations in the electric organ. Specimens were identified from the descriptions of Bigelow & Schroeder (1953) and Wheeler & du Heaume (1964). Weights of organs are given in Table 1.

Preparation of vesicles

Extraction of vesicles from electric tissue. Electric tissue is gelatinous and slippery; it is best removed from the body cavity by scraping or blunt dissection after stripping away the dorsal skin. Various methods of comminution and various types of suspension medium were tried (see the Results section and Table 3) including homogenization in a glass-and-Perspex homogenizer (Aldridge *et al.*, 1960) or a domestic blender for 1.5 min: the most successful method was to freeze the tissue in 30–70 g batches with liquid Freon 12 in a large ceramic mortar and crush it to a coarse powder with a pestle; this was particularly effective with *Narcine* and *T. nobiliana*, whose organs appear to have a higher collagen content than *T. marmorata*. The powdered tissue was allowed to warm to 0°C and was extracted with about 2 vol. of ice-cold 0.2M-sucrose–0.3M-NaCl (cf. Israël *et al.*, 1970): this process was usually assisted by a brief homogenization (six up-and-down strokes) in an Aldridge homogenizer at a speed of 900 rev./min

Table 1. *Weights of electric organs and associated cholinergic innervation of Torpedinidae*For more than one determination, values are means $\pm$ range (2) or S.E.M. (3 or more).

Species	Weight (g)		
	Electric lobes	Nerve trunks	Electric organs
<i>N. brasiliensis</i>	0.12 $\pm$ 0.02 (4)	0.51 $\pm$ 0.10 (4)	56 $\pm$ 7 (14)
<i>T. marmorata</i>	0.21 $\pm$ 0.05 (3)	0.61 $\pm$ 0.24 (3)	68 $\pm$ 16 (6)
<i>T. torpedo</i>	—	0.13 (1)	34.1 (1)
<i>T. nobiliana</i>	1.67 $\pm$ 0.26 (3)	21.4 $\pm$ 1.5 (2)	1600 (1)
<i>T. californica</i>	1.09 (1)	7.4 (1)	454 $\pm$ 59 (3)

and with a pestle diameter of 0.8% of that of the mortar; after some minutes the slurry was pressed through a double layer of cheese-cloth. The tissue fragments left in the cloth were re-extracted with a further small volume of cold sucrose to give a combined 'cytoplasmic extract' of which 2.5–5.0 ml was equivalent to 1 g of original tissue. These and subsequent operations were performed in a cold laboratory at 0–4°C. Electron microscopy of frozen tissue and tissue fragments (see the Appendix; Soifer & Whittaker, 1972) showed that freezing did not destroy the vesicles; on storage at –20° to –60°C, acetylcholine was lost, though vesicles could still be isolated on the basis of their density.

The tissue extract so obtained was centrifuged, unless otherwise stated, at 10000g for 30 min (r_{av} , 7.8 cm) to remove coarse membrane fragments, nuclei and mitochondria; the pellet (fraction P₁₂) was suspended in 0.2M-sucrose–0.3M-NaCl (about 0.7 ml/amount of pellet derived from 1 g of tissue) for assay. The supernatant (fraction S₁₂) was in some experiments centrifuged at 150000g (r_{av} , 5.4 cm) or 270000g (r_{av} , 5.7 cm) for 2 h to give a pellet (P₃) and a supernatant (S₃).

Separation of vesicles by zonal centrifuging. Separation of synaptic vesicles from soluble proteins and larger membrane fragments ('microsomes') also present in fraction S₁₂ was carried out at 0°C in Beckman Ti-14 or Ti-15 zonal rotors by using a model L2 65B preparative ultracentrifuge modified for zonal centrifuging. Fraction S₁₂ (30–100 ml) was layered against a continuous sucrose density gradient containing from 0.2M- to 1.4M- (sometimes 1.45M-) sucrose and containing enough NaCl to ensure that the osmolarity of the gradient did not fall below 800 mosmol/l (cf. Israël *et al.*, 1970). The gradient was formed from 'light' and 'heavy' sucrose solutions by using a Beckman gradient maker with a cam that was cut to deliver a gradient whose concentration was proportional to the distance from the centre of the rotor rather than to the volume. The relationship between concentration *C* and volume *V* was:

$$C = C_0 + (C_m - C_0) \sqrt{(V/V_m)}$$

where *C*₀ and *C*_m were the concentrations of light and heavy sucrose solutions and *V*_m was the total volume of the gradient (550 ml for the Ti-14 rotor and 1100 ml for the Ti-15 rotor). The light solution contained 0.2M-sucrose and 0.3M-NaCl; initially the heavy solution was 0.5M-sucrose–0.15M-NaCl, but after 200 ml (Ti-14 rotor) or 400 ml (Ti-15 rotor) of gradient had been delivered, the 0.5M-sucrose–0.15M-NaCl solution was replaced by 1.4M (or 1.45M)-sucrose: this caused an S-shaped inflexion in the gradient which was complete in 200 ml. Pumping was continued until the rotor was filled; supernatant was then injected through the line to the centre of the rotor, and was followed by an overlay of 0.2M-sucrose–0.3M-NaCl, bringing the total volume of supernatant and overlay to 100–120 ml (Ti-14 rotor) or 200–265 ml (Ti-15 rotor).

After centrifuging at 115000g for 3 h (r_{av} , 4.34 cm) (Ti-14 rotor) or 78000g for 4 h (r_{av} , 5.51 cm) (Ti-15 rotor), the rotor was emptied by pumping 1.6M-sucrose through the line to the rim of the rotor and collecting 10 ml (Ti-14 rotor) or 15 ml (Ti-15 rotor) fractions. Peaks of soluble protein, vesicles and membrane fragments were localized by measurements of protein, bound acetylcholine and marker enzymes as detailed below. Recoveries averaged 71% (acetylcholine) and 75% (protein); losses may have resulted from a small amount of leakage at the upper-bearing assembly of the rotor.

Gel filtration of vesicles. Vesicles were separated from soluble proteins of mol. wt. below 5×10^6 by gel filtration through columns of Bio-Gel agarose 5m (100–200 mesh) (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The gel (4g) was suspended in the elution medium (0.4M-sucrose–0.2M-NaCl); the suspension was evacuated to remove air bubbles and poured into a 2 cm-diameter glass column to form a gel column of 40 ml bed volume. After the column had been washed with medium (200 ml), samples (4 ml) of vesicle preparations were submitted to gel filtration. The eluent was collected in 2 ml fractions and analysed for acetylcholine and protein.

Counting of vesicles. Vesicles were counted by the

technique described by Whittaker & Sheridan (1965) and Sheridan *et al.* (1966); diluted polystyrene latex (Dow Chemical Co., Midland, Mich., U.S.A.), estimated to contain 6.25×10^{11} 88 nm (880 Å)-diameter latex beads/ml, was added to vesicle suspensions fixed with glutaraldehyde and the mixture was examined in the electron microscope after negative staining as described in the Appendix (Soifer & Whittaker, 1972). The ratio of the number of vesicles to the number of beads in several fields when multiplied by 6.25×10^{10} gave the number of vesicles/ml.

Other methods

Protein. The method of Lowry *et al.* (1951) was used. The E_{280} and E_{410} were also measured but were unreliable as a measure of protein concentration in turbid solutions.

Acetylcholine. Acetylcholine was extracted from tissue samples with ice-cold 10% (w/v) trichloroacetic acid (MacIntosh & Perry, 1950) and from suspensions of subcellular particles by heating them at pH 4.0 and 100°C for 10 min (Hebb & Whittaker, 1958); it was assayed on small (8 mm $\times$ 0.25 mm) slips of the dorsal muscle of the leech by the method of Szerb (1962) (compare also Dudar & Szerb, 1970), as modified by Whittaker *et al.* (1964), or on the morphine-treated ileum of the guinea pig; a detailed description of the methods used has been given by Whittaker & Barker (1971). When suspensions were assayed by both methods, assays agreed to $\pm 5\%$, confirming previous chromatographic evidence (Sheridan *et al.*, 1966) that the cholinergic activity is due to acetylcholine and not to a related ester (for discussion of the identification of acetylcholine in tissue extracts see Whittaker, 1963).

The bound acetylcholine of tissues (electric lobes, nerves and electric organs) was defined as that portion of the total (i.e. trichloroacetic acid-extractable) acetylcholine remaining after homogenizing the tissue, usually in 9 vol. of 0.2 M-sucrose–0.3 M-NaCl, but in a few experiments in 0.32 M-sucrose containing 0.33 M-urea, by using an Aldridge-type glass-and-Perspex homogenizer with a pestle rotating at 840–900 rev./min and of a diameter about 0.8% less than the internal diameter of the mortar. Nerves and electric tissue required more vigorous and prolonged homogenization than electric lobes, which readily disintegrated during the 2 min of homogenization (12 up-and-down strokes of the mortar punctuated by a 15 s cooling period) used as a routine for mammalian brain (Gray & Whittaker, 1962). Acetylcholine freed by homogenization is destroyed by the tissue cholinesterases.

Enzymes. Acetylcholinesterase was measured by the method of Ellman *et al.* (1961), choline acetyltransferase by the radiochemical method of Fonnum

(1966) and lactate dehydrogenase as described by Johnson (1960).

Results and Discussion

Total and bound acetylcholine in the neurons innervating the electric organ

Table 2 shows the total and bound acetylcholine content of the tissues containing the three main portions of the neurons innervating the electric organ: the electric lobes, nerve trunks and electric organs containing their cell bodies, axons and terminals respectively. It will be seen that acetylcholine is distributed throughout the length of the neurons, though the concentration in the terminal region is very much higher than in the rest of the neuron. These comparisons are only semi-quantitative since all three regions contain non-neural tissue; probably if allowance were made for this the difference would be even more striking. It is also difficult, especially in the smaller species, to exclude small amounts of the acetylcholine-rich electric tissue from the samples of nerve trunk. This may account for the large variance in the acetylcholine content of these samples.

Most of the acetylcholine in the nerve trunks is in a form that is released by homogenization (and then destroyed by the cholinesterases present in the preparation). This implies that the acetylcholine is not sequestered within storage particles in the axon, but is free in the cytoplasm; this cytoplasmic acetylcholine may represent acetylcholine synthesized by choline acetyltransferase during its passage down the axon (Hebb & Waites, 1956). The enzyme is soluble in this species (see below, also Israël *et al.*, 1970) as it is in mammalian brain tissue (Fonnum, 1967).

Much of the acetylcholine in the electric lobes is also in the free, cytoplasmic form as judged by the criterion of destruction by homogenization and possibly represents the somatic extension of the axonal cytoplasmic pool already mentioned. However, significant quantities of the acetylcholine of the electric lobes are bound and this most likely represents acetylcholine sequestered within cholinergic nerve terminals that have become pinched-off during homogenization to form synaptosomes as is known to happen with neural tissues in a wide variety of other species. In one experiment nearly 70% of the bound acetylcholine of the electric-lobe homogenate sedimented in a relatively low-centrifugal field (10000g for 30 min), suggesting that most of the bound acetylcholine of the lobe is indeed synaptosomal. The incoming tracts to this part of the brain are not thought to be cholinergic (Bennett, 1970), but the existence of recurrent collaterals terminating in cholinergic boutons remains a possibility.

By analogy with areas of the mammalian brain

Table 2. Total and bound acetylcholine content of cell bodies, axons and terminals of the cholinergic nerves supplying the electric organs of *Torpedinidae*
For experimental details see the text. Values for more than one specimen are means $\pm$ S.E.M. (3 or more) or range (2); the numbers of specimens assayed are given in parentheses.

Species	Acetylcholine content							
	Electric lobes			Nerve trunks			Electric organ	
	Total (nmol/g)	Bound		Total (nmol/g)	Bound		Total (nmol/g)	Bound (nmol/g)
		(nmol/g)	(% of total)		(nmol/g)	(% of total)		(% of total)
<i>N. brasiliensis</i> (4)	14.1 $\pm$ 3.5	3.5 $\pm$ 1.9 (2)	25	29.9 $\pm$ 15.9	0.85 $\pm$ 0.15 (2)	3	148 $\pm$ 43	52 $\pm$ 13
<i>T. marmorata</i> (3)	17.7 $\pm$ 6.8	—	—	12.3 $\pm$ 8.0	—	—	313 $\pm$ 72 (7)	170 $\pm$ 78 (7)
<i>T. torpedo</i> (2)	—	—	—	13.8	—	—	140 $\pm$ 2	153 $\pm$ 84
<i>T. nobiliana</i> (3)	55.4 $\pm$ 4.4 (2)	19.1 $\pm$ 1.15	34	9.6 $\pm$ 3.8 (2)	0.50 $\pm$ 0.50 (2)	5	229 $\pm$ 41 (2)	119 $\pm$ 18 (4)
<i>T. californica</i> (2)	—	—	—	—	—	—	192	64
							50	50
							111 $\pm$ 46	18 $\pm$ 2
							18 $\pm$ 2	10*§

* 20–25% homogenate.
† Homogenate in 0.32M-sucrose–0.33M-urea.
‡ Speed of pestle, 1400rev./min.
§ Cytoplasmic extract of frozen and powdered tissue.

(e.g. cerebral cortex, caudate nucleus) in which cholinergic neurons are mainly represented by their terminals, one might have expected to find most of the acetylcholine of the electric tissue in a bound state after homogenization (Lavery *et al.*, 1963; for further discussion see Whittaker, 1969, 1971*b*). However, a high yield of bound acetylcholine is only obtained under these conditions when most of the terminals pinch off to form synaptosomes: and synaptosome formation occurs only to a small extent in electric tissue (Sheridan *et al.*, 1966). The bound acetylcholine in electric-tissue homogenates thus probably represents mainly acetylcholine sequestered in synaptic vesicles. Again, by analogy with mammalian tissue, vesicular acetylcholine would be expected to account for about half of the total acetylcholine in the terminal; the somewhat lower yield of bound acetylcholine in three of the five species examined may well reflect differences in technique and tissue consistency, since side-by-side comparisons were not feasible. Cytoplasmic extracts of freshly frozen and powdered tissue gave somewhat lower mean values for the concentration of bound acetylcholine than homogenates of fresh tissue in 0.32M-sucrose–0.33M-urea.

Fractionation of tissue

Supplies of fish were not adequate to make extensive studies of the optimum conditions for fractionation. Of the five species examined, *T. marmorata* gave preparations of highest specific acetylcholine concentration and the electric tissues from this species were the easiest to comminute, apparently because of a lower collagen content and softer consistency.

Electron microscopic examination of the powdered frozen tissue (Soifer & Whittaker, 1972) showed that it consisted essentially of fragments of electroplaques in which the membranes of the non-innervated face, the postsynaptic membrane and the pre-synaptic membrane adjacent to it were well preserved, but in which the portions of the nerve-ending membranes away from the synaptic cleft were largely disrupted, leaving a layer of cytoplasm containing numerous synaptic vesicles. Extraction with sucrose-saline caused a proportion of vesicles to become detached, leaving empty nerve terminals, and these were recovered in the supernatant (S_{12}) fraction (Table 3). In a preliminary series of experiments in which the tissue was homogenized in the sucrose-saline medium (or, in some experiments, in a sucrose-

Table 3. *Distribution of bound acetylcholine in subcellular fractions of electric tissue of Torpedinidae*

For experimental details see the text. Values for two or more experiments are means $\pm$ range (2) or S.E.M. (3 or more); the number of animals is given in parentheses.

Species	Method of disruption	Acetylcholine distribution between fractions			
		Homogenate or cytoplasmic extract (nmol/g)	P_{12} (% of total recovered)	S_{12} (% of total recovered)	Recovery (% of column 3)
<i>N. brasiliensis</i>	Homogenization of fresh tissue (4)*	52 $\pm$ 13	84 $\pm$ 3	16 $\pm$ 3	66 $\pm$ 18
	Extraction of tissue comminuted while frozen (1)	30	39	61	117
<i>T. marmorata</i>	Homogenization of fresh tissue (2)†	320 $\pm$ 60	70 $\pm$ 2	30 $\pm$ 2	99 $\pm$ 4
	Homogenization of fresh tissue (1400 rev./min) (3)†	153 $\pm$ 84	40 $\pm$ 10	60 $\pm$ 10	77 $\pm$ 6
	Blending (1)†	240	40	60	70
	Extraction of tissue comminuted while frozen (4)	119 $\pm$ 18	33 $\pm$ 7	68 $\pm$ 7	69 $\pm$ 20
	Extraction of frozen comminuted tissue (2)	18 $\pm$ 2	31 $\pm$ 2	69 $\pm$ 2	115 $\pm$ 15
<i>T. californica</i>	Extraction of frozen comminuted tissue (2)	18 $\pm$ 2	31 $\pm$ 2	69 $\pm$ 2	115 $\pm$ 15

* 20–25% homogenate.

† P_{12} sedimented at 3500g for 10 min (r_{av} 7.8 cm); medium contained 0.09M-NaCl, 0.33M-urea and 0.12M-sucrose in place of 0.3M-NaCl.

saline-urea medium of almost equivalent osmolarity), with an Aldridge-type glass-and-Perspex homogenizer (pestle speed 840–900 rev./min), the yield of bound acetylcholine in the supernatant fraction was much lower (Table 3, compare lines 1 and 2, also 3 and 6). More vigorous homogenization (pestle rotating at 1400 rev./min, Table 3, line 4) or the use of a domestic blender (Table 3, line 5) released a larger proportion of bound acetylcholine into the supernatant, but also larger amounts of small membrane fragments which tended to contaminate the synaptic vesicle fraction at a later stage. The main series of experiments were therefore done with extracts of powdered tissue, in spite of the somewhat lower yields of bound acetylcholine in the initial extract.

Zonal separation of vesicles

On submitting fraction S_{12} to density-gradient fractionation in a zonal rotor (Fig. 2, Table 3), the bound acetylcholine migrated as a sharp peak (VP) with a density (Ti-14 runs) at the peak equivalent to 0.37–0.39M-sucrose containing 0.225–0.215M-NaCl and well separated from a more slowly moving peak of soluble protein (SP), which showed little tendency to migrate into the gradient during a run. The fractions containing soluble protein were often slightly pink because of traces of haemoglobin ($\lambda_{\max}$. 410nm). The peak acetylcholine fractions were rich in mono-disperse synaptic vesicles (Plate 1 of the Appendix; Soifer & Whittaker, 1972) and were remarkably free from larger membrane fragments.

Fig. 2. Typical separation of bound acetylcholine (peak VP) from soluble protein (peak SP) by using a Ti-14 zonal rotor

At the arrow, 1.4M-sucrose replaced 0.5M-sucrose–0.15M-NaCl as the dense solution in the gradient former. Recoveries: acetylcholine, 1.12 μ mol (50%); protein, 291 mg (65%). Acetylcholine concentration at peak: 560 nmol/mg of protein.

Table 4. *Density and acetylcholine content of the cholinergic vesicle fraction from the electric organ of T. marmorata*

For experimental details see the text. Each line refers to a separate run. Densities are given as the concentration of sucrose in a sucrose-saline mixture of constant osmolality of 800 mosmol/l and are the theoretical values calculated from the equation given in the text. The range relates to the width of the peak at half the maximum acetylcholine concentration. The volumes in column 1 are measured from the beginning of the gradient and do not include the volumes of sample and overlay, which varied slightly from one run to another. The portions of peak VP pooled and sampled for gel filtration were: A, three peak fractions; B, fractions containing acetylcholine to half or more of the maximum (peak) concentration of acetylcholine. Volume of fractions: 10 ml (Ti-14 rotor), 15 ml (Ti-15 rotor).

	Vol. of gradient to peak (ml)	Density of peak tube (and range)	Recoveries (%)		Specific concn. of acetylcholine in peak (nmol/mg of protein)	Portion of peak taken for gel filtration	Estimated proportion of protein in void-volume peak after gel filtration (%)	Corrected specific concn. of acetylcholine (nmol/mg of protein)
			Acetylcholine	Protein				
Ti-14 rotor	210	0.39 (0.35-0.40)	51	91	250	A	37	680
	210	0.39 (0.35-0.395)	63	64	583	—	—	—
	200	0.38 (0.36-0.39)	50	65	560	B	85 ± 2 (3)	660
	180	0.37 (0.33-0.38)	98	—	300	B	82	366
Mean ± s.e.m. ...	200 ± 7	0.38 ± 0.01	66 ± 11	73 ± 9	423 ± 81	—	—	—
Ti-15 rotor	220	0.34 (0.30-0.36)	65	85	363	—	—	—
	215	0.33 (0.31-0.35)	100	72	441	A	79 ± 2 (2)	558
	200	0.33 (0.32-0.34)	—	—	348	—	—	—
	212 ± 6	0.33 ± 0.01	83 ± 17	79 ± 7	384 ± 29	—	—	566 ± 72 (all)

Table 5. *Acetylcholine content of vesicles*

For details see the text.

Expt.	Fraction no.	Acetylcholine content (nmol/ml)	Total no. of beads counted	No. of vesicles/ no. of beads* (b)	10 ⁻¹⁰ × No. of beads/ml (c)	10 ⁻¹⁰ × No. of vesicles/ml (d = bc)	Acetylcholine content (molecules/10 ³ vesicles) (= 60a/d)
Z16	30†	5.0	700	0.18 ± 0.05 (2)	12.5	2.2	137
Z17	26	5.2	192	6.27 ± 0.47 (3)	6.25	39	8
	30‡	7.5	27	0.91 ± 0.17 (2)	6.25	5.7	79
	34	4.0	82	0.95 ± 0.15 (2)	6.25	5.9	41
Mean ± s.e.m. 66 ± 30 (4)							

* Mean ± range (2) or s.e.m. (3); no. of fields counted given in parentheses.

† Peak fraction.

‡ One of two peak fractions; fractions are numbered according to increasing density. Fraction 26 appeared to have many more vesicles than would be expected from its acetylcholine content and the vesicle content of other fractions.

Fig. 3. Gel filtration of zonal peaks

(a) Gel filtration of zonal-density-gradient peaks VP and SP (see Fig. 2) from *T. marmorata* through a column of Bio-Gel agarose 5m (bed volume, 40ml). In the case of peak VP, the three peak fractions (30ml in all) were pooled and 6ml was taken for gel filtration. In the case of peak SP, the entire peak was pooled and 1ml taken for gel filtration. Recoveries: (peak VP) acetylcholine, 43nmol (71%); protein, 191 μ g (88%); (peak SP) protein, 1.70mg (62%). Note the association of bound acetylcholine with the void volume peak derived from peak VP and the presence of a small amount of soluble protein in peak VP with a retention volume similar to that of the main peak derived from peak SP. (b) Gel filtration of the pooled void volume peaks from two runs of peak VP showing the absence of soluble protein. Recovery: 105 μ g (80%).

The best results were obtained with preparations from *T. marmorata*. In the runs of lower integrated centrifugal field in the Ti-15 rotor ($1.87 \times 10^7 g\text{-min}$ compared with $2.07 \times 10^7 g\text{-min}$ in the Ti-14 rotor) the vesicles did not migrate so far, but were nearly as well separated from soluble protein (Table 4). Centrifuging for longer than 3–4h did not improve the preparation. Runs were highly reproducible, the peak acetylcholine fraction falling within a range of only 2–3 fractions. Lower down the gradient there was

a small usually double peak (MP) of membranous material with a density equivalent to 0.8–1.2M-sucrose. This peak was considerably larger when fraction S_{12} was prepared from tissue homogenates made in an Aldridge-type homogenizer or a blender, and then sometimes contained acetylcholine in low specific concentration, presumably because of the presence of vesicles in incompletely disrupted terminals. A comparison of Tables 3, 4 and 6 shows that the mean yield of bound acetylcholine in peak VP based

on the original extract is 33% and that the enrichment factor (relative to protein) is 71, most of the enrichment occurring as a result of the density-gradient separation.

When fraction P₃ was used as starting material instead of fraction S₁₂, the peak acetylcholine concentration occurred at about the same density. However, perhaps because of the difficulty in redispersing the pellet, the acetylcholine peak was much smaller and more spread out relative to the membrane peak.

Gel filtration of vesicles

When peak VP from the zonal density gradient was passed through a Bio-Gel agarose 5m column, most of the protein came through in the void volume and was associated with acetylcholine (Fig. 3). However, further elution with 0.4M-sucrose–0.2M-NaCl usually gave a small peak of soluble protein with a retention volume equal to that of the broad soluble-protein peak obtained when peak SP from the zonal run was similarly passed through the gel column. This small peak of soluble protein presumably represents contamination of the vesicle fraction by soluble protein that has diffused down the gradient. There was less diffusion during short (3h) runs than during overnight runs. By gel filtration it is thus possible to determine the proportion of protein in peak VP that is present in the vesicles (and any contaminating membrane fragments). The concentration of acetylcholine in nmol/mg of protein did not actually increase as a result of gel filtration, however, because of loss of bound acetylcholine during manipulation.

In all cases the protein concentration of the fractions pooled for gel filtration (Table 4, column 6) was almost the same, i.e. there was no evidence from the protein distribution in this part of the gradient that

the lighter fractions were more contaminated with soluble protein than the heavier ones, though presumably the contamination was not completely uniform. Thus the application of a correction factor for soluble protein derived from a pooled sample to an acetylcholine concentration derived from a peak fraction probably does not involve much error.

Acetylcholine content of vesicles

The acetylcholine concentration (nmol/mg of protein) of the peak fraction of VP in a number of experiments is given in Table 4. If allowance is made for the soluble protein present in the fraction as measured by gel filtration, a limiting value of about 680nmol of acetylcholine/mg of vesicle protein is obtained. Occasionally values of up to about 1000nmol/mg were obtained from fractions on the leading edge of peak VP, containing small amounts of acetylcholine and protein, but since these values are the ratio of two small quantities and were not reproducible, they are not considered very reliable. However, acetylcholine leaks out of isolated vesicles rather quickly (the half-life is about 3h) and it is possible that the acetylcholine content of the vesicle *in situ* is higher than the mean value of 566 ± 72 (4) nmol/mg of protein given in Table 4.

The acetylcholine content per vesicle was determined in suspensions of known acetylcholine content whose vesicle content was estimated by 'tagging' with polystyrene beads. Perhaps because of the need for repeated application of suspensions to the grids, the variance in the vesicle counts was considerable, but the mean value for the number of acetylcholine molecules/vesicle was 66000 (Table 5), and is of the same order of magnitude as that found by Sheridan *et al.* (1966) of 40000. For a vesicle of mean

Table 6. *Distribution of marker enzymes and protein in the primary fractions derived from sucrose extracts of comminuted electric organs*

For experimental details and nomenclature of fractions see the text. Enzyme units are μmol of substrate transformed/min.

Component	Species	Concn. in extract (units/g of tissue)	Distribution between fractions (% of recovered component)		Recovery (% of suspension)
			P ₁₂	S ₁₂	
Acetylcholinesterase	<i>T. torpedo</i>	127	45	55	73
Acetylcholinesterase	<i>T. marmorata</i>	330	46	54	61
Lactate dehydrogenase	<i>T. marmorata</i>	540	17	83	89
Choline acetyltransferase	<i>T. marmorata</i>	16.2	5	95	91
Protein (mg/g of tissue)	<i>T. torpedo</i>	17.0	24	76	70
Protein (mg/g of tissue)	<i>T. marmorata</i>	15.2	38	62	124

Fig. 4. Centrifugal-density-gradient separation of fraction *S*₁₂ from *T. marmorata* by using a Ti-14 zonal rotor

A vesicle-rich peak containing bound acetylcholine (peak VP) (■), is separated from a peak (SP) of soluble protein (●) and a peak (MP) of larger membrane fragments rich in acetylcholinesterase (X). The lactate dehydrogenase (○) and choline acetyltransferase (+) present in fraction *S*₁₂ behave like soluble protein; acetylcholinesterase is partially soluble and partially bound to the membrane fragments sedimenting beyond the synaptic vesicles to the region of peak MP. The concentration of each component in the fractions is expressed as a percentage of the total amount of the component recovered at the end of the experiment. The results of two almost identical runs, with two samples of the same preparation of fraction *S*₁₂, are superimposed. In the second run (in which choline acetyltransferase was measured) the sample volume was 60ml and the overlay 40ml; the plots of enzyme activity have accordingly been displaced to the right by 10ml. The amounts (percentage recoveries in parentheses) were: protein, 183mg (91%); acetylcholine, 612nmol (51%); lactate dehydrogenase, 8360 units (78.5%); choline acetyltransferase, 177 units (110%); acetylcholinesterase, 1530 units (56%); for definition of enzyme units see Table 6.

Table 7. Distribution of acetylcholinesterase in sucrose density gradients after zonal centrifuging of fraction *S*₁₂ from electric tissue

For details see the text. Values for more than one experiment are means±s.e.m. (3) or range (2).

Species	Acetylcholinesterase recovered in fraction SP (%)	Concn. of sucrose of second peak (MP)	Acetylcholinesterase recovered in gradient (% of parent sample)
<i>T. nobiliana</i>	45 ± 9 (2)	0.88 ± 0.08 (2)	—
<i>T. torpedo</i>	67	1.11 ± 0.02 (2)	50 ± 0 (2)
<i>T. marmorata</i>	56 ± 8 (3)	0.92 ± 0.07 (3)	88 ± 32 (2)

diameter 84 nm (840 Å) (Sheridan *et al.*, 1966) this corresponds to 5.2 g of acetylcholine cation/100 ml of vesicles or 360 nmol/ μ l of vesicles. For an acetylcholine content of 566 nmol/mg of protein, this implies a vesicle protein content of about 64% (w/v). Since the 8 nm (80 Å)-thick membrane of an 84 nm (840 Å)-diameter vesicle accounts for 45% of the vesicle volume and may contain say, 20% of protein (cf. myelin; Gent *et al.*, 1971), this would leave about 55% (w/v) of protein to be accommodated within the core of the vesicle, which accounts for 55% (v/v) of the volume. This protein, together with acetylcholine, might thus account for most of the vesicle core.

Distribution of marker enzymes

A few experiments were done to determine the sub-cellular distribution of lactate dehydrogenase, choline acetyltransferase and acetylcholinesterase (Table 6, Fig. 4). The first two enzymes have the distribution expected of a soluble cytoplasmic enzyme. Most (83–93%) of the activity of the initial tissue extract is recovered in fraction S_{12} , and on the density gradient (Fig. 4) almost all the activity was recovered in the SP peak. These results confirm those of Israël *et al.* (1970). The virtual absence of choline acetyltransferase from the vesicle fraction is noteworthy, showing that the high local concentration of acetylcholine in the vesicle is not caused by local synthesis but presumably by the uptake of acetylcholine synthesized in the cytoplasm.

By contrast, acetylcholinesterase (Table 6) is mainly a membrane-bound enzyme; nearly half is recovered in the low-speed pellet (P_{12}), and of the remainder, over half (Fig. 4, Table 7), after density-gradient centrifuging, is recovered in a second particulate peak of membrane fragments (MP), sedimenting beyond the vesicles to a region of the gradient corresponding to 0.80–1.20 M-sucrose. This peak was heterogeneous and usually showed a shoulder or subsidiary peak extending into the denser sucrose. This was particularly evident in the Ti-14 runs. The remainder appears to be soluble; the vesicle fraction as in mammalian brain (Whittaker *et al.*, 1964) has very little activity.

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APPENDIX

Morphology of Subcellular Fractions Derived from the Electric Organ of *Torpedo*

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Several studies have been carried out on the fine structure of the electric organs of torpedine fish (Luft, 1956, 1958; Wachtel *et al.*, 1961; Sheridan, 1965) and of subcellular fractions derived from them (Frontali & Toschi, 1958; Sheridan *et al.*, 1966; Israël *et al.*, 1970). However, because the conditions used to prepare the subcellular fractions studied in the accompanying paper (Whittaker *et al.*, 1972) differ significantly from those used by previous workers, a fresh investigation was made and is reported in this Appendix.

Methods

Negative staining

Vesicle preparations were fixed by the addition of 0.1 vol. of glutaraldehyde (5% in 0.4M-sucrose–0.2M-NaCl) and were applied to carbon-coated electron-microscope specimen grids by the 'double-droplet' technique (Whittaker *et al.*, 1964) for negative staining with 1% ammonium molybdate (Horne & Whittaker, 1962). Repeated applications of vesicle preparations were necessary because of the low concentration of vesicles. Spreading was promoted by coating the grids before use with 1% bovine serum albumin.

Positive staining, embedding and sectioning

Tissue fragments or suitable volumes (usually about 0.2 vol.) of suspension were placed in cold 2.5% glutaraldehyde containing 0.1M- NaH_2PO_4 – Na_2HPO_4 buffer, pH 7.4, to which had been added enough sucrose to give a final concentration of 0.2M. This buffer was approximately iso-osmotic with elasmol-

branch plasma. The tissue fragments were added to the buffered glutaraldehyde while still frozen and were allowed to thaw in the fixative. After 0.5–2 h the fixed material was collected from the glutaraldehyde by centrifugation at 5000g for 10 min; small pieces of the resultant pellet were stained with 1% OsO_4 in 0.1M-sodium phosphate buffer–0.2M-sucrose, before being dehydrated in ethanol and embedded in Araldite. Sections were cut with a diamond knife on a Sorvall Porter–Blum MT II ultramicrotome; then they were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. Material was examined from fractions derived from both *Torpedo nobiliana* and *Torpedo marmorata*, with similar results.

Results

After being frozen and comminuted to a coarse powder, the fragments of electroplaques retain enough basic organization to be readily identifiable (Plate 1b). The innervated surface of the electroplaque, which is lined with nerve endings and subsynaptic invaginations, may be distinguished on almost every fragment. A striking feature of many of the nerve endings is the distortion, disruption and occasional absence of the nerve terminal membrane on the side away from the cleft. Parts of the endings themselves may be distorted or retracted from the electroplaque surface. The extracellular basal lamina material, which normally lies over the nerve ending surface, often appears consolidated in aggregates along the disrupted neuronal surface, mixed with remnants of the Schwann cell covering of the nerve endings. Nevertheless, numerous synaptic vesicles