ISOLATION OF PURE CHOLINERGIC NERVE ENDINGS FROM *TORPEDO* ELECTRIC ORGAN

Evaluation of Their Metabolic Properties

NICOLAS MOREL, MAURICE ISRAEL, ROBERT MANARANCHE, and PAULE MASTOUR-FRACHON

From the Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire, Centre National de la Recherche Scientifique, 91190 Gif sur Yvette, France

ABSTRACT

Pure cholinergic nerve endings (synaptosomes) were isolated from the electric organ of *Torpedo* by a rapid procedure. These synaptosomes are ~3 µm in diameter. They contain an occasional mitochondrion, numerous synaptic vesicles, and sometimes an active zone is observed. No postsynaptic membrane attachment is found. This nerve ending fraction is extremely pure as shown by morphological controls and biochemical data. It is rich in choline acetyltransferase (450 nmol/h per mg protein) and acetylcholine (ACh) (130 nmol/mg protein). The isolated endings retain their cytoplasmic components and they synthesize ACh and are stable in vitro for several hours, as shown by biochemical measurements and morphological analysis.

KEY WORDS   synaptosomes · acetylcholine ·
Torpedo · electric organ

Nerve ending particles (synaptosomes) have been extensively used since their isolation from brain (35, 2). During mild homogenization, the pinched-off nerve terminals reseal, retaining their cytoplasm. Synaptosomes have proven to be valuable tools for in vitro studies of presynaptic metabolism. Recently, Jones (25) reviewed this field and gave useful information on the different preparations that are available. Unfortunately, these preparations originate from tissues containing different types of synapses and transmitters. To avoid such a disadvantage, fractionation studies (33, 14) have been performed on a homogeneous cholinergic material such as the electric organ of *Torpedo* which is particularly rich in nerve endings (11). However, initial attempts to purify synaptosomes or vesicles from this tissue were unsuccessful (33).

It was only in 1968 that a pure fraction of synaptic vesicles rich in acetylcholine (ACh) could be obtained from this tissue (20, 18, 21, 36). Indeed, nerve endings (pseudo-boutons) were found in some fractions, but they were altered and had lost most of their cytoplasmic content (33, 19). The isolation of *Torpedo* synaptosomes has been rendered possible by the observation that chopping the electric organ leaves the innervated face of the electroplaques intact (31). A preliminary report on the purification technique has already been published (22). The present work describes the isolation procedure and tests the physiological value of the resulting, purely cholinergic nerve ending fraction.

MATERIALS AND METHODS

Fractionation Procedure

Torpedo specimens were obtained from the Marine Station (Arcachon, France). The fish are first chilled for

10-15 min on ice. Slices of electric organ (15-20 g) are finely chopped with a scalpel until the macroscopic structure of the tissue is no longer visible. The mince is suspended in 200 ml of *Torpedo* physiological medium consisting of: 280 mM NaCl, 3 mM KCl, 1.8 mM $MgCl_2$, 3.4 mM $CaCl_2$, 1.2 mM sodium phosphate buffer (pH 6.8), 5.5 mM glucose, 300 mM urea, and 100 mM sucrose. When equilibrated with O_2, $NaHCO_3$ (4-5 mM) is added to adjust the solution to pH 7.0-7.1. All further steps are carried out in a cold room at + 4°C. After stirring for 30 min (magnetic stirrer), the suspension is forced through stainless steel grids, which were purchased from Tripette et Renaud (Paris, France). Grids (3 cm diam.) are stuck, by heating, to the openings of 50-ml plastic syringes whose ends have been sawed off. The suspension is successively passed through three grids with square openings of 1,000-, 500-, and 200-μm side length. After each step, the tissue adherent to the grid is recovered by scraping it off with a scalpel blade. The suspension is then filtered through a nylon gauze (50-μm square openings) under slight suction. The nylon cloth is washed with 50 ml of physiological medium. The filtrate (fraction F) is then centrifuged at 6,000 g for 20 min. The supernate (fraction S) is discarded. The pellet (fraction P) is resuspended in 20-25 ml of physiological medium and vigorously shaken. Then 6 ml of P are layered onto a discontinuous sucrose gradient prepared 1^1/$_2$-2 h before. This gradient has already been described in a previous report (22). The fractions obtained are, from the top to bottom of the tube: a clear supernate (A), three bands at each interface (B, dense; C, wider; D, hazy), and a thick pellet (E).

Incubation of Synaptosomes

Fraction C (40 ml, when prepared from six gradients) is twice diluted in the physiological medium without sucrose. The synaptosomes are allowed to warm up to 20°C for 15 min. Two precursors of ACh are used: [1-^{14}C]acetate (Amersham CFA 13, specific radioactivity 58 mCi/mmol, Amersham/Searle Corp., Arlington Heights, Ill.), methyl-[^{14}C]choline (Amersham CFA 424, specific radioactivity 58 mCi/mmol) at a final concentration of 1 μCi/ml, or methyl-[^{3}H]choline (Amersham TRK 179, specific radioactivity 10.1 Ci/mmol) at a final concentration of 5 μCi/ml. Unlabeled acetate or choline is added to the incubation medium as indicated for each experiment. At the end of the incubation period, samples are subjected to filtration. Millipore filters (0.65 μm, MF Millipore DAWP 02500, Millipore Corp., Bedford, Mass.) are placed into filtration chambers (Swinnex 25, SX 0002500, Millipore Corp.) mounted on 10-ml syringes. Filtration is performed by suction under vacuum. Before a 1-ml sample of synaptosomes is passed through it, the filter is saturated with 1 ml of 10 mM unlabeled precursor in the physiological medium. Then the filter is washed with 10 ml of physiological medium kept in ice. The filter is dried on What-man 42 paper and then extracted immediately in ice-cold solvent.

Extraction Procedure

Extraction of acetyl-labeled ACh is achieved by dissolving the filter in 3 ml of allyl cyanide containing Na^+-tetraphenylborate (10 mg/ml). Total radioactivity is measured in an aliquot of 1 ml. The remaining 2 ml are washed in 8 ml of Na-phosphate buffer (10 mM, pH 7.4). After centrifugation, the organic phase is collected and counted. The specificity of the procedure when applied to the electric organ has been tested (23).

Extraction of choline-labeled ACh is achieved by dissolving the filter in 2 ml of ethylbutylketone containing Na^+-tetraphenylborate (10 mg/ml). Total radioactivity is measured in an aliquot of 0.5 ml. An internal standard (ACh labeled on the choline moiety) is added to the remaining 1.5 ml. After washing in 8 ml of Na-phosphate buffer (10 mM, pH 7.4) and centrifugation, the organic phase is shaken with 0.5 ml of 1 N HCl and discarded. The aqueous phase is washed with 5 ml of ethylbutylketone without tetraphenylborate, and several times with ether (to remove the white precipitate). After rapid evaporation, the sample is dissolved in 20 μl of methanol containing a mixture of ACh and choline (5 mM) and chromatographed on thin-layer chromatography (TLC) cellulose plates (DC-Fertigplatten Cellulose F, Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.) with the following solvent (butanol, ethanol, acetic acid, water: 100, 20, 17, 33 vol/vol). Spots are stained, scraped, and counted as described earlier (see references 17 and 28).

Biochemical Estimations

Choline acetyltransferase (EC 2.3.1.6) is measured as described by Fonnum (13). Acetylcholinesterase (EC 3.1.1.7) is measured by the method of Ellman et al. (10). Lactate dehydrogenase (EC 1.1.1.27) is determined as described by Johnson and Whittaker (24) and proteins by the method of Lowry et al. (26). ACh is determined by the eserinized frog rectus technique as summarized by McIntosh and Perry (27). ATP is measured by the luciferine-luciferase method (34) using a photomultiplier unit built in the laboratory (Meunier & Lesbats, unpublished method).

Morphological Observations

The tissue is fixed for 90 min in 5% (vol/vol) glutaraldehyde in 0.4 M cacodylate buffer (pH 7.4) while synaptosome pellets (see figure legends) are fixed for 30 min in 3% (vol/vol) glutaraldehyde in 0.5 M cacodylate buffer (pH 7.4). They are postfixed in 2% (wt/vol) OsO_4, in 0.5 M cacodylate buffer (pH 7.4), dehydrated, and embedded in Araldite. Sections are cut on an LKB microtome (LKB Produkter, Bromma, Sweden) and stained with uranyl acetate and lead citrate.

RESULTS

Description of the Purified Synaptosomes

As much as 4% of the electric organ consists of nerve endings: they cover almost the whole surface of the ventral faces of the electroplaques. Because of its large surface, the postsynaptic cell must be open when the tissue is comminuted during the initial phase of the isolation procedure. Cleavage through the electroplaque cytoplasm occurs during this first step. Ventral faces covered with intact nerve endings can be seen without postsynaptic cytoplasm. Fig. 1a shows four stacked electroplaques (500 of them would constitute the electrogenic prism) (32). Ventral faces can be identified under the light microscope by the adhering nerve branches (Fig. 2a) and in the electron microscope by the nerve endings (Fig. 1b).

At this stage of the procedure, >50% of the cytoplasmic marker lactate dehydrogenase is no longer retained while up to 85% of choline acetyltransferase remains sedimentable (31). When forced through calibrated grids, the nerve branches of the ventral faces are opened as suggested by the liberation of choline acetyltransferase, presumably from axoplasm. Fig. 2b shows that the nerve terminals are no longer visible on the ventral faces which can still be identified by the nerve branches. Electron microscope observations have confirmed the presence of pinched-off nerve endings in the suspension. At this stage, up to 50% of choline acetyltransferase and 35% of lactate dehydrogenase are still retained. Large membrane fragments are eliminated by filtration through the 50-μm nylon cloth while the nerve endings are recovered in the filtrate. These particles are purified on discontinuous sucrose gradients. Because of the high osmolarity of Torpedo plasma, we were able to increase the density of the fractionation media (up to 0.7 M sucrose) without altering the ionic composition of the physiological solution; osmolarity was kept in a physiological range by reducing urea. After 40 min at 64,000 g (differential centrifugation), a fraction (C) of pure synaptosomes is found at the 0.3-0.5 M sucrose interface. Table I shows the distribution of some characteristic markers. Fraction C contains the cytoplasmic marker lactate dehydrogenase, and peaks of choline acetyltransferase, ACh, and ATP clearly separated from the amount found in fraction E. The pellet E, rich in proteins and acetylcholinesterases, is heterogeneous and contains fragments of postsynaptic membranes with damaged nerve endings, nuclei and erythrocytes.

Fig. 3 shows a representative field of fraction C. The synaptosomes, about 3 μm in diam, contain numerous synaptic vesicles, glycogen granules, and an occasional mitochondrion, like nerve endings examined in situ. In contrast to brain synaptosomes, postsynaptic membranes are never found attached, not even in areas facing the so-called active zone. The homogeneity of fraction C is evident in the large field of this figure. Four independent experiments were carried out and gave similar results, throughout the thickness of the pelleted fraction. This is in accordance with the high specific activity for ACh (130 ± 9 nmol/mg protein; mean of specific activity from 17 experiments) and choline acetyltransferase (450 ± 40 nmol/h per mg protein; mean of specific activity from 14 experiments). It is of interest to notice that the distribution of ATP closely follows that of ACh. We indeed know that synaptic vesicles contain ATP, the ratio ACh/ATP being close to 5.3 (4). This ratio for whole synaptosomes was of the same order (5.60 ± 0.49; 11 experiments).

Stability of Isolated Synaptosomes

To evaluate an eventual alteration of incubated synaptosomes, three biochemical parameters were measured. A morphological control after 3 h of incubation (at 20°C) was compared to the initial fraction. Table II shows that the amount of ACh remained constant for as long as 3 h. Choline acetyltransferase, a marker of the synaptosome cytoplasm, remains occluded as shown by the fact that up to 90% of it can still be sedimented with the synaptosomes after an incubation period of 3 h. An initial decrease of ATP is observed when the fraction is warmed and diluted. Then, ATP declines by only 18% within 3 h (Table II). Fig. 4 shows synaptosomes incubated for 3 h. No significant differences from the control were noticed.

ACh Synthesis by Incubated Synaptosomes

When radioactive choline is added to the incubation medium, the synaptosomes become labeled and a fraction of the total radioactivity is found as ACh. Fig. 5 shows a linear increase of total incorporated radioactivity and of labeled ACh. Two other experiments have confirmed the result in Fig. 5. The incorporation of choline in 30 min is shown as a function of choline concentration in Fig. 6. The maximum velocity for ACh labeling
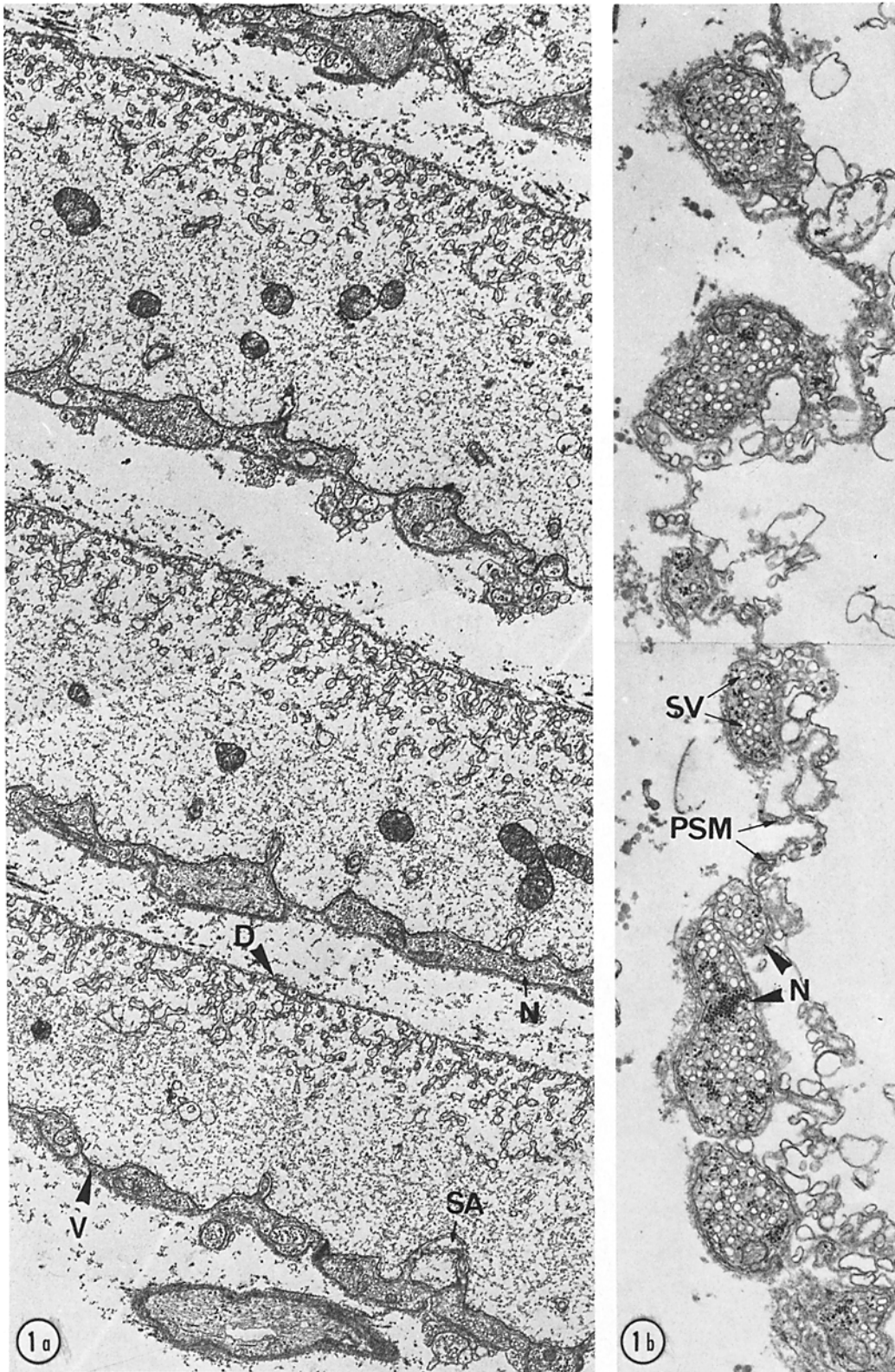
FIGURE 1 (a) Four stacked electroplaques shown at low magnification (× 6,300). V: ventral innervated face; D: dorsal face; N: nerve ending; SA: subneural arch. (b) Electron microscopy of the ventral innervated face found after comminuting the tissue (× 10,800). N: nerve endings; PSM: postsynaptic membrane; SV: synaptic vesicles.
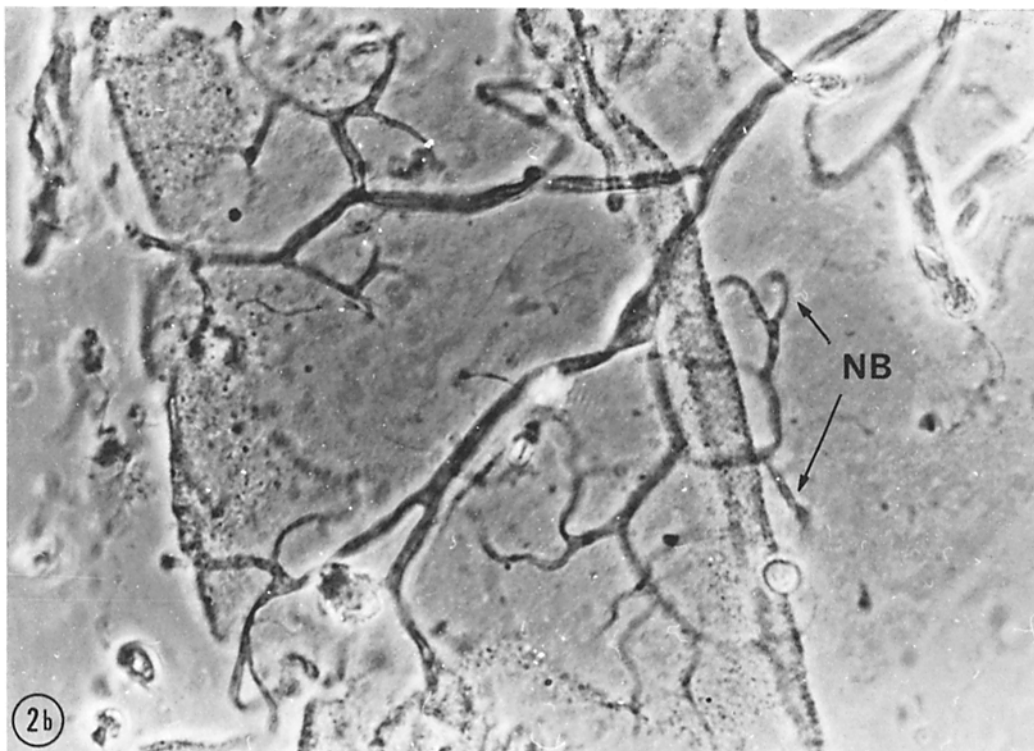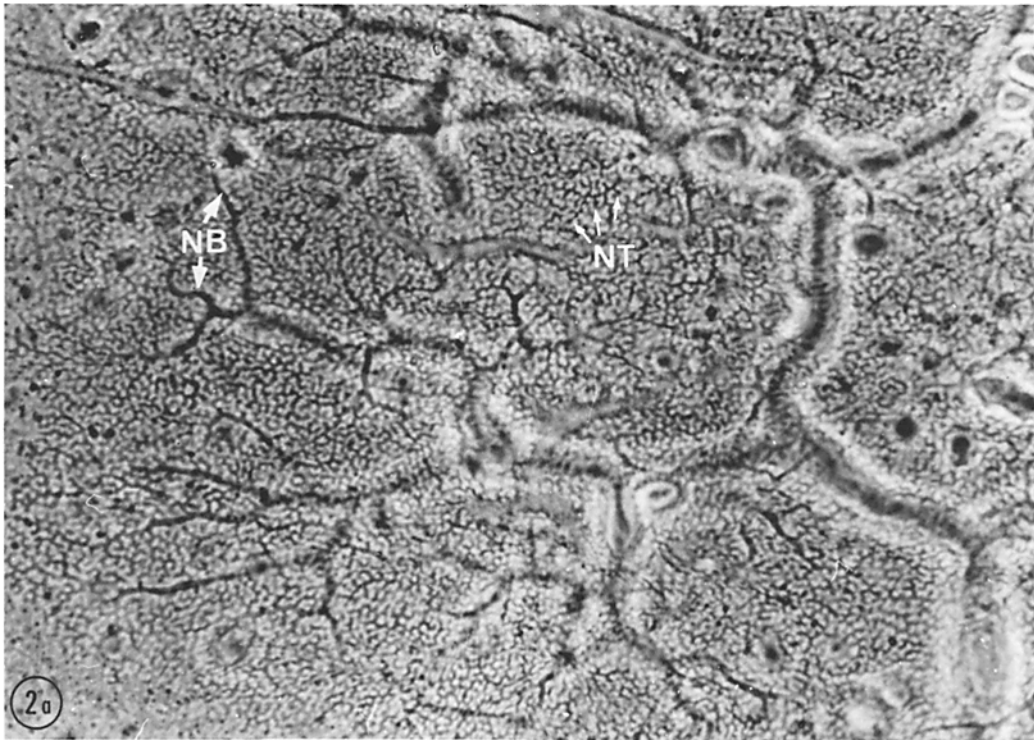
FIGURE 2 Ventral innervated face seen in light microscopy (phase-contrast). × 400. (a) After the comminution step, nerve branches (NB) and nerve terminals (NT) cover the ventral face. (b) After passage through the grids, the nerve terminals are no longer present on the ventral face identified by the nerve branches (NB).

TABLE I

Subcellular Distribution of Biochemical Markers

	No. of experiments	F	S	P	R_r	A	B	C	D	E	R_a
					%						%
Proteins (mg/g)	19	15.6 ± 0.6	11.3 ± 0.4	4.4 ± 0.2	100	0.18 ± 0.02	0.43 ± 0.03	0.35 ± 0.02	0.20 ± 0.01	3.00 ± 0.20	95
ACh (nmol/g)	17	210 ± 18	56 ± 8	131 ± 10	89	1 ± 0	11 ± 2	46 ± 5	14 ± 1	54 ± 6	96
ATP (nmol/g)	11	47.7 ± 7.3	8.6 ± 2.1	31.6 ± 4.4	84	0	1.6 ± 0.3	8.6 ± 1.9	2.4 ± 0.3	10.2 ± 1.5	72
Choline acetyltransferase (nmol/h per g)	14	1,824 ± 170	1,026 ± 108	489 ± 33	83	25 ± 5	69 ± 9	170 ± 21	43 ± 6	128 ± 20	89
Acetylcholinesterases (mmol/h per g)	5	12.5 ± 1.7	1.3 ± 0.2	6.5 ± 1.2	62	0.01 ± 0.001	0.14 ± 0.01	0.26 ± 0.05	0.09 ± 0.01	6.64 ± 1.61	110
Lactate dehydrogenase (Δ E/min per g per ml)	5	33.2 ± 13.8	21.3 ± 10.2	12.0 ± 0.9	100	0.19 ± 0.11	1.46 ± 0.18	2.68 ± 0.32	0.97 ± 0.19	6.23 ± 1.03	96

The results are mean ± SEM of the number of experiments indicated. For each experiment, three to six gradients were pooled. For nomenclature of fractions, see Materials and Methods. R_r is recovery in primary fractions (S + P); R_a is recovery on gradients (A + B + C + D + E). P is the parent fraction of the gradient, and C is the synaptosome fraction showing a peak of ACh and choline acetyltransferase.
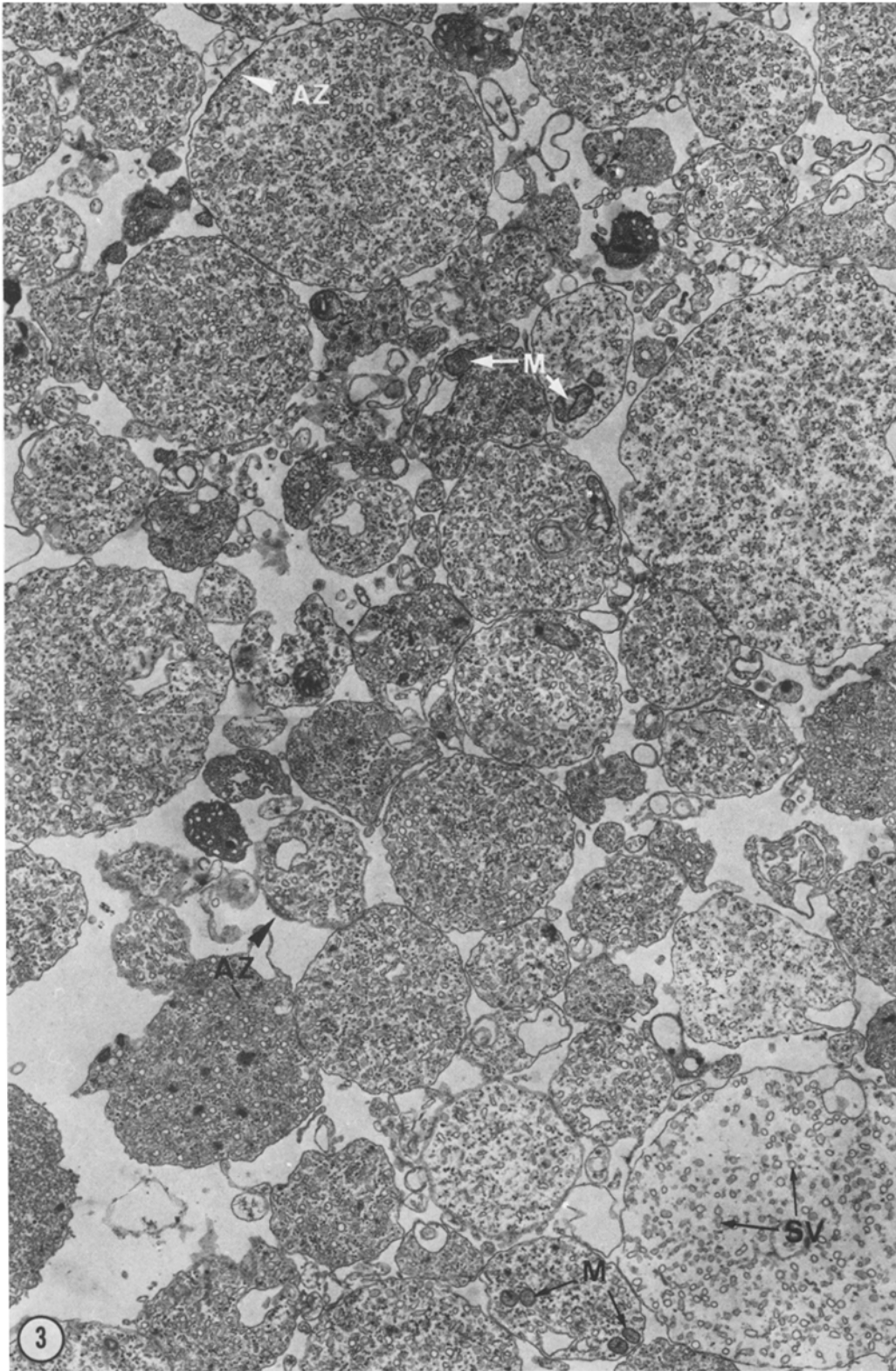
FIGURE 3 Electron micrograph of fraction C. The synaptosomes were collected with a Pasteur pipet from the C band, centrifuged down at 9,500 rpm × 20 min, and the pellet was fixed. This large field is representative of the whole pellet. About 50 synaptosomes can be counted. *AZ*: active zone; *M*: mitochondrion; *SV*: synaptic vesicle. × 12,000.

Effect of Incubation on the ACh, ATP, and Choline Acetyltransferase Content of Synaptosomes

	Fraction C content	After warming and dilution	% of C recovered after:		
			60 min	120 min	180 min
		% of C			
ACh	47.5 ± 5.6 (13) nmol/g	95.5 ± 7.6 (4)	98.4 ± 4.0 (8)	93.4 ± 3.8 (8)	87.6 ± 3.8 (7)
ATP	10.6 ± 2.6 (7) nmol/g	77.7 ± 3.2 (6)	69.0 ± 2.1 (7)	61.0 ± 2.6 (6)	59.3 ± 3.1 (6)
Choline acetyltransferase sedimentable	176 ± 17 (4) nmol/h per g	76.4 ± 11.9 (4)	82.3 ± 16.6 (4)	72.9 ± 17.7 (4)	81.9 ± 20.2 (4)

Results are the mean ± SEM of the indicated number of experiments (in parentheses). The percentage of sedimentable choline acetyltransferase is determined in an aliquot of fraction C centrifuged at 13,000 g for 20 min. The pellet is resuspended in an aliquot of physiological medium.

(dotted line) is reached for 100 μM choline (905 ± 156 pmol/h per mg protein, five experiments). It represents 25 ± 5% (five experiments) of the total incorporated radioactivity. The Lineweaver-Burk plot shows two components for the incorporation of choline (insert, Fig. 6). In spite of our longer incubation period, the kinetic parameters determined are in good agreement with previous metabolic studies (16, 37, 5, 15). Nevertheless, it must be emphasized that <1% of the total ACh in the fraction (0.79 ± 0.14%, five experiments) is renewed in 1 h (at rest).

As found for slices of electric organ (23) or minced electric tissue (31), acetate is a good precursor of ACh in synaptosomes isolated from this tissue. Fig. 7 shows that radioactive acetate is linearly incorporated and very efficiently converted into ACh. (The specificity of the synaptosomal ACh extraction was tested by TLC separation.) Results were confirmed by three other experiments. The incorporation of acetate in 30 min was measured as a function of acetate concentrations (Fig. 8). At a concentration of about 100 μM, the rate of ACh labeling is maximal (1,780 ± 320 pmol/h per mg protein, six experiments). At this acetate concentration (117 μM), radioactive ACh represents 69 ± 9% (six experiments) of the total incorporated radioactivity. For lower acetate concentrations, the percentage is even higher (87 ± 5% at 37 μM, four experiments). The Lineweaver-Burk plot (insert, Fig. 8) shows one component for acetate incorporation into ACh having an apparent K_m value of 10 μM. As for choline,

the percentage of the total ACh of fraction C labeled in 1 h is low (1.26 ± 0.23% at 117 μM acetate, six experiments).

ACh Compartments in the Synaptosomes

When the electric organ of Torpedo is homogenized (Potter-Elvehjem or Waring blender, Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), the pool of ACh not hydrolysed by cholinesterases, i.e., "bound" ACh, has been shown to be associated with synaptic vesicles (20, 29). No sealed synaptosomes are found during such homogenizations and therefore only vesicular ACh is protected from cholinesterases. The amount of ACh hydrolyzed is usually called "free" ACh. To find out whether, as in minced electric tissue (31), synaptosomes from the electric organ still contain the two physiological pools of ACh, we looked for a method for rupturing their membrane without altering the vesicular pool. Freezing the electric organ does not alter vesicles since their isolation may still be performed (36), with a yield comparable to that obtained with other procedures (20, 21). Fig. 9a plots the ACh content of fraction C as a function of the number of successive freezing-and-thawing steps. After the 40% drop seen after the first thawing, the amount of ACh declines regularly with a slower slope. Fig. 9b demonstrates that the amount of ACh remaining after the first thawing declines with a constant specific radioactivity, and is therefore homogeneous. The amount of ACh lost before the first thawing has a higher specific
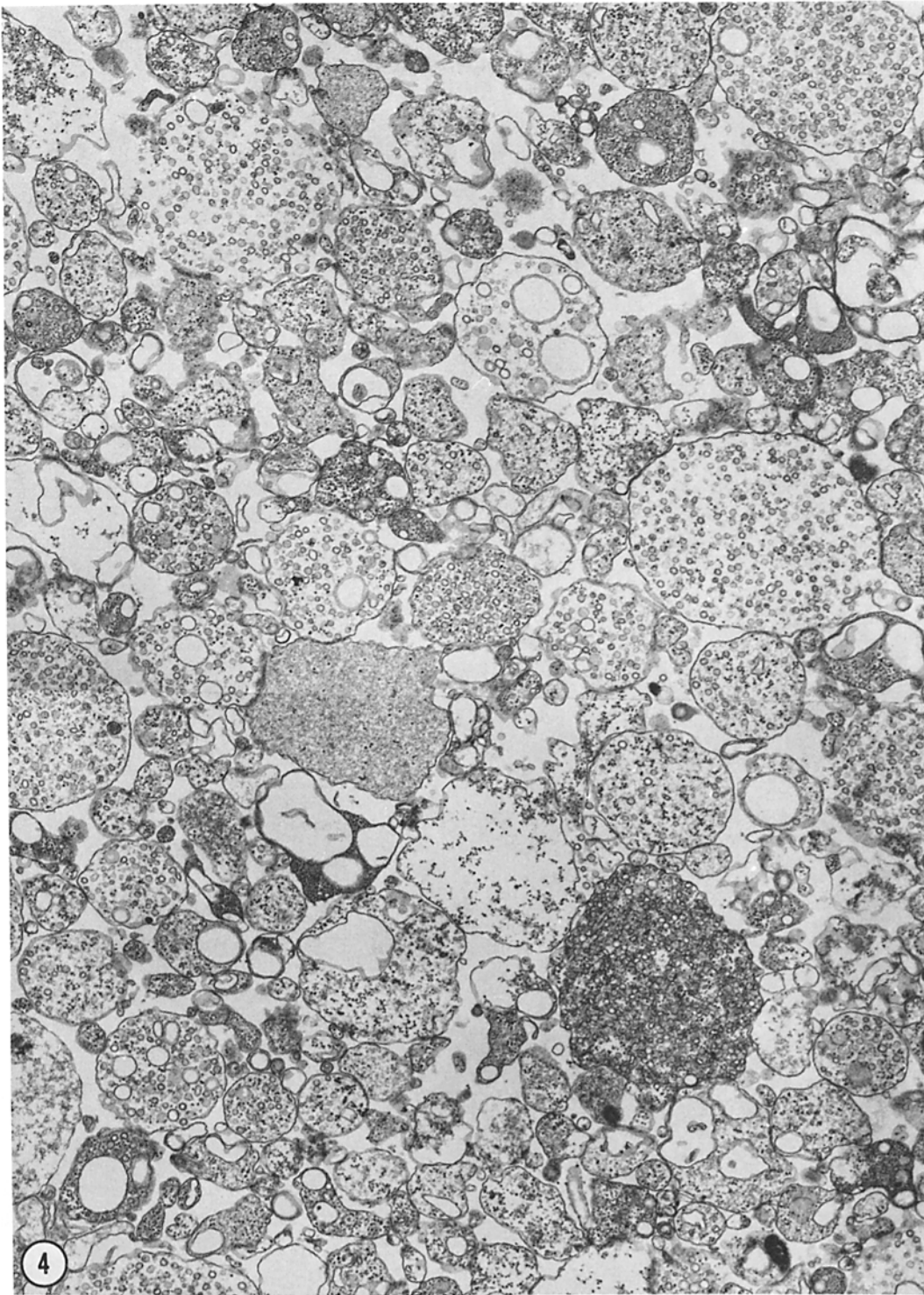
FIGURE 4   Electron micrograph of fraction C incubated for 3 h at 20°C. The whole fraction C (taken with a tube slicer) was centrifuged down at 13,000 rpm × 20 min, and the pellet was fixed. No significant difference was found with a nonincubated sample. × 11,400.
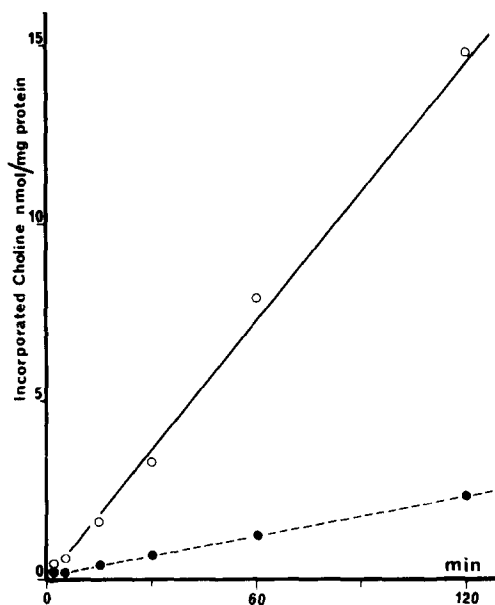
FIGURE 5 Time-course of choline incorporation into synaptosomes: total incorporation (O) and into ACh (●). Radioactivity was converted to nanomoles using the specific radioactivity of precursor.

radioactivity. Three other experiments confirmed these results. The specific radioactivity of the compartment hydrolyzed after the first thawing is 2.46 ± 0.17 times higher (three experiments) than that of the remaining pool. Free ACh measured in total tissue (23) or in minced tissue (31) was found to be 2.8 times more radioactive than bound ACh. Therefore, ACh compartmentation is maintained in purified and incubated synaptosomes.

DISCUSSION

Validity of the Isolation Procedure

As pointed out by Sheridan et al. in 1966 (33), attempts to fractionate the electric organ failed "to concentrate nerve endings or synaptic vesicles in any one fraction ... " Although further efforts succeeded in isolating synaptic vesicles (20, 21, 36), a nerve ending fraction, i.e., synaptosomes, was not available until 1976 (22). Early observations had indeed shown that, when electric tissue was homogenized in sucrose (33, 19) or glycine (19, 21), the presynaptic membrane sequestered a few vesicles. Recently, the glycine and sucrose-glycine media were re-used in an attempt to purify these particles — called T-sacs by Dowdall et al. (3) and Dowdall and Zimmerman (6). Unfortunately, these authors have not yet published a detailed

description of the isolation procedure or of their fraction. Michaelson and Sokolovsky (30), using similar but Ca^{++}-free media, described in a preliminary account a method for preparing synaptosomes. These particles are recovered at an interface where they are likely to be associated with free synaptic vesicles and soluble choline acetyltransferase diffusing from the supernate. This disadvantage should be overcome by adding an intermediate layer between the supernate and their fraction. The leakage curve of ACh (30% in 30 min) renders the synaptosomes isolated by Michaelson and Sokolovsky inconvenient for metabolic studies.

By using physiological media and mild mechanical comminution of the electric tissue, we were able to overcome most of the difficulties pointed out above. The fractionation procedure described is rapid ($2^{1}/_{2}$-3 h are necessary for the whole procedure). The synaptosome fraction is highly purified as shown by its biochemical composition
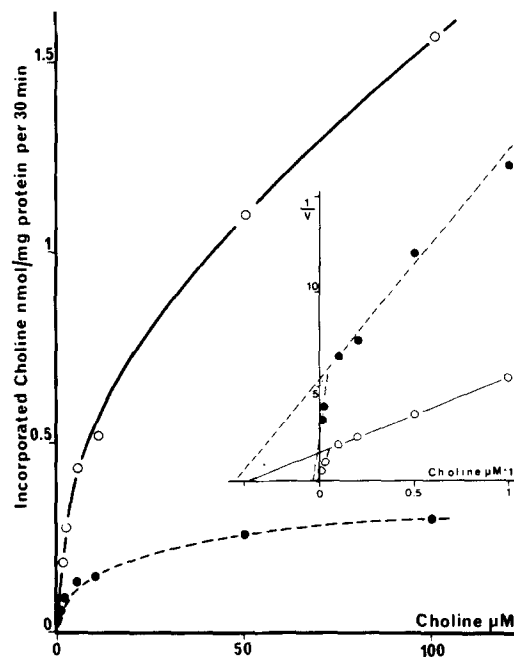

FIGURE 6 Rate of choline incorporation into synaptosomes: total incorporation (O) and into ACh (●) as a function of choline concentration. The Lineweaver-Burk plot (insert) shows two components for choline incorporations (a high affinity component— K_m, 2 μm— and a low affinity component K_m about 50 μm). For choline uptake, two V_{max} are deduced: 0.7 and 5 nmol/mg protein per 30 min. For choline incorporation into ACh, the V_{max} are 0.2 and 0.4 nmol/mg protein per 30 min.
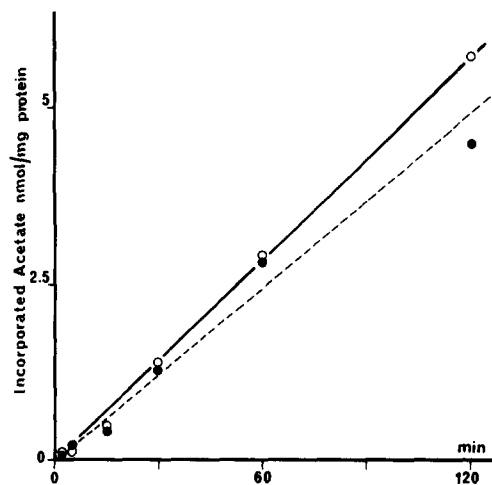
Figure 7 Time-course of acetate incorporation into synaptosomes: total incorporation (O) and into ACh (●). Radioactivity was converted to nanomoles using the specific radioactivity of precursor.
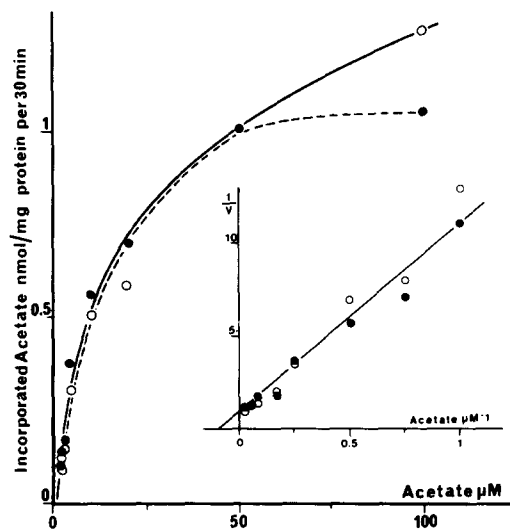

Figure 8 Rate of acetate incorporation into synaptosomes: total incorporation (O) and into ACh (●) as a function of acetate concentration. The Lineweaver-Burk plot (insert) shows one component for acetate incorporation. An apparent K_m of 10 μM can be deduced (V_{max} $\simeq$ 1 nmol/mg protein per 30 min).

and morphological analysis. The nerve endings remain in physiological media during the whole process. After dilution and warming, they retain all their ACh and choline acetyltransferase for at least 3 h so that metabolic studies are possible. The yield of the procedure is not very high, since we can estimate that we isolate 5-10% of the

nerve endings of the tissue. Because the starting material is abundant and richly innervated, we have not tried to increase the yield at the expense of purity.

Synthesis of ACh by Synaptosomes

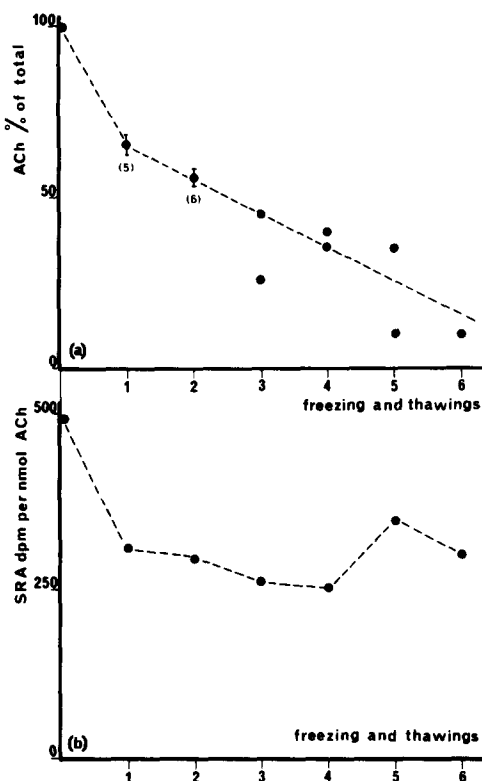When incubated with radioactive precursors of ACh, synaptosomes are able to take up these

Figure 9 (a) Effect of successive freezings and thawings on total ACh content of synaptosomes. After collection, samples were frozen at −80°C. They were thawed at room temperature and immediately frozen again. This procedure was repeated, the number of trials is indicated on the abscissa. A 40% drop occurs after the first trial followed by a slower decay of about 10% after each freezing. The two first points are the mean ± SEM. Numbers in parentheses are the numbers of experiments. (b) Effect of successive freezings and thawings on the specific radioactivity (SRA) of ACh. Synaptosomes were labeled with [1-^{14}C]acetate for 1 h. They were frozen and thawed as described above. Total ACh content was measured. [^{14}C]ACh was extracted by the tetraphenylboron procedure, [^{3}H]acetyl ACh being used as an internal standard. The SRA drops after the first trial (50%) and remains constant after the next five trials. This figure shows a typical experiment which has been confirmed twice more.

precursors and incorporate them efficiently into ACh. The rate of synthesis is linear for at least 2 h, and maximum incorporation rates are reached for concentrations of choline or acetate of about 100 μM. More radioactive choline than acetate accumulates in the synaptosomes, but the rate of acetate incorporation into ACh is twice that of choline. This might be explained by a dilution of radioactive choline into an endogenous pool. Of particular interest is the observation that up to 90% of the incorporated acetate is converted to ACh. Since the tetraphenylboron extraction procedure is specific of quaternary ammonium (12), it is easy to separate precursor and product. The high percentage of conversion makes acetate a valuable precursor for studying the synthesis and release of ACh. Kinetic parameters for choline uptake are similar to those determined by other authors (see Results). It is of interest to notice that acetate is also taken up by an apparent high affinity process. As for intact or minced tissue, labeled ACh in synaptosomes represents only a few percent of the total ACh after several hours of incubation under resting conditions.

ACh Compartments

The definitions of free ACh is operational. It represents the difference between total ACh and ACh bound to vesicles that can be isolated and observed in electron microscopy. Free ACh might represent the cytoplasmic pool where ACh synthesis occurs or a pool associated with fragile vesicles which have still to be characterized. Whatever free ACh means, it represents a physiologically active compartment directly involved during stimulation of the electric organ (7-9). It is interesting to find, as for intact tissue or minces (31), that this compartment represents ~40% of the total ACh content of synaptosomes and that its specific radioactivity is about 2.5 times that of bound, i.e., vesicular, ACh. These results were obtained regardless of the disruption procedure used: homogenization (intact tissue) or freeze-thawing (synaptosomes). Both procedures respect the integrity of synaptic vesicles.

CONCLUSION

The present work demonstrates that it is possible to obtain synaptosomes from the electric organ of Torpedo by selecting an adequate disruption procedure. The resulting fraction is pure and exclusively cholinergic and therefore represents a useful tool for further studies. The good physiological preservation of the synaptosomes and their stability in vitro permit the labeling of the transmitter. Acetate appears to be a suitable precursor for further studies on synthesis and release of ACh. Because of its high purity and the absence of postsynaptic elements, this fraction is a good starting material for the isolation of homogeneous presynaptic membranes.

REFERENCES

1. BENNETT, M. R., and E. M. McLACHLAN. 1972. An electrophysiological analysis of the synthesis of acetylcholine in preganglionic nerve terminals. J. Physiol. (Lond.). 221:669.
2. DE ROBERTIS, E., A. P. DE IRALDI, G. RODRIGUEZ, and J. GOMEZ. 1961. On the isolation of nerve endings and synaptic vesicles. J. Biophys. Biochem. Cytol. 9:229.
3. DOWDALL, M. J., F. J. BARRANTES, W. STENDER, and T. M. JOVIN. 1976. Inhibitory action of 1-pyrene butyryl-choline and related compounds on choline uptake by cholinergic nerve endings. J. Neurochem. 26:1253.
4. DOWDALL, M. J., A. F. BOYNE, and V. P. WHITTAKER. 1974. Adenosine triphosphate: a constituent of cholinergic synaptic vesicles. Biochem. J. 140:1.
5. DOWDALL, M. J., and E. J. SIMON. 1973. Comparative studies on synaptosomes: uptake of (N-Me ^{3}H) choline by synaptosomes from squid optic lobes. J. Neurochem. 21:969.
6. DOWDALL, M. J., and H. ZIMMERMAN. 1976. The preparation and properties of "nerve terminals sacs" from the electric organ of Torpedo. Exp. Brain Res. 24:8.
7. DUNANT, Y., J. GAUTRON, M. ISRAEL, B. LESBATS, and R. MANARANCHE. 1972. Les compartiments d'acétylcholine de l'organe électrique de la Torpille et leurs modifications par la stimulation. J. Neurochem. 19:1987.
8. DUNANT, Y., M. ISRAEL, B. LESBATS, and R. MANARANCHE. 1976. Loss of vesicular acetylcholine in the Torpedo electric organ on discharge against high external resistance. J. Neurochem. 27:975.
9. DUNANT, Y., M. ISRAEL, B. LESBATS, and R. MANARANCHE. 1977. Oscillation of acetylcholine during nerve activity in the Torpedo electric organ. Brain Res. 125:123-140.
10. ELLMAN, G. L., K. D. COURTNEY, V. ANDRES, JR., and R. M. FEATHERSTONE. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88.

11. FELDBERG, W., A. FESSARD, and D. NACHMAN-SOHN. 1940. The cholinergic nature of the nervous supply to the electrical organ of the Torpedo (Torpedo marmorata). J. Physiol. (Lond.). **97**:3.

12. FONNUM, F. 1969. Isolation of choline esters from aqueous solutions by extraction with sodium tetra-phenylboron in organ solvents. Biochem. J. **113**:291.

13. FONNUM, F. 1975. A rapid radiochemical method for the determination of choline acetyltransferase. J. Neurochem. **24**:407.

14. FRONTALI, N., and G. TOSCHI. 1958. Subcellular fractions from the electric tissue of Torpedo: morphological aspects and cholinesterase content. Exp. Cell Res. **15**:450.

15. GUYENET, P., P. LEFRESNE, J. ROSSIER, J. C. BEAUJOUAN, and J. GLOWINSKI. 1973. Effect of sodium, hemicholinium 3 and anti-parkinson drugs on (^{14}C)acetylcholine synthesis and ^{3}H choline uptake in rat striatal synaptosomes. Brain Res. **62**:523.

16. HAGA, T., and H. NODA. 1973. Choline uptake systems of rat brain synaptosomes. B. B. A. Biochim. Biophys. Acta. Libr. **291**:564.

17. HEMWORTH, B. A., and D. MORRIS, 1964. A comparison of the N-alkyl group specificity of choline acetyltransferase from different species. J. Neurochem. **11**:793.

18. ISRAEL, M. 1970. Localisation de l'acétylcholine des synapses myoneurales et nerf-électroplaque. Arch. Anat. Micros. Morphol. Exp. **59**:67.

19. ISRAEL, M., and J. GAUTRON. 1969. Cellular and subcellular localization of acetylcholine in electric organs. Symp. Int. Soc. Cell Biol. **8**:137.

20. ISRAEL, M., J. GAUTRON, and B. LESBATS. 1968. Isolement des vésicules synaptiques de l'organe électrique de la Torpille et localisation de l'acétylcholine à leur niveau. C. R. Acad. Sci. (Paris). **266**:273.

21. ISRAEL, M., J. GAUTRON, and B. LESBATS. 1970. Fractionnement de l'organe électrique de la Torpille: localisation subcellulaire de l'acétylcholine. J. Neurochem. **17**:1441.

22. ISRAEL, M., R. MANARANCHE, P. MASTOUR-FRACHON, and N. MOREL. 1976. Isolation of pure cholinergic nerve endings from the electric organ of Torpedo marmorata. Biochem. J. **160**:113.

23. ISRAEL, M., and S. TUČEK. 1974. Utilization of acetate and pyruvate for the synthesis of "total", "bound" and "free" acetylcholine in the electric organ of Torpedo. J. Neurochem. **22**:487.

24. JOHNSON, M. K., and V. P. WHITTAKER. 1963. Lactate dehydrogenase as a cytoplasmic marker in brain. Biochem. J. **88**:404.

25. JONES, D. G. 1975. Synapses and Synaptosomes. Chapman and Hall Ltd., London.

26. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265.

27. MCINTOSH, F. C., and W. L. M. PERRY. 1950. Biological estimation of acetylcholine. Methods Med. Res. **3**:78.

28. MARCHBANKS, R. M., and M. ISRAEL. 1971. Aspects of acetylcholine metabolism in the electric organ of Torpedo marmorata. J. Neurochem. **18**:439.

29. MARCHBANKS, R. M., and M. ISRAEL, 1972. The heterogeneity of bound acetylcholine and synaptic vesicles. Biochem. J. **129**:1049.

30. MICHAELSON, D. M., and M. SOKOLOVSKY. 1976. Neurotransmitter release from viable purely cholinergic Torpedo synaptosomes. Biochem. Biophys. Res. Commun. **73**:25.

31. MOREL, N. 1976. Effect of choline on the rates of synthesis and of release of acetylcholine in the electric organ of Torpedo. J. Neurochem. **27**:779.

32. SHERIDAN, M. N. 1965. The fine structure of the electric organ of Torpedo marmorata. J. Cell Biol. **24**:129.

33. SHERIDAN, M. N., V. P. WHITTAKER, and M. ISRAEL. 1966. The subcellular fractionation of the electric organ of Torpedo. Z. Zellforsch. Mikrosk. Anat. **74**:291.

34. STREHLER, B. L. 1974. Adenosine 5'triphosphate and creatine phosphate: determination with luciferase. In Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. **4**:2112.

35. WHITTAKER, V. P. 1959. The isolation and characterization of acetylcholine-containing particles from brain. Biochem. J. **72**:694.

36. WHITTAKER, V. P., W. B. ESSMAN, and G. H. C. DOWE. 1972. The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpedinidae. Biochem. J. **128**:833.

37. YAMAMURA, H. I., and S. H. SNYDER. 1973. High affinity transport of choline into synaptosomes of rat brain. J. Neurochem. **21**:1355.