THE ASSOCIATION OF ACETYLCHOLINESTERASE AND MEMBRANE IN SUBCELLULAR FRACTIONS OF THE ELECTRIC TISSUE OF *ELECTROPHORUS*

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ABSTRACT

Subcellular fractions of the electric tissue of the main organ of the eel *Electrophorus electricus* were prepared in sucrose media by differential centrifugation and differential discontinuous gradient centrifugation. The distributions of acetylcholinesterase, cytochrome oxidase, DNA, and protein were determined. The appearance of the fractions was determined by phase contrast microscopy and by electron microscopy. A fraction prepared by differectial centrifugation at 30,000 *g* for 20 minutes in 0.89 M sucrose contained 63 per cent of the total acetylcholinesterase activity at 4 times the specific activity of that of the tissue homogenate. A subfraction prepared by centrifugation in a discontinuous density gradient showed a peak of total and relative specific acetylcholinesterase activity of 35 per cent and 1.9, respectively. The average over-all purification was 7 times. The acetylcholinesterase peak was below the cytochrome oxidase peak and above the DNA peak in the density gradient. The presence of acetylcholinesterase in the fractions was correlated with the presence of large fragments of the cell membrane; however, the presence of other tissue components was noted. The acetylcholinesterase associated with membrane was found to be activated by incubation with sodium deoxycholate. The possible use of the peak fraction containing membranes rich in acetylcholinesterase for the investigation of other components of the acetylcholine system and of other properties of the membrane is discussed.

INTRODUCTION

Acetylcholine (ACh) is thought to be involved in the changes in the permeability of the cell membrane that are the bases of electrogenesis (1, 2). Because of the brief duration of these permeability changes, it is assumed that the component(s) with which ACh interacts in initiating the permeability change, referred to as the ACh receptor, and acetylcholinesterase (ACh-esterase), which removes ACh by hydrolysis, are located in close proximity at the cell membrane.

The purpose of the work described in this paper has been to isolate from electrogenic tissue subcellular fractions rich in ACh-esterase by techniques likely to preserve the assumed association between ACh-esterase and the ACh receptor. By these means, we would test the assumption of the surface localization of ACh-esterase and at the same time provide valuable material for the study of other components interacting with ACh.

ACh-esterase has been localized at the surface

of electrogenic cells in a variety of tissues both by biochemical techniques (1) and by histochemical techniques (3, 4). ACh-esterase has been concentrated in subcellular fractions of brain containing nerve ending ghosts (5). Also, in subcellular fractions of the electric tissue of *Torpedo ocellata* the presence of ACh-esterase has been correlated with the presence of membrane (6). This enzyme has been localized by histochemical methods in the innervated membrane of the electroplax of *Electrophorus electricus* (7) and similarly in the electroplax of the torpedine fishes (8).

This paper describes the isolation of a subcellular fraction of the electric tissue of *Electrophorus electricus* characterized by a specific activity of ACh-esterase about seven times that of the tissue homogenate and by the presence of large fragments of membrane.

MATERIALS AND METHODS

Tissue Fractionation

Sucrose media containing 1 mM disodium ethylenediaminetetraacetate (9), adjusted to pH 7.4 with NaOH, were used throughout. All solutions and suspensions were kept as close to 0°C as possible.

Specimens of the eel *Electrophorus electricus* 60 to 100 cm in length were used. A section 7 to 8 cm long was cut from the rostral part of the main organ. The dissection yielded 10 to 12 gm of electric tissue, which was freed of superficial connective tissue, washed in the suspension medium, blotted, and weighed.

The suspension medium was 0.89 M sucrose. The tissue was added to 5 volumes of medium (50 to 60 ml) in a 250-ml flask of a VirTis "45" homogenizer (The VirTis Co., Inc.), minced with a pair of scissors, and then homogenized for 10 seconds at a speed setting of 75 (on a scale from 0 to 100). The resulting brei was added to a homogenizer consisting of a smooth-walled glass tube fitted with a Teflon pestle, clearance 150 to 230 μ, capacity 50 ml (A. H. Thomas Co.). A powerful homogenizer drive was constructed by attaching a belt-driven spindle (A. H. Thomas Co.), adapted with a 1/4-inch Jacobs chuck (1B) to a Bodine motor, model 104, 1/18 H.P. (Talboys Instrument Corp.). The homogenizer pestle rotated at 3200 RPM under full load. The brei was homogenized by 10 up-and-down strokes of the pestle, and then filtered with stirring for 3 to 4 minutes through a 160-mesh stainless steel sieve, openings 96 μ (Newark Wire Cloth Co.). The white fibrous residue was not washed. The filtered brei was then further homogenized by 10 strokes of the homogenizer pestle.

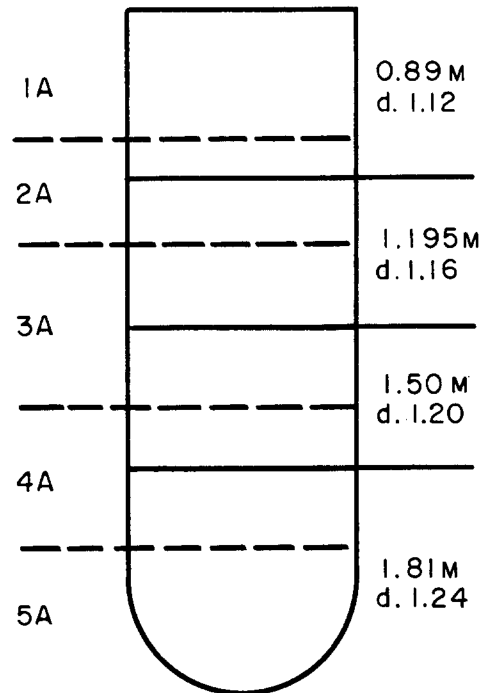

FIGURE 1 The discontinuous density gradient. After differential centrifugation of the total homogenate, the fraction sedimented at 30,000 g for 20 minutes was resuspended in 0.89 M sucrose and carefully layered on top of the gradient shown in the figure, in which the top layer consists of 8 ml of the resuspended sediment; the next layer, 7 ml of 1.195 M sucrose; the next, 8 ml of 1.50 M sucrose; and the next, 8 ml of 1.81 M sucrose. The densities (d.) are given in the figure. After 1- to 2-hour centrifugation in a swinging-bucket rotor, bands formed at the three interfaces and a pellet at the bottom of the tube. The tube was sectioned as indicated by the dashed lines. Each fraction consisted of all the material between the dashed lines.

The material was considered the total homogenate (referred to as H).

The total homogenate (H) was centrifuged in a Lourdes refrigerated centrifuge, in a 9RA-24 rotor, at 30,000 g (bottom of tube) for 20 minutes. The sediment was resuspended by 10 strokes in 50 ml of 0.89 M sucrose, and recentrifugated at 30,000 g for 20 minutes. The sediment was resuspended in 20 to 25 ml of 0.89 M sucrose by 10 to 20 strokes (fraction A). The combined supernatants were called fraction B. A discontinuous density gradient of sucrose was formed in 1 × 3 inch lusteroid tubes by carefully layering, successively, 8 ml of 1.81 M sucrose (1.24 gm/ml at 0°C), 8 ml of 1.50 M sucrose (1.20 gm/ml), 7 ml of 1.195 M sucrose (1.16 gm/ml), and finally 8 ml of fraction A or, in the dummy tubes, 8 ml of 0.89

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M sucrose (1.12 gm/ml) (densities taken from reference 10). The gradients were formed either immediately before, or 4 to 5 hours before, the addition of fraction A and in the latter case were stored at 2°C.

The tubes were centrifuged in a Spinco Model L Ultracentrifuge, set at 25,000 RPM (64,000 g at R_{av}) for 1 hour, brake off, in a precooled swinging-bucket rotor (SW 25.1). The rotor took 55 minutes to decelerate. The temperature in the tubes at the end of a run was 2°C. After centrifugation the tube contained three narrow opalescent bands, one at each of the original interfaces, and a pellet at the bottom of the tube. The tube was sectioned with a Spinco tube slicer (11) into five sections: one slice just above the uppermost band, the second between the uppermost and middle bands, the third between the middle and lowermost bands, the fourth between the lowermost band and the bottom of the tube (Fig. 1). After each slice, the material above the blade was removed with a pipette, the section was washed with a few milliliters of medium, and the tube was moved up for the next slice. The fractions were homogenized by 5 strokes and labeled 1A to 5A (top to bottom). All fractions were frozen in solid CO_2 and stored at −20°C. The time lapse between the dissection of the eel and the freezing of the fractions was 6 to 7 hours.

All fractions were examined as prepared, unfixed and unstained, with positive phase contrast optics, using a Zeiss microscope with an achromatic-aplanatic phase condenser V/Z, Neofluar phase objectives, and Complan eyepieces, routinely with 40× objective and 12.5× oculars.

A preliminary fractionation scheme will be described under Results.

Electron Microscopy

Aliquots of the freshly prepared fractions were diluted and centrifuged in a Spinco Model L Ultracentrifuge in a 40 rotor at 105,000 g (40,000 RPM) for 60 minutes. The pellets were fixed in 1 per cent OsO_4 in 0.9 M sucrose overnight at 4°C, dehydrated in increasing concentrations of ethanol, and embedded in Epon 812. The sections were stained with uranyl acetate and photographed with an RCA 3F electron microscope.

Assays

Each fraction was assayed for ACh-esterase, cytochrome oxidase, protein, and DNA.

ACh-esterase was assayed by determining the unhydrolyzed ACh by the colorimetric method of Hestrin (12). The fractions were diluted so that about 20 per cent of the total ACh was hydrolyzed in 3 minutes at 30°C. The fractions were diluted in H_2O and in 1 per cent sodium deoxycholate (Difco). In the latter case, the deoxycholate precipitated upon addition of acid in the determination of ACh, and the assay mixture was rapidly filtered through a Millipore filter (0.45 μ pore) before determining its absorbancy at 540 mμ. Neither the presence of deoxycholate nor the filtration had any significant effect on the assay for ACh.

Cytochrome oxidase was determined by the spectrophotometric method of Cooperstein and Lazarow (13). Tissue fractions were diluted 1:1 with 5 per cent sodium taurocholate (14) and preincubated for 5 minutes at room temperature. The fractions were added to a solution of reduced cytochrome c in a cuvette, and the absorbancy at 550 mμ was read at 30-second intervals for 2 minutes. A few crystals of potassium ferricyanide were added to the cuvette and the absorbancy of the completely oxidized cytochrome c was determined. The first order rate constant was calculated. The units of activity are decade log unit × (gm wet tissue)$^{-1}$ × min.$^{-1}$.

Protein was determined by the method of Lowry *et al.* (15) for insoluble protein, using human serum albumin (Pentex) as a standard.

DNA was determined by separating the nucleic acids and assaying the DNA pentose by the Dische reaction (16). Salmon sperm DNA (Calbiochem Co., A grade) was used as a standard.

RESULTS

Preliminary Fractionation

Preliminary experiments in which the electric tissue was homogenized in 0.25 M sucrose and separated by differential centrifugation into a heavy fraction (700 g 10 minutes), a medium fraction (5000 g 20 minutes), a light fraction (105,000 g 30 minutes), and a final supernatant fraction demonstrated that about one-half of the total ACh-esterase is associated with the heavy ("nuclear") fraction (Table I; *cf.* reference 6). In these experiments the electric tissue was homogenized as described under Methods. When less rigorous means of homogenization were used, poor dispersion of the tissue resulted. Upon homogenization, the multinucleated cells of which the electric tissue is composed break up into nucleated fragments that are themselves extremely resistant to further dispersion. Only by driving the homogenized pestle at high speed (3200 RPM) was it possible to disperse the tissue to such an extent that no nucleated fragments remained. After homogenization, sedimentation, resuspension, resedimentation, and resuspension, the heavy fraction was seen by phase microscopy to contain large fragments of cell membrane, free nuclei, mitochondrion-sized particles, nerve fibers, and connec-

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TABLE I

Distribution of ACh-esterase and of Protein in Fractions Prepared by Differential Centrifugation in 0.25 M Sucrose

ACh-esterase was assayed in a mixture of 0.1 ml of diluted tissue fraction and 1.0 ml of 3 mM acetylcholine bromide in buffer containing 0.1 M NaCl, 10 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0), incubated for 3 minutes at 30°C. Enzyme activity is given as mmole ACh hydrolyzed per hour per gram wet tissue. Specific activity is expressed in mmole ACh hydrolyzed per hour per milligram protein. Relative specific activity is (percentage of total activity)/(percentage of total protein). Total activity is taken as the sum of the activities of the individual fractions, and similarly for total protein.

Fraction	ACh-esterase				Protein	
	Activity	% total	Relative specific activity	Specific activity	mg protein/gm tissue	% total
	mmole/hr/gm			*mmole/hr/mg*		
Heavy, (700 *g*, 10 min.)	8.9	46	2.2	2.9	3.08	20.3
Medium, (5000 *g*, 20 min.)	2.8	14	1.8	2.2	1.25	8.2
Light, (105,000 *g*, 30 min.)	3.5	18	1.1	1.4	2.44	16.1
Supernatant	4.3	22	0.4	0.5	8.40	55.4
Total	19.5				15.17	
Homogenate	19.0			1.3	15.0	

TABLE II

Distribution of ACh-esterase in Final Fractionation

Fractions were preincubated in 1 per cent sodium deoxycholate for 5 minutes at 25°C. Incubation mixture contained 0.1 ml of fraction in 1 per cent sodium deoxycholate and 1.0 ml of 3 mM acetylcholine bromide in 0.1 M NaCl, 10 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0). Incubation was at 30°C for 3 minutes. Units are as in Table I. The mean and range (4 experiments) is given. The time setting for the density gradient run was 1 hour.

Fraction	Activity	% total	Relative specific activity	Specific activity
	mmole/hr/gm			*mmole/hr/mg*
A	*11.4*(9.4–15.0)	*63.1*(58.0–68.5)	*3.5*(3.1–4.0)	*3.6*(2.9–4.2)
B	*6.6*(5.2–7.4)	*36.9*(31.5–38.5)	*0.45*(0.4–0.5)	*0.5*(0.4–0.6)
Total	*18.0*(14.6–21.9)			
H	*19.4*(16.9–22.0)			*0.9*(0.88–0.96)
1A	*1.4*(0.8–2.0)	*12.2*(8.4–16.0)	*0.5*(0.5–0.5)	*1.9*(1.5–2.2)
2A	*0.9*(0.75–1.0)	*8.0*(7.2–9.6)	*0.4*(0.3–0.4)	*1.3*(1.1–1.6)
3A	*2.9*(2.3–3.9)	*26.5*(24.3–30.7)	*1.5*(1.2–1.8)	*5.4*(3.4–7.3)
4A	*3.9*(3.0–4.3)	*35.2*(32.2–42.1)	*1.9*(1.7–2.4)	*7.0*(5.0–9.3)
5A	*2.0*(1.1–3.0)	*18.0*(10.6–24.9)	*1.1*(0.9–1.3)	*3.7*(2.7–4.5)
Total	*11.1*(9.2–12.6)			

tive tissue fibers. These preliminary experiments were consistent with the idea that ACh-esterase is associated with the cell membrane. The next step was to try to separate these membrane fragments from the other components of the heavy fraction.

Final Fractionation

For convenience in preparing the fractions for density gradient centrifugation, the suspension medium was changed to 0.89 M sucrose. It was soon discovered that the dispersion of the tissue

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was enhanced by the more concentrated sucrose solution. The membrane fragments obtained in 0.89 M sucrose appeared less contaminated by adhering cytoplasm than those obtained in 0.25 M sucrose, and relatively more protein was solubilized (see below). The fraction (A) which was finally taken for subfractionation on a density gradient was obtained by centrifugation of the homogenate at 30,000 *g* for 20 minutes. This fraction was found to combine a high recovery of ACh-esterase with a large increase in specific activity (Table II). This fraction (A) might be expected to contain the combined particulate matter of the heavy and medium fractions of the preliminary fractionation (rat liver mitochondria in 0.88 M sucrose are sedimented by 24,000 *g* for 20 minutes; reference 17). It actually contained a slightly larger fraction of the total ACh-esterase (Table II) and an appreciably smaller fraction of the total protein (Table V) than the heavy and medium fractions combined.

TABLE III

Distribution of ACh-esterase after a 2-Hour Density Gradient Run

Preparation and assay of fractions were as in Table II except that the density gradient run was set at 2 hours.

Fraction	Activity	% total	Relative specific activity	Specific activity
	mmole/hr./gm			*mmole/hr./mg*
A	12.7	65.0	3.8	4.7
B	6.8	35.0	0.4	0.5
Total	19.5			
H	19.2			1.4
1A	0.8	8.0	0.4	1.9
2A	0.6	6.0	0.3	1.2
3A	2.6	24.6	1.4	6.2
4A	3.1	29.0	2.0	9.0
5A	3.5	32.8	1.1	5.1
Total	10.6			

The method used for the subfractionation of fraction A was differential centrifugation on a discontinuous density gradient (18). This is a relatively rapid and simple method. The gradient used was selected after preliminary experiments with two-layer gradients, fraction A in 0.89 M sucrose forming the upper layer. The final procedure is given under Methods (see Fig. 1).

Distribution of ACh-esterase

Fraction A contained 63 per cent of the total ACh-esterase activity (59 per cent of the activity of the total homogenate, H) at a fourfold increase

TABLE IV

Distribution of Cytochrome Oxidase in Final Fractionation

Fractions were diluted 1:1 with 5 per cent sodium taurocholate and preincubated for 5 minutes at 25°C. 0.4 ml of this mixture was added to 3.0 ml of 17 μM reduced cytochrome *c* in 0.03 M potassium phosphate buffer (pH 7.4), and the oxidation of cytochrome *c* was followed spectrophotometrically at 550 mμ. The mean and range (4 experiments) is given.

Fraction	Activity	% total	Relative specific activity
	min⁻¹gm⁻¹		
A	*1.3*(0.4–2.1)	*44*(30–53)	*2.4*(1.8–2.9)
B	*1.7*(0.7–3.1)	*56*(46–70)	*0.7*(0.6–0.8)
Total	*2.9*(1.1–4.4)		
H	*3.5*(1.3–5.7)		
1A	*0.04*(0–0.08)	*3*(0–8)	*0.1*(0–0.4)
2A	*0.22*(0.08–0.34)	*20*(15–23)	*0.9*(0.7–1.1)
3A	*0.61*(0.27–0.96)	*55*(42–61)	*3.0*(2.6–3.6)
4A	*0.18*(0.08–0.28)	*17*(16–18)	*1.0*(0.7–1.3)
5A	*0.06*(0–0.12)	*5*(0–12)	*0.3*(0.1–0.5)
Total	*1.1*(0.4–1.8)		

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TABLE V

Distribution of Protein in Final Fractionation

The units are milligrams of protein per gram of wet tissue. The mean and the range is given for 4 experiments with a density gradient run of 1 hour. The results of 1 experiment with a density gradient run of 2 hours is given.

Fraction	1-hour run		2-hour run	
	mg/gm	% total	mg/gm	% total
A	*3.1*(2.5–3.6)	*18.1*(17.0–19.9)	*2.7*	17.3
B	*14.1*(12.0–17.4)	*81.9*(81.0–83.0)	*13.0*	*82.7*
Total	*17.2*		*15.7*	
H	*19.0*(15.9–23.0)		*13.8*	
1A	*0.71*(0.50–0.93)	*23.7*(16.1–30.0)	*0.43*	*18.3*
2A	*0.69*(0.47–0.88)	*22.6*(17.4–28.0)	*0.50*	*21.0*
3A	*0.55*(0.51–0.68)	*18.4*(16.1–21.6)	*0.42*	*17.6*
4A	*0.55*(0.45–0.62)	*18.6*(14.0–23.0)	*0.34*	*14.3*
5A	*0.51*(0.33–0.75)	*16.6*(11.0–23.8)	*0.68*	*28.8*
Total	*3.01*(2.7–3.2)		*2.37*	

TABLE VI

Distribution of DNA in Final Fractionation

The units are micrograms of DNA-phosphorus per gram of wet tissue. The mean of 2 experiments is given.

Fraction	μg/gm	% total	Relative specific activity
A	8.0	43	2.6
B	10.5	57	0.6
Total	18.5		
H	14.7		
1A	0.3	6	0.3
2A	0.4	8	0.4
3A	0.7	13	0.7
4A	1.8	35	1.8
5A	2.0	38	1.9
Total	5.2		

of specific activity over that of H (Table II). Subfractionation of A by centrifugation in the density gradient for 1 hour resulted in a broad distribution of ACh-esterase over fractions 3A, 4A, and 5A, with a peak of percentage of total activity (35 per cent) and of relative specific activity (1.9) in 4A. The average over-all purification of ACh-esterase in 4A was 7 times. The average over-all recovery was 21 per cent.

Increasing the duration of the density gradient run to 2 hours resulted in a shift of ACh-esterase activity toward the bottom of the tube, nearly 33 per cent of the total activity passing through the 1.20–1.24 density interface into fraction 5A (Table III). The relative specific activities were nearly identical with those of the 1-hour run, owing to a compensating movement of protein toward the bottom of the tube (Table V).

Distribution of Cytochrome Oxidase

The absolute amounts of cytochrome oxidase activity in the fractions varied considerably among the different preparations; nevertheless, the distribution of activity among the fractions had the same shape in all the preparations (Table IV). The distribution showed a sharp peak in fraction 3A, with an average of 55 per cent of the total activity at a relative specific activity of 3.0 (*cf.* reference 18).

Distribution of Protein

After a 1-hour density gradient run the protein was distributed almost equally among the subfractions of A (Table V). After a 2-hour density gradient run there was a shift of protein toward the bottom of the tube, fraction 5A containing 29 per cent of the total.

Distribution of DNA

The high percentage of total DNA found in fraction B (57 per cent) indicates that a large frac-

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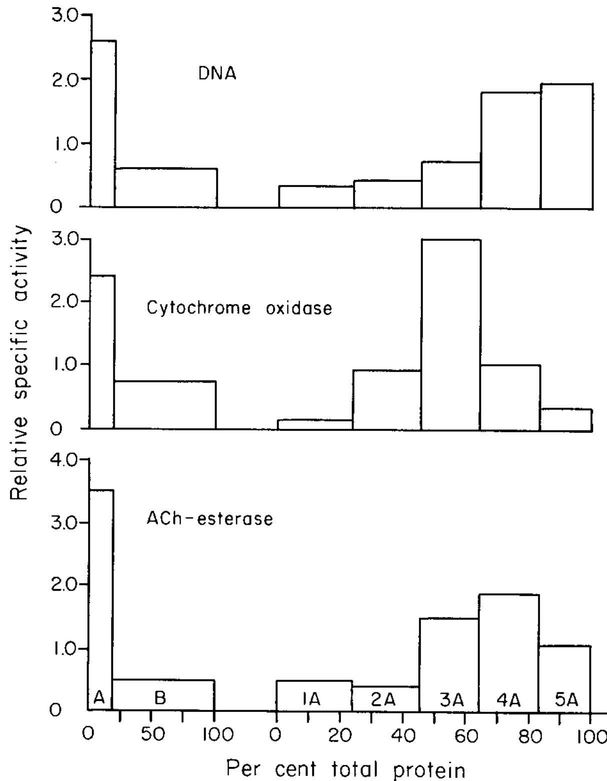

FIGURE 2 Summary of the distributions of ACh-esterase, cytochrome oxidase, and DNA in the final fractionation procedure. The abscissa gives the percentage of total recovered protein. The ordinate gives the relative specific activity, which is the ratio of the percentage of total recovered activity in a fraction to the percentage of total recovered protein in that fraction. It measures the extent of purification of a fraction over the starting material. The *area* under a bar is equal to the percentage of total recovered activity in that fraction.

To the left, the distributions of activities in the two fractions, A and B, are given. To the right, the distributions of activities in the subfractions of A are given. In the first step, ACh-esterase was purified in A about 3.5 times. In the second step, ACh-esterase was purified in subfraction 4A about 2 times. The over-all purification was about 7 times. The peak of relative specific activity as well as of percentage of total activity was in 3A for cytochrome oxidase, in 4A for ACh-esterase, and in 5A for DNA.

tion of the nuclei did not remain intact in the homogenization procedure (Table VI). After fractionation of A, DNA was found mainly in fractions 4A, and 5A, with slightly more in 5A. In summary, the peak of the distribution of cytochrome oxidase was in 3A, that of ACh-esterase in 4A, and that of DNA in 5A (Fig. 2).

The Activation of ACh-esterase by Sodium Deoxycholate

The ACh-esterase of the fractions was consistently activated by sodium deoxycholate (Table VII). Preincubation of the fractions with concentrations of sodium deoxycholate from 0.1 to 2.0 per cent caused nearly equal activation, 1 per cent giving slightly greater activation than the other concentrations. Sodium taurocholate also caused activation, whereas polyoxyethylene sorbital monolaurate (Tween 20), a non-ionic surface-active agent, had little effect. The fractions containing the most ACh-esterase, *i.e.*, fractions A, 3A, 4A, and 5A, were strongly activated, whereas fractions B, 1A, and 2A were activated very little. Sodium deoxycholate has been used to solubilize cholinesterases from brain and heart with some

TABLE VII

Activation of ACh-esterase by Sodium Deoxycholate

Fractions prepared by the final fractionation procedure were diluted in either water or 1 per cent sodium deoxycholate. The diluted fractions were preincubated for 5 minutes at 25°C. 1.0 ml of 3 mM acetylcholine bromide in 0.1 M NaCl, 10 mM $MgCl_2$, and 20 mM potassium phosphate (pH 7.0) was added to 0.1 ml of diluted fraction. Incubation was at 30°C for 3 minutes. The ratio of the activity after dilution in 1 per cent sodium deoxycholate to the activity after dilution in water is given (3 experiments).

Fraction	Ratio of activities	
	Mean	Range
H	1.6*	
A	2.4	1.5–3.9
B	1.1	1.0–1.2
1A	1.1	1.1–1.2
2A	1.1	1.1–1.2
3A	1.6	1.5–1.8
4A	1.9	1.7–2.2
5A	1.9	1.4–2.3

* One experiment.

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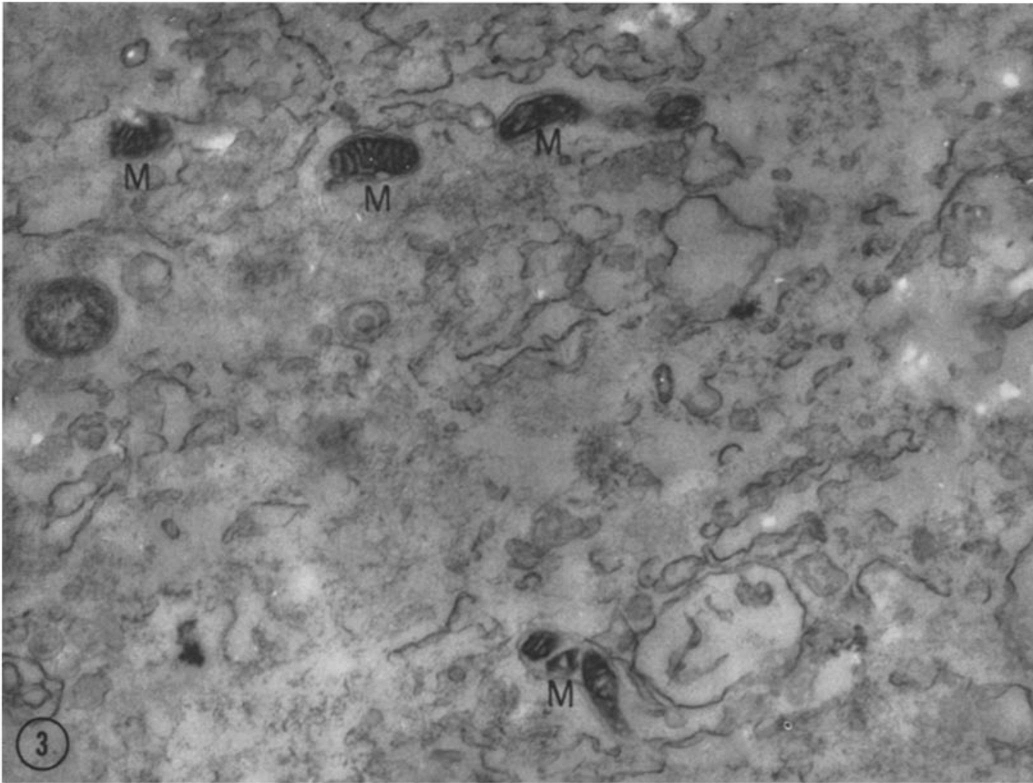

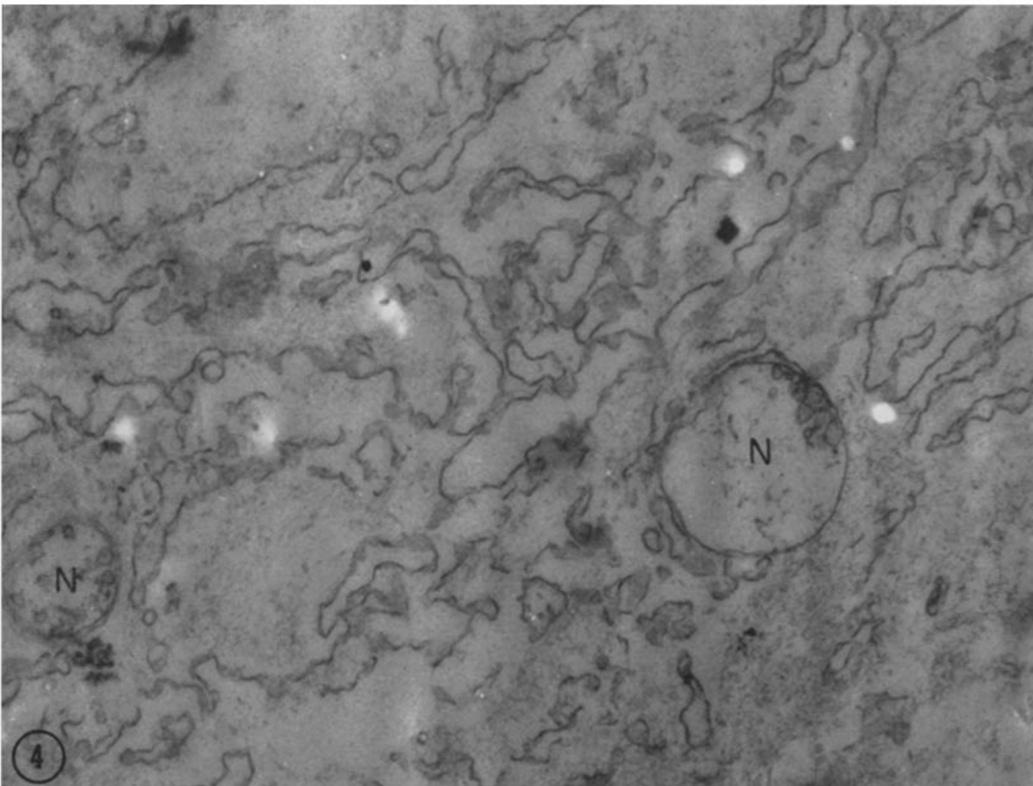

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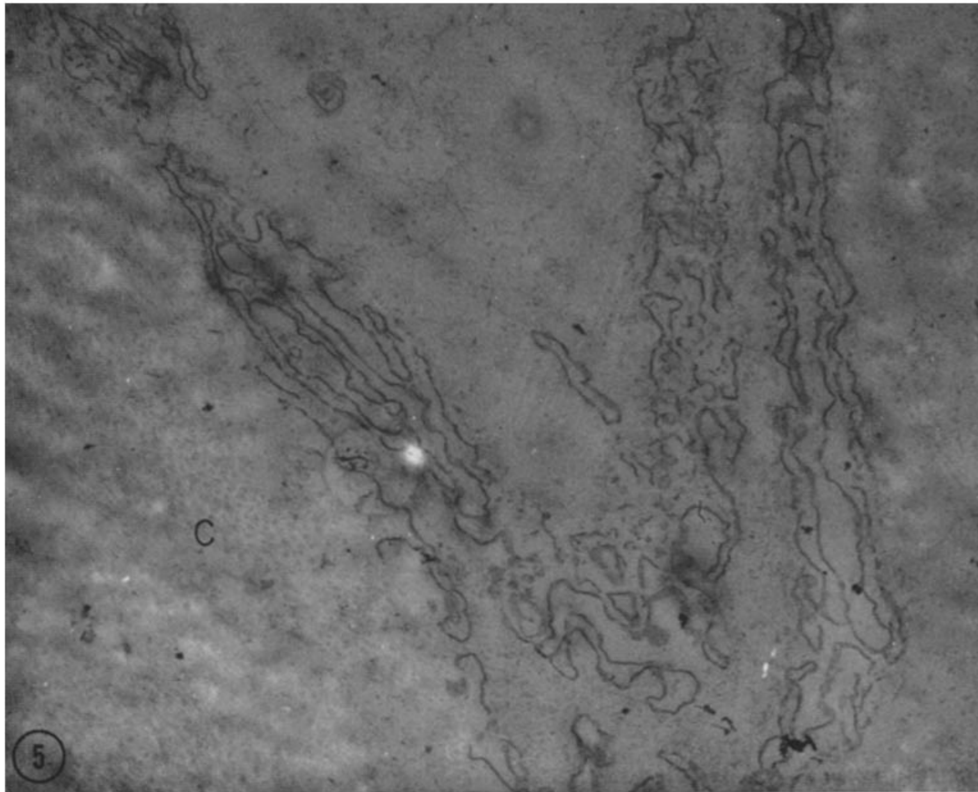

FIGURES 3 to 5 Electron micrographs of fractions 3A, 4A, and 5A. These fractions were prepared by sedimentation in a discontinuous density gradient of sucrose. The fractions were centrifuged at 100,000 g for 60 minutes and the resultant pellets were fixed in 1 per cent OsO_4. Pieces of the fixed pellets were dehydrated and embedded in Epon 812. Sections were stained with uranyl acetate. All figures × 18,500.

All three fractions show large, folded membrane fragments. In addition, mitochondria (*M*) are seen in Fig. 3. Circular profiles are seen in fraction 4A (Fig. 4, *N*) that could be cross-sections through nerve endings (note the enclosed vesicles); but cross-sections through folded cell membranes or through swollen mitochondria might have a similar appearance. Fibrous material probably of connective tissue origin appears in all the fractions. Material seen in Fig. 5 has the appearance of cross-sections of collagen fibers (*C*). The electron micrographs were taken by Dr. Philip W. Brandt.

loss of activity (19), and saponin has been found to activate, and sodium taurocholate and Tween 20 to inhibit, the ACh-esterase of bovine erythrocytes (20). On the other hand, Tween 20 has been used to extract ACh-esterase from human erythrocytes, with little loss in activity (21). It was found, however, that even rigorous homogenization of fraction 4A in 1 per cent sodium deoxycholate did not solubilize the ACh-esterase activity. Seventy per cent of the initial activity sedimented after 30 minutes at 105,000 g; 20 per cent was recovered in the supernatant; and 10% was lost. Activation was not therefore due primarily to solubilization of the enzyme.

Electron Microscopy

The electron micrographs of fractions 3A and 4A show that these fractions contained numerous large membrane fragments (Figs. 3 and 4). Continuous membrane profiles 1 to 10 μ in length were commonly seen. In addition, these fragments appear extensively folded, as is the cell membrane *in situ* (22). Membrane fragments similar in appearance to those in fractions 3A and 4A, but larger and occurring less frequently, were seen in fraction 5A (Fig. 5). There seemed to be a gradient in the size of the membrane fragments, an impression received also from phase microscopic observations of these fractions, the fragments in 4A being

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larger than those in 3A, and those in 5A larger than those in 4A. This finding is of course consistent with the position of these fractions in the density gradient.

In addition to membrane fragments, fraction 3A contained mitochondria, apparently well preserved structurally. Mitochondria were also seen in fraction 4A, although less frequently than in 3A. The electron micrographs of fractions 3A and 4A showed circular profiles about 1 μ in diameter enclosing presumably vesicles 50 to 100 mμ in diameter (shown in Fig. 4 but not in Fig. 3). These may indicate the presence of nerve endings in fractions 3A and 4A, although cross-sections through folded cell membranes or through swollen mitochondria might have a similar appearance. All three fractions contained fibrous material which in some micrographs showed cross-striations with about a 70-mμ periodicity which suggested collagen or collagen-like material. Some intact nuclei were seen in fraction 5A (not shown in Fig. 5).

DISCUSSION

Biochemical, histochemical, and physiological evidence supports the idea that ACh-esterase is associated with the cell membrane of electrogenic cells (see Introduction). The term "cell membrane" as used here represents (following Neville, 23) the most peripheral, visible portion of the cell which has a membrane structure. The cell membrane of the electroplax has been called the electrolemma (24). In the work described here, an attempt has been made to isolate the cell membrane of the electroplax using ACh-esterase as a marker. For comparison with a discrete particle, the distribution of the mitochondria was followed using cytochrome oxidase as a marker. In a similar way, the distribution of the nuclei was to be followed using DNA as a marker. However, the number of intact nuclei progressively declined during the course of fractionation procedure, owing no doubt to the presence of ethylenediaminetetraacetate in the media (*i.e.*, the absence of protective divalent ions, reference 25) and to the high shearing forces used in homogenization. Therefore the distribution of DNA represents that of intact nuclei, nuclear fragments, and probably DNA secondarily adsorbed.

In the first step of the fractionation procedure, ACh-esterase was found to be strongly concentrated (4 times) in a heavy fraction (A) which contained large fragments of membrane. Subfractionation of A by differential gradient centrifugation resulted in a broad distribution of ACh-esterase activity, with a peak of activity in a fraction (4A) containing 35 per cent of the activity of A at about 2 times the specific activity of A. The peak of the ACh-esterase activity fell below that of the cytochrome oxidase activity and above that of the DNA in the density gradient (Fig. 2). The lack of sharpness in the distribution of ACh-esterase is what might be expected if the ACh-esterase were associated with the cell membrane, which fragmented into pieces of a wide range of sizes with more or less adhering cytoplasm and extracellular material. In contrast, the distribution of cytochrome oxidase is quite sharp, reflecting the relatively narrow range of sizes and densities of the mitochondria.

The three subfractions with significant ACh-esterase activity, 3A, 4A, and 5A, contained large fragments of membrane (Figs. 3, 4, and 5), 3A and 4A containing both more enzyme and more membrane than 5A. The cell membrane with its numerous invaginations and foldings comprises the bulk of the membrane of the intact electroplax (22, 24). Internal membrane systems (*e.g.* endoplasmic reticulum) are not prominent. Therefore the only major source for the membrane fragments seen in 3A, 4A, and 5A is the cell membrane.

The ACh-esterase associated with membrane in fractions A, 3A, 4A, and 5A was strongly activated by preincubation with sodium deoxycholate (Table VII). The ACh-esterase in fractions B, 1A, and 2A, which was either soluble or associated with particles of microsomal size, was not appreciably activated by deoxycholate. Preliminary work indicated that the activation was not due to solubilization of the enzyme, which remained sedimentable, but may have been due to the breakdown of the large membrane fragments to smaller pieces. Analogous results are found with mitochondria, where cytochrome oxidase is activated by surface-active agents without being solubilized (14). Use of deoxycholate and similar agents may lead to a method for the further purification of bound ACh-esterase and possibly to the separation of a simple structural unit bearing this enzyme.

In conclusion, ACh-esterase has been found to be associated with fragments of the cell membrane, and most probably is tightly bound to these fragments. On the other hand, some of the ACh-esterase of the membrane-rich fractions might have been associated with fragments of other electric tissue components such as nerve fibers and glial

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cells, which, however, appeared to be minor components. One fraction in particular, 4A, contained a profusion of membrane fragments as well as ACh-esterase at an average specific activity of 7 mmole/hr./mg (7 times that of the tissue homogenate). The most highly purified preparation of *Electrophorus* ACh-esterase so far had a specific activity of 660 mmole/hr./mg (26). Assuming that the enzyme has equivalent activity whether in solution or bound to membrane, it can be concluded that 1% of the protein in 4A is ACh-esterase. This seems remarkable considering the great amount of structural material present in 4A. This fraction in particular should prove useful for further investigations of the components involved in the action of ACh and of other properties closely associated with membrane function.

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