Biochem. J. (1976) **160**, 113–115
Printed in Great Britain

Isolation of Pure Cholinergic Nerve Endings from the Electric Organ of *Torpedo marmorata*

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(*Received* 12 *July* 1976)

A rapid method for the preparation of highly purified cholinergic nerve endings from the electric organ of *Torpedo* is described. The endings retain their cytoplasmic components, as shown by biochemical and morphological observations. The homogeneity of these synaptosomes make them a useful tool for further studies.

Whittaker (1959) and De Robertis *et al.* (1961) pioneered the isolation of nerve-ending particles from guinea-pig and rat cortex. The nerve endings become pinched off during homogenization, forming sealed synaptosomes that contain specific cytoplasmic markers, for example choline acetyltransferase in those derived from the cholinergic synapses. They also contain synaptic vesicles and sometimes a mitochondrion. The synaptosomes are able to respire, synthesize and release transmitter, take up precursors, and they also exhibit a membrane potential [for reviews, see Jones (1975) and Marchbanks (1975)]. They are therefore very useful for the study of pre-synaptic metabolism and mechanisms of transmitter release.

Unfortunately, the synaptosomal preparations at present available are obtained from various materials, such as rodent cortex, squid ganglia or *Octopus* brain, that are heterogeneous, since they contain nerve endings with different transmitters (see Jones, 1975).

We report here a method for the isolation of pure cholinergic nerve endings. The electric organ of *Torpedo marmorata* was chosen because of its homogeneity with respect to the transmitter acetylcholine and because of its numerous nerve terminals. Previous attempts with this tissue led only to damaged nerve-ending preparations (Israël & Gautron, 1969) and to the isolation of synaptic vesicles only (Israël, 1970; Israël *et al.*, 1968, 1970; Whittaker *et al.*, 1972).

Materials and Methods

The isolation procedure was rendered possible by the observation that chopping the electric organ preserves intact the innervated face of the electroplax (Morel, 1976). When other homogenization procedures are used, nerve endings get damaged.

Torpedoes were obtained from the Marine Station of Arcachon, France. A fragment of electric organ (20g) was finely chopped with a razor blade and suspended in 200ml of *Torpedo* physiological medium, which consists of 280mM-NaCl, 3mM-KCl, 1.8mM-$MgCl_2$, 3.4mM-$CaCl_2$, 5mM-$NaHCO_3$, 1.2mM-sodium phosphate buffer (pH6.8), 5.5mM-glucose, 300mM-urea and 100mM-sucrose. When equilibrated with O_2, its final pH is 7–7.2. All further steps were carried out at +4°C. After stirring for 30min, the suspension was forced through three stainless-steel grids mounted on syringes. The grids were purchased from Tripette et Renaud (Paris, France) and had square meshes of 1000, 500 and 200μm side, used in that order. The suspension was then filtered through a nylon gauze (square of 50μm side) under slight suction. The nylon cloth was washed with 50ml of physiological medium. The filtrate (fraction F) was then centrifuged at 6000*g* for 20min. The supernatant (fraction S) was discarded and the pellet (fraction P) resuspended in 20–25ml of physiological medium. Then 6ml was layered on to a discontinuous sucrose gradient (prepared 2h before). This gradient was composed, from bottom to top, of 8ml of physiological medium without urea but containing 0.7M-sucrose (final concentration), 8ml of physiological medium without urea but with 0.5M-sucrose (final concentration) and 12ml of physiological medium with 0.1M-urea and 0.3M-sucrose (final concentrations). It was centrifuged for 40min in an SW27 Beckman rotor at 63900*g* ($r_{av.}$ 11.8cm). The Beckman LS-65 centrifuge was set at its maximum acceleration rate, with its brake on. A tube slicer was used to collect the fractions, which were, from the top to the bottom of the tube, a clear supernatant (A), three bands at each interface (B, dense; C, wider; D, hazy) and a thick pellet (E).

Choline acetyltransferase (EC 2.3.1.6) was measured as described by Fonnum (1975). Acetylcho!-

esterase (EC 3.1.1.7) was measured by the method of Ellman *et al.* (1961). Acetylcholine was determined by the eserinized frog rectus technique as summarized by McIntosh & Perry (1950). Lactate dehydrogenase (EC 1.1.1.27) was determined as described by Johnson & Whittaker (1963), and proteins by the method of Lowry *et al.* (1951).

For morphological observations, fractions were half-diluted with the physiological medium and spun down (11000*g* for 20min). Pellets were fixed in 3% (w/v) glutaraldehyde in 0.5M-cacodylate buffer (pH7.4), post-fixed in 2% (w/v) OsO_4, dehydrated and embedded in Araldite.

Results and Discussion

Special attention was given for preparing a fraction of nerve endings of high purity and for maintaining intact their cytoplasmic content. Plate 1 shows a representative electron micrograph, at low magnification, of fraction C. Three independent experiments showed the same homogeneity. These endings contain numerous synaptic vesicles, glycogen granules, and, as in the intact tissue, few mitochondria are to be seen. The post-synaptic membrane does not remain adherent to the nerve endings. Table 1 shows that the fraction of pure nerve endings (C) contains the cytoplasmic marker lactate dehydrogenase, and a peak of acetylcholine and choline acetyltransferase clearly separated from the amounts found in fraction E. The pellet E, in which acetylcholinesterase is abundant, is heterogeneous and contains fragments of post-synaptic membranes with partially attached and damaged nerve endings, nuclei and erythrocytes.

The fact that 50% of the acetylcholine content of the filtrate (F) sediments in pellet P with only 25% of the choline acetyltransferase activity is expected, since the enzyme is present in the axoplasm of nerve branches (Israël, 1970). These are opened by the chopping and filtration procedures and account for about 50% of the total choline acetyltransferase activity.

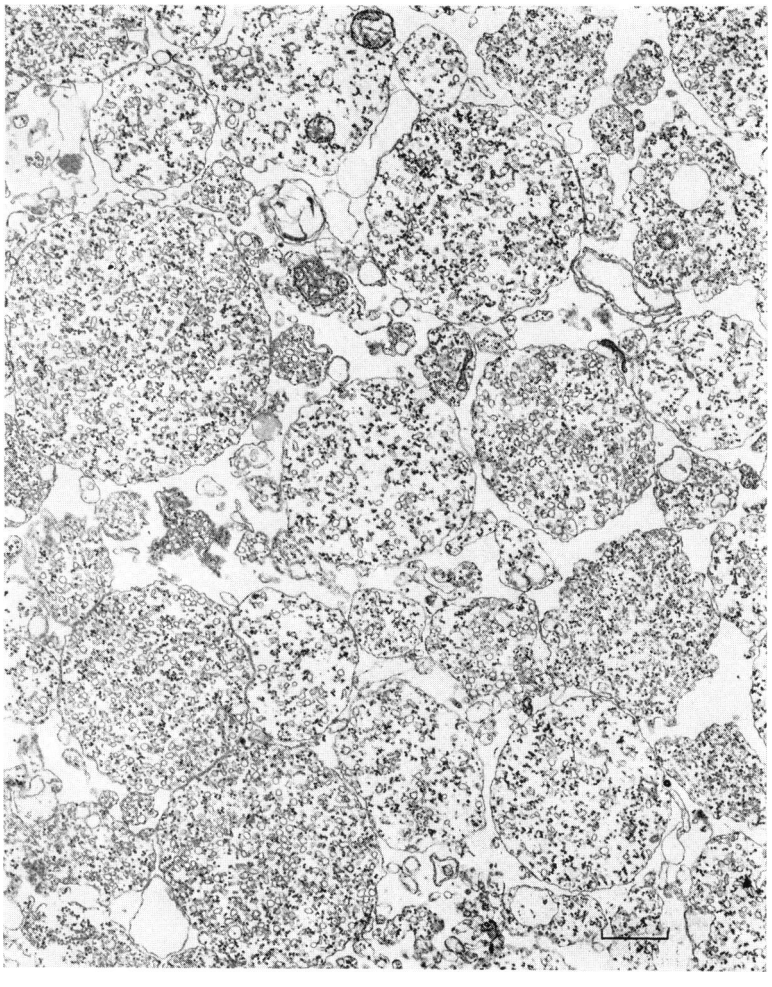
The ratio choline acetyltransferase/acetylcholine in the pure fraction C is higher than that in the pellet E, showing that the endings lost in pellet E have probably been damaged, loosing some cytoplasm. On the whole, we may calculate, by comparing the acetylcholine content of the filtrate (F) and fraction C, that the procedure purifies 20% of the intact nerve endings.

From electron-microscopic measurements it can be calculated that nerve endings represent about 11% of the electroplax volume; therefore we may attribute to them 1.66mg of the proteins present in the filtrate (F). If all the acetylcholine (271nmol/g) is occluded in these endings, a maximum specific activity of 163nmol of acetylcholine/mg of protein would be reached in a pure fraction. As the specific activity of

Table 1. *Isolation of cholinergic nerve endings: biochemical markers*

The results are means± S.E.M. of five experiments (three pooled gradients per experiment). Rf is recovery in primary fractions (S+P); Rg is recovery in gradients. For nomenclature of fractions, see the Materials and Methods section.

	Homogenate	F	S	P	A	B	C	D	E	Rf (%)	Rg (%)
Proteins (mg/g)	23.38±1.67	15.10±0.65	10.85±0.88	4.35±0.50	0.14±0.01	0.38±0.03	0.37±0.03	0.20±0.02	2.99±0.45	101	94
Acetylcholine (nmol/g)	485±49	271±17	79±12	150±5	2.1±0.1	13.1±1.7	57.2±4.8	15.1±2.0	72.1±5.4	84	106
Choline acetyltransferase (nmol/h per g)	2372±208	2137±315	1134±153	539±25	22±4	77±17	207±41	33±9	170±21	78	94
Acetylcholinesterase (mmol/h per g)	24.6±3.2	12.5±1.7	1.3±0.2	6.5±1.2	0.01±0.001	0.14±0.01	0.26±0.05	0.09±0.01	6.64±1.61	62	110
Lactate dehydrogenase (ΔE/min per g per ml)	44.7±14.9	33.2±13.8	21.3±10.2	12.0±0.9	0.19±0.11	1.46±0.18	2.68±0.32	0.97±0.19	6.23±1.03	100	96



EXPLANATION OF PLATE 1

Electron micrograph of the pure nerve endings (fraction C)

For details, see the text. The scale bar represents 1 μm.

fraction C is 154 nmol of acetylcholine/mg of protein, we may consider that its purity is in accordance with the electron-microscopic observations.

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