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FRACTIONATION BY VELOCITY SEDIMENTATION OF *TORPEDO* NICOTINIC POST-SYNAPTIC MEMBRANES

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Velocity sedimentation on sucrose gradients containing *Torpedo* physiological saline has been utilized to fractionate *Torpedo* (*Torpedo californica* and *T. nobiliana*) post-synaptic membranes isolated initially on the basis of their density by equilibrium centrifugation. Membranes are separated into two populations: (1) those retained within the gradient (referred to as gradient pool); and (2) membranes sedimenting rapidly through the gradient (referred to as f 22, fraction 22 of the gradient). Comparison of their polypeptide compositions by sodium dodecyl sulfate/polyacrylamide gel electrophoresis indicates that the gradient pool consists of highly purified nicotinic post-synaptic membranes containing the peptides of the acetylcholine receptor and a peptide of M_r 43 000, while f 22 contains the contaminating membranes present in the initial suspension as well as a small fraction of the nicotinic post-synaptic membranes. On the basis of the kinetics of efflux of $^{22}Na^+$ from the membrane fractions, it is concluded that the gradient pool contains most of the sealed vesicles with functional nicotinic receptors. The internal volume (μl/mg protein) of those membranes exceeds that of f 22 by a factor of 4, and greater than 85% of that internal volume is equilibrated by the nicotinic agonist carbamylcholine, while for f 22 only 40% is equilibrated. Thin-section electron microscopy has been used to estimate the distribution of vesicle sizes. The observed distribution for the gradient pool indicates that these vesicles are a size homogeneous population of diameter 0.3 μm, while f 22 contains a number of smaller and larger vesicles. *Torpedo* post-synaptic membranes have been treated with alkali to remove the non-receptor peptide of M_r 43 000. After alkaline extraction, velocity sedimentation permits the isolation of a population of size-homogeneous and well-sealed vesicles containing only the peptides of the nicotinic receptor. It is concluded that upon homogenization, the innervated surface of the *Torpedo* electroplax tends to form vesicles of uniform size (0.3 μm) which can be readily isolated by velocity sedimentation and that the peptide of M_r 43 000 is not required for the maintenance of bilayer structure.

Introduction

Biochemical analysis of the structure and function of the nicotinic acetylcholine receptor is facilitated by the fact that it is possible to isolate from *Torpedo* electric tissue post-synaptic membranes highly enriched in nicotinic receptors (for reviews, see Refs. 1 and 2). As a result of their unusually high protein to lipid ratio (about 2 : 1), those membranes can be isolated from homogenates of electric tissue by equilibrium centrifugation on sucrose density gradients. The most highly purified preparations bind 3–4 μmol α-bungarotoxin/g protein, and the receptor constitutes about 50% of the protein in these membranes. Analysis of their peptide composition by polyacrylamide gel electrophoresis in the presence of SDS reveals the presence of the peptides of the receptor itself (M_r 41 000(α), 50 000(β), 60 000(γ), 65 000(δ)) and a non-receptor peptide of M_r 43 000

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(43 K protein [3,4] or ν protein [5]), which is a peripheral protein that can be removed without significant effect on the nicotinic permeability response [6,7].

Torpedo post-synaptic membranes exist primarily as unilamellar vesicles with diameters ranging from less than 0.1 to 1 μm [8–10]. Some of those vesicles are sealed to $^{22}Na^+$, and the nicotinic permeability response has been measured in terms of the agonist-induced flux of $^{22}Na^+$ [6,11–15]. In the absence of agonist, the observed $^{22}Na^+$-flux kinetics are characterized by multiple rate constants reflecting a heterogeneity of vesicle sealing or of size. About 50% of the internal $^{22}Na^+$ is contained within well-sealed vesicles for which the efflux half time is about 180 min [15]. Rapid-mixing and quenched-flow techniques have been used to measure at millisecond times the kinetics of agonist-induced $^{22}Na^+$ efflux from these well-sealed vesicles [15]. While these studies are possible with membrane suspensions containing vesicles of various sizes and of different degrees of sealing, it would be desirable to further fractionate the *Torpedo* post-synaptic membranes on the basis of permeability to Na^+ or alternatively on the basis of size. Hartig and Raftery [16] reported that it was possible to isolate a preparation of vesicles sealed to low molecular weight solutes from homogenates of *Torpedo* electric tissue, but no data were presented concerning the sealing to $^{22}Na^+$ or the size of the vesicles.

We examined different techniques to further fractionate *Torpedo* post-synaptic membranes which had been isolated initially on the basis of equilibrium density, and we report here velocity sedimentation conditions that provide a rapid, simple method to isolate highly purified preparations of *Torpedo* membranes that are apparently homogeneous in size and also well sealed to $^{22}Na^+$. A preliminary report of these studies has appeared [17].

Materials and Methods

Isolation of nicotinic post-synaptic membranes. Membranes enriched in acetylcholine receptors were isolated from freshly dissected *Torpedo* electric organs according to the method of Sobel et al. [3] with the following modifications. Minced electric tissue (up to 2 kg) was homogenized at 4°C in an equal volume of water containing 0.1 mM phenylmethylsulfonylfluoride and 0.02% NaN_3. A one gallon Waring blender was used, and the tissue was homogenized for 4×1 min at maximal speed with cooling intervals to assure that the temperature remained below 8°C. The homogenate was centrifuged at $5000 \times g$ for 10 min, and the pellet was homogenized again in a Virtis homogenizer at 95% of maximal speed for two times 90 s with a 60-s rest interval. The supernatants from these homogenizations were combined and centrifuged at $10000 \times g$ for 120 min. The supernatant was then discarded, and the pellet resuspended in 32% (w/w) sucrose and then fractionated by centrifugation on discontinuous and continuous sucrose gradients as described [3]. Fractions from the continuous sucrose gradient were collected and assayed for the binding of α-[^{3}H]bungarotoxin by the method of Neubig and Cohen [18], and for protein by the method of Lowry et al. [19]. For 11 fish (nine *Torpedo californica*, two *T. nobiliana*) the specific activity (μmol α-toxin binding per gram protein) of the most enriched (peak) fraction (38% w/w sucrose) was 2.1 ± 0.5, and membranes were combined from gradient fractions containing 36%–40% sucrose. The combined fractions were characterized by specific activities (1.5 ± 0.5), about 2/3 that of the corresponding peak fractions. Membrane suspensions in 38% sucrose/0.02% NaN_3 were stored at 4°C.

Removal of peptides from nicotinic receptor-enriched membranes. Non-receptor peptides were extracted from the isolated nicotinic post-synaptic membranes by alkaline treatment at 4°C according to the method of Neubig et al. [6].

Velocity centrifugation fractionation of receptor-enriched membranes. Receptor-enriched membranes in 38% sucrose were diluted 10-fold in *Torpedo* saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0) and centrifuged at $110000 \times g$ for 30 min. The pellets were resuspended in *Torpedo* saline at a concentration of 3 mg protein/ml, and incubated at 4°C for 12 h to allow equilibration of the vesicle internal medium. That suspension was layered (1 ml per tube) on a sucrose gradient containing *Torpedo* saline (0.5 ml of 54% (w/w) sucrose, 10 ml of a linear gradient 20–8% sucrose, and 0.1 ml 2% sucrose) and centrifuged at 4°C at 20000 rev./min for 10 min in a Beck-

man SW 41 rotor. Fractions (0.5 ml) were collected from the top of the gradients.

Measurement of $^{22}Na^+$ efflux. The efflux of $^{22}Na^+$ from the vesicles was measured at 4°C by a filtration assay according to the method of Neubig and Cohen [15]. Membranes from sucrose gradients were diluted 10-fold in Torpedo saline, pelleted, and resuspended at a concentration of 3 mg protein/ml in *Torpedo* saline. These membranes were incubated overnight with $^{22}Na^+$ (40 μCi/ml). To initiate $^{22}Na^+$ efflux, the concentrated suspension was passed over an ion-exchange column to remove external and readily exchangeable $^{22}Na^+$ and further diluted to a final protein concentration of 0.1 mg/ml. $^{22}Na^+$ retention within the vesicles was determined by filtration of 1 ml aliquots on glass fiber (Whatman GF/F) filters. Non-specific retention of $^{22}Na^+$ was determined by incubating membrane suspensions with gramicidin (10 μg per ml) for 5 min before filtration to release $^{22}Na^+$ from sealed vesicles. That background was due to $^{22}Na^+$ retention by the filters themselves, since the same background was obtained when control solutions containing no membranes were filtered. The internal volume of the vesicles was determined from the amount of gramicidin-releasable $^{22}Na^+$ and the $^{22}Na^+$ specific activity in the concentrated membrane suspension before passage over the ion-exchange column, and that internal volume was expressed in terms of μl of $^{22}Na^+$ trapped per mg of protein. To measure the fraction of $^{22}Na^+$ inside vesicles containing functional nicotinic receptors, membrane suspensions were exposed to 0.1 mM carbamylcholine for 20 s before filtration [15].

Electron microscopy. Thin-section electron microscopy was used to determine the sizes of the vesicles. Samples containing 40–100 μg of protein were taken directly from the sucrose gradients and prepared for electron microscopy without additional pelleting. Each sample was diluted with iso-osmotic sucrose to a final volume of 0.2 ml, then mixed with 0.1 ml of fixative (4% glutaraldehyde, 50 mM sodium cacodylate, pH 7.4) made iso-osmotic with sucrose. Samples were incubated for 30 min at room temperature in small plastic capsules with a hemihyperboloid tip (BEEM Products) and then centrifuged to flat pellets in a Beckman SW-41 rotor (20 000 × *g* for 20 min). Pellets were removed from the capsules, dehydrated in a graded series of ethanols, transferred to polypropylene oxide, and embedded in Epon 812.

For thin sectioning, pellets were oriented with the axis of centrifugation parallel to the block face, so that each section sampled through the thickness of the pellet. Silver sections were post-stained with uranylacetate and lead citrate and were examined and photographed in a Siemens Elmiskop 101 operating at 80 kV. Randomly chosen fields were photographed at magnifications of 11 000 to 30 400. Negatives were printed at final magnifications of 32 900 to 89 700, and diameters of vesicles were measured on the prints. Vesicles in which the trilaminar ultrastructure of the membrane was not apparent were not measured, since they were presumed to have been cut in a grazing section which would not provide a valid indicator of the diameter.

Other assays. Acetylcholinesterase and $(Na^+ + K^+)$-ATPase activities were measured by the methods of Ellman et al. [20] and Robinson [21], respectively. Electrophoresis in the presence of SDS was performed by the method of Laemmli [22] with 9% acrylamide (Sigma) in the separating gel. Gels were stained with Coomassie Brilliant Blue R.

Results

Fractionation of receptor-enriched membranes by velocity sedimentation

Receptor-enriched membranes isolated at their equilibrium density were resuspended in *Torpedo* saline and subjected to velocity fractionation. In preliminary experiments, the time and velocity of centrifugation were varied, and we found that a brief centrifugation at intermediate forces resulted in a useful fractionation. After centrifugation for 10 min at 50 000 × *g*, visual inspection of the gradient revealed that the membranes were separated into two populations: those that sedimented rapidly and were recovered as a dense layer at the 54% sucrose cushion (fraction 22); and a population of membranes retained within the gradient between 10 and 16% sucrose (fractions 3–15). In Fig. 1 is shown the distribution of ^{3}H-labelled α-toxin binding sites and of membrane protein in a typical experiment in which the initial membrane suspension contained about 1.8 μmol α-toxin sites/g protein. Membranes isolated at 10% sucrose contained about 2.6 μmol α-toxin sites/g protein, and 80% of the α-toxin sites but only 60%

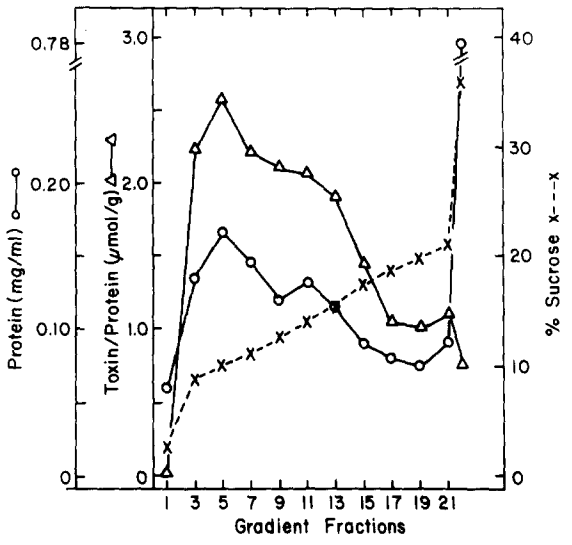

Fig. 1. Distribution of α-toxin binding (△———△) and membrane protein (○———○) in a sucrose gradient after a suspension of *Torpedo* post-synaptic membrane (1.8 μmol α-toxin sites/g protein) was subjected to velocity sedimentation as described in Methods.

of the protein was recovered within the gradient (f 3–15). On the other hand, the material which sedimented rapidly contained 40% of the membrane protein but only 20% of the α-toxin sites. Typical preparations of nicotinic post-synaptic membranes contain less than 2% of the acetylcholinesterase and $(Na^+ + K^+)$-ATPase activities present in homogenates of *Torpedo* electric tissue, and when those membranes were subjected to velocity fractionation, greater than 90% of those residual enzyme activities was recovered in f 22 (data not shown).

The purification of the post-synaptic membrane preparation achieved by the velocity sedimentation procedure was readily revealed when the polypeptide composition of the gradient fractions was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). In the membranes retained within the linear sucrose gradient (Fig. 2b), the peptides of the nicotinic post-synaptic membrane (M_r 41 000, 43 000, 50 000, 60 000, 65 000) were present with few contaminating peptides. The membranes recovered in f 22 (Fig. 2c) were enriched in a peptide of 90 kdaltons as well as the other non-receptor peptides contaminating the preparation isolated by equilibrium centrifugation (Fig. 2a). The peptides of 37 and 38 kdaltons present

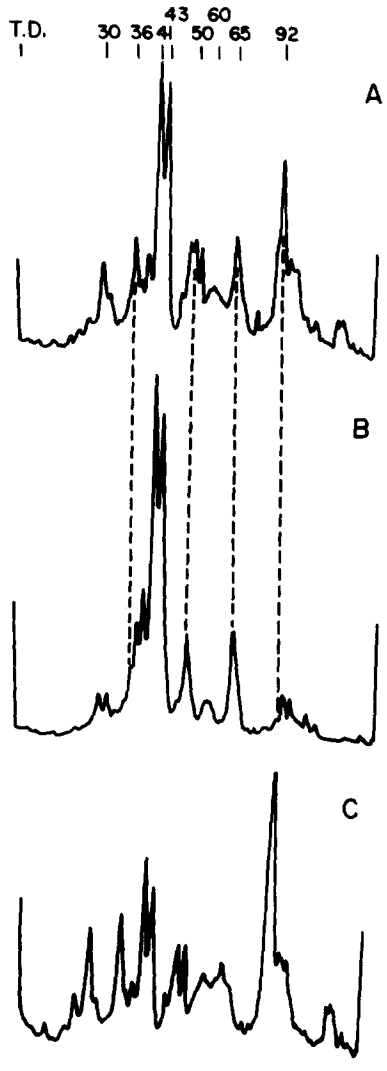

Fig. 2. Polypeptide composition of *Torpedo* post-synaptic membranes purified by velocity sedimentation. A membrane suspension (1.1 μmol of α-toxin sites/g protein) was fractionated as described in Methods. Membranes containing 15% of the α-toxin sites and 50% of the proteins sedimented rapidly and were recovered in f 22, while the rest of the membranes were retained within the gradient. Membranes recovered between 8 and 15% sucrose were combined (gradient pool). Densitometric scans of Coomassie Blue-stained, SDS-polyacrylamide gels are presented for: A, the initial membrane suspension; B, the gradient pool (1.8 μmol of α-toxin sites/g protein); C, f 22 (0.2 μmol of α-toxin sites/g protein). Numbers shown on the top represent molecular weights in thousands, and the dashed lines connect polypeptides characterized by the same relative mobilities, T.D., tracking dye.

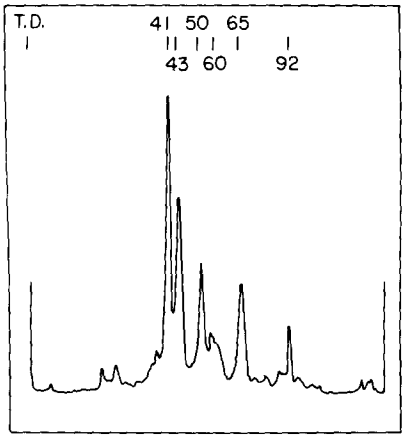

Fig. 3. Polypeptide composition of *Torpedo* post-synaptic membranes purified by velocity sedimentation: effect of EDTA. Minced electric organ was homogenized in an equal volume of water containing 1 mM EDTA, pH 7.0 in addition to 0.1 mM phenylmethylsulfonyl fluoride and 0.02% NaN_3. Membranes were isolated initially by equilibrium centrifugation and then refractionated by velocity sedimentation as described in Methods. Densitometric scan of Coomassie Blue-stained, SDS-polyacrylamide gel is presented for an aliquot of the membranes recovered within the linear sucrose gradient.

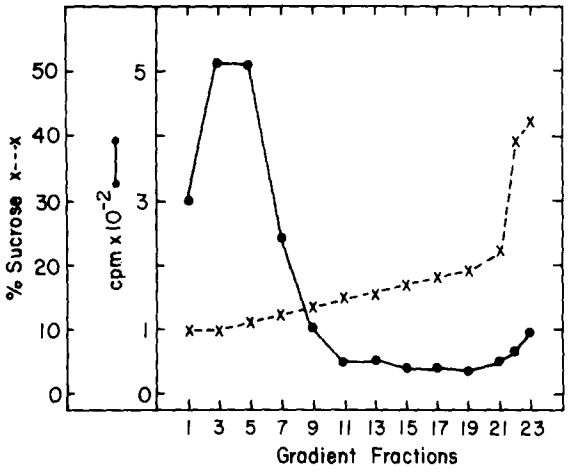

Fig. 4. Recentrifugation of *Torpedo* membranes on a 10–20% (w/w) sucrose gradient. Membranes were labelled with ^{3}H-labelled *Naja nigricollis* α-toxin and subjected to velocity sedimentation as in Fig. 1. Membranes collected in fractions 4 and 5 (8–10% sucrose) were combined and centrifuged in an SW 41 rotor for 10 min at 20 000 rev./min on a sucrose *Torpedo* saline gradient.

in the post-synaptic membrane suspension (Fig. 2b) are also present in nicotinic receptors purified in detergent solution from those membranes (unpublished observations). They are not present in post-synaptic membranes isolated in the presence of EDTA (Fig. 3) and are probably products of Ca^{2+}-stimulated proteolysis of receptor peptides.

The membranes retained within the 8–20% sucrose gradient are not at their equilibrium density, since after centrifugation for 25 min at 150 000 × *g*, all the α-toxin sites were recovered at the 54% sucrose cushion (unpublished data). They do represent, however, a distinct, stable population that can be isolated and recentrifuged. This property was demonstrated in an experiment where membranes were subjected to velocity fractionation as in Fig. 1, and the membranes isolated in 10% sucrose were then applied to a 10–20% sucrose-saline gradient and centrifuged for 10 min at 50 000 × *g*. After recentrifugation, greater than 95% of the α-toxin sites were recovered at 10% sucrose (Fig. 4).

Various factors were examined that might alter the observed distribution of *Torpedo* post-synaptic membranes on the linear, sucrose gradient. First, membranes suspended in 200 mM CsCl (1.024 g/ml) or 200 mM NaCl (1.006 g/ml) were centrifuged for 10 min at 50 000 × *g* on linear 10–20% sucrose gradients containing *Torpedo* saline. The resultant distribution of α-toxin binding sites and membrane protein did not differ significantly, and in both cases most of the α-toxin binding was recovered between 10 and 15% sucrose. In a second experiment, membranes in *Torpedo* saline were exposed to 0.3% saponin before velocity fractionation. That treatment, which makes the vesicles permeable to macromolecules such as lactoperoxidase [23,24], did not affect the observed distribution of post-synaptic membranes in the gradient (data not shown).

Permeability properties of fractionated post-synaptic membranes

The permeability properties of the membranes fractionated by velocity sedimentation were characterized by two parameters: (1) the internal volume defined by the amount of $^{22}Na^+$ enclosed within the vesicles and released by gramicidin; (2) the fraction of the internal $^{22}Na^+$ released upon exposure to the nicotinic agonist, carbamylcholine. Table I contains

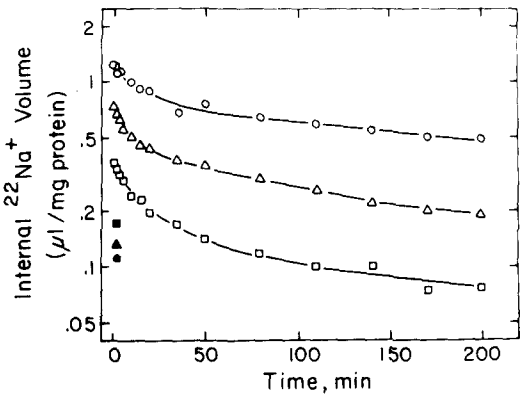

Fig. 5. Kinetics of $^{22}Na^+$ efflux from *Torpedo* vesicles after fractionation by velocity sedimentation. After fractionation as shown in Fig. 1, about 40% of the membrane protein and 80% of the α-toxin were retained within the linear, sucrose gradient (○, ●, gradient pool, containing 1.5 μmol of α-toxin sites/g protein) while 60% of the membrane protein sedimented rapidly and was recovered in f 22 (□, ■, containing approx. 0.3 μmol of α-toxin sites/g protein). The initial membrane suspension (△, ▲) isolated by equilibrium centrifugation contained approx. 1 μmol of α-toxin sites/g protein. For each membrane fraction, a concentrated membrane suspension was incubated overnight with $^{22}Na^+$ (40 μCi/ml) in *Torpedo* saline. $^{22}Na^+$ retention was determined as a function of time after conditions for unidirectional efflux were established. Efflux was measured in the absence (open symbols) or presence of 0.1 mM carbamylcholine (closed symbols).

parameters characterizing the gradient fractions obtained in the experiment summarized in Fig. 1. Sealed vesicles were present within all gradient fractions, with about 60% of the recovered volume within the 8–20% sucrose gradient and 40% in the rapidly sedimenting material (f 22). The membranes retained within the gradient contained larger specific volumes (μl/mg protein) than the initial membrane suspension, while f 22 contained only a small proportion of sealed vesicles. For gradient fractions 3–7, greater than 85% of the internal $^{22}Na^+$ was released by agonist, while for f 22 only 25% of the $^{22}Na^+$ was in vesicles containing functional receptors. Membranes in gradient fractions 9–21 were usually characterized by internal volumes similar to the starting material, but only a small fraction of the $^{22}Na^+$ was released by carbamylcholine. These results indicated that after velocity sedimentation gradient fractions 3–8 contained a population of highly enriched nicotinic post-synaptic membranes that were sealed to $^{22}Na^+$. Those fractions were combined for further characterization, and we refer to them as the gradient pool.

Permeability properties of the gradient pool and of membranes within f 22 were characterized in terms of the kinetics of $^{22}Na^+$ efflux in the absence of cholinergic agonist (Fig. 5). About 50% of the internal volume of the *Torpedo* vesicles isolated by equilibrium centrifugation was associated with well-sealed vesicles for which the half time ($T_{1/2}$) of $^{22}Na^+$ efflux was about 180 min in the absence of agonists, while for the remaining vesicles the $T_{1/2}$ was about 5–10 min. The efflux of $^{22}Na^+$ from vesicles of the gradient pool and f 22 indicated that both populations contained well-sealed vesicles characterized by $T_{1/2}$ of about 180 min. In seven experiments with membranes prepared from four fish, for the gradient pool 73 ± 13% of the internal volume was in well-sealed vesicles, while for f 22 it was only 37 ± 13%. For the vesicles of the gradient pool, f 22, and the initial membrane suspension, these volumes were equivalent 0.68 ± 0.18, 0.16 ± 0.08, and 0.36 ± 0.09 μl/mg protein, respectively.

TABLE I

DISTRIBUTION OF SEALED *TORPEDO* VESICLES AFTER VELOCITY SEDIMENTATION

After velocity sedimentation, membranes from each fraction of the gradient shown in Fig. 1 were resuspended in *Torpedo* saline and incubated with $^{22}Na^+$ (40 μCi/ml) at 4°C. $^{22}Na^+$ retention was determined by filtration on glass fiber filters as described in Methods. When added, the concentration of carbamylcholine was 0.1 mM. The initial membrane suspension was characterized by an α-toxin specific activity of 1.8 μmol/g protein.

Fraction number	3	5	7	9	22	Starting material
Internal $^{22}Na^+$ volume (μl/mg protein)	0.66	0.48	0.32	0.35	0.19	0.26
Total internal volume (μl)	0.13	0.11	0.07	0.05	0.44	1.72
$^{22}Na^+$ released by carbamylcholine (%)	90	85	73	55	25	68

Electron microscopy

We used thin-section electron microscopy to characterize the size distribution of the vesicles after velocity fractionation. Membranes isolated by equilibrium centrifugation from two fish were used. For preparation I the initial suspension was relatively pure as judged by binding of α-toxin (2.5 μmol α-toxin/g protein), while preparation II was characterized by a binding of 0.7 μmol α-toxin/g protein. Visual inspection of random fields of the different samples suggested that vesicles within the gradient pool were relatively homogeneous in size, while f 22 contained many of the small and large vesicles present within the starting suspension. To quantify the vesicle size distribution, the diameters of the vesicles within the different fractions were measured. In Fig. 6 is presented the distribution of vesicle diameters determined for the different fractions. For both preparations the initial membrane suspensions were characterized by a broad distribution of vesicle diameters. The gradient pools contained neither the small nor large vesicles, while the vesicles within f 22 contained both small and large vesicles. Data characterizing the size distributions of the various membrane fractions are presented in Table II.

Velocity fractionation of alkaline-extracted membranes

Torpedo post-synaptic membranes were treated with alkali [6] to remove the non-receptor 43-kdalton peptide. When those alkaline-extracted membranes were subjected to velocity fractionation, the resultant distribution (Fig. 7) of membranes was similar to that observed for native membranes. Analysis of the peptide composition of the different gradient fractions revealed that velocity fractionation provided further purification of the post-synaptic membranes (Fig. 8). The residual quantities of non-receptor peptides (30 and 90 kdaltons) remaining in the

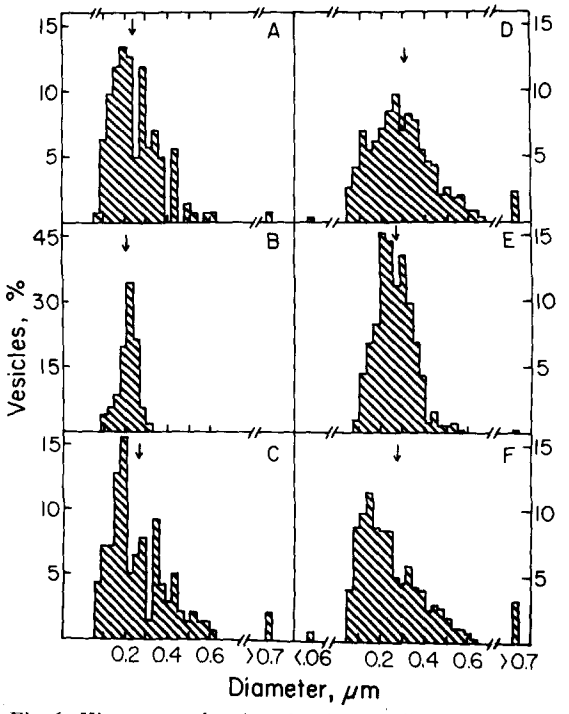

Fig. 6. Histograms showing the size distribution of *Torpedo* membranes after fractionation by velocity sedimentation. The two membrane suspensions studied were characterized by α-toxin specific activities of 2.5 (Prepn. I) and 0.7 (Prepn. II) μmol of α-toxin binding/g protein. Size distributions were determined for the starting material (A and D), the gradient pools (B and E) and f 22 (C and F). For preparation I (A–C) about 100 vesicles were scored in each fraction; for preparation II (D–F) the number was about 400. Arrows indicate the mean diameter of each vesicle population.

TABLE II

SIZE DISTRIBUTION OF *TORPEDO* MEMBRANES FRACTIONATED BY VELOCITY SEDIMENTATION.

Diameters were estimated by thin section-electron microscopy as described in Methods. The α-toxin binding specific activities of the initial membrane suspension for preparations I and II were 2.5 and 0.7 μmoles α-toxin sites/gm protein, respectively. N, number of vesicle profiles measured.

	Mean vesicle diameter ± S.D., μm (*N*)		
	Gradient pool	f22	Initial suspension
Preparation I	0.22 ± 0.05 (108)	0.27 ± 0.15 (141)	0.24 ± 0.12 (143)
Preparation II	0.27 ± 0.09 (440)	0.28 ± 0.20 (378)	0.31 ± 0.15 (392)

TABLE III

$^{22}Na^+$ EFFLUX PARAMETERS FOR ALKALINE-EXTRACTED *TORPEDO* POST-SYNAPTIC MEMBRANES AFTER FRACTIONATION BY VELOCITY SEDIMENTATION

Membranes were treated at pH 11 for 1 h (4°C) according to Ref. 6, and then fractionated by velocity sedimentation. The fraction of the sealed-vesicles containing functional receptor was assessed by determining percent $^{22}Na^+$ released by exposure to 0.1 mM carbamylcholine for 1 min. $^{22}Na^+$ retained in vesicles after 20 min of efflux is expressed as percent internal volume measured at 2 min after passage through the ion-exchange column.

	Gradient fraction						Alkaline extracted membranes	Untreated membranes
	f5	f7	f9	f11	f13	f22		
Internal volume (μl/mg protein)	0.35	0.45	0.61	0.42	0.35	0.15	0.14	0.70
$^{22}Na^+$ released by carbamylcholine (%)	92	90	88	87	78	54	87	66
$^{22}Na^+$ retained after 20 min (%)	72	81	77	61	55	60	61	65

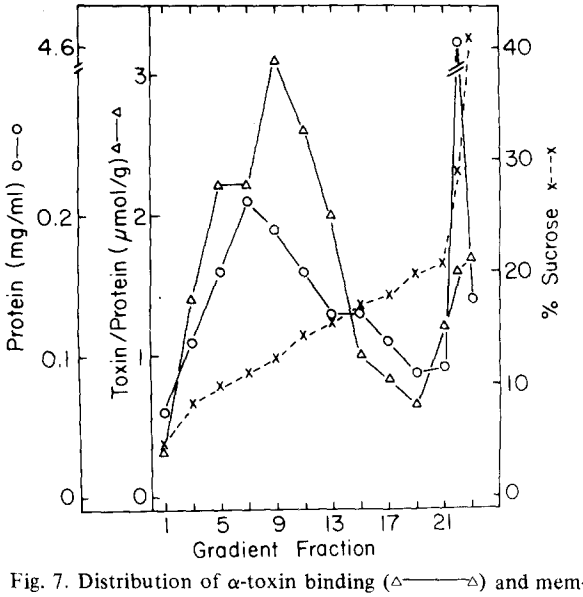

Fig. 7. Distribution of α-toxin binding (Δ———Δ) and membrane protein (o———o) after velocity sedimentation of a suspension of alkaline-extracted *Torpedo* membranes.

alkaline-extracted membranes were recovered in the rapidly sedimenting membranes (f 22) along with about 50% of the receptor (results not shown), while the membranes of the gradient pool contained only peptides of the nicotinic receptor itself *.

* As discussed above, peptides of 37 and 38 kdaltons are probably proteolytic products of receptor peptides (see also Refs. 25 and 26).

The permeability properties of the alkaline-extracted membranes were characterized by the use of $^{22}Na^+$. Although the M_r 43 000 protein was removed, the membranes retained within the linear sucrose gradient contained significant internal volume

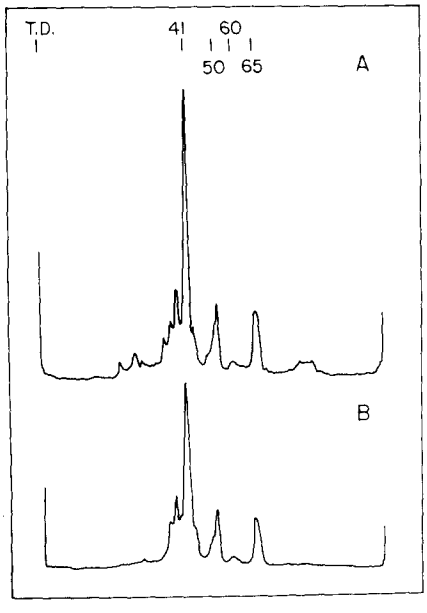

Fig. 8. Polypeptide composition of alkaline-extracted *Torpedo* membranes after purification by velocity sedimentation. Densitometric scans of Coomassie Blue-stained, SDS-polyacrylamide gels are presented for: A, the initial membrane suspension; B, the gradient pool.

(Table III). The kinetics of efflux of $^{22}Na^+$ from the alkaline-extracted membranes of the gradient pool were similar to those of the native membranes: about 80% of the internal volume was associated with well-sealed vesicles ($T_{1/2}$ approx. 3 h). Furthermore, the sizes of vesicles of the gradient pool prepared from the alkaline-extracted membranes appeared to be homogeneous (results not shown).

Discussion

Fractionation by velocity sedimentation of the *Torpedo* vesicles, whether 'native' or depleted of the M_r 43 000 protein by alkaline extraction, provides a simple method to further purify the post-synaptic membranes from suspensions containing contaminating membrane fractions. Membranes purified by velocity sedimentation are virtually devoid of the 90-kdalton peptide as well as other peptides contaminating the initial membrane suspension (Fig. 2). After velocity sedimentation, some of the membranes containing α-toxin binding sites do sediment rapidly, and it is not possible to determine whether those membranes possess the same peptide composition as the fractions retained within the gradient.

The membranes retained within the sucrose gradient after velocity fractionation are not at equilibrium, and this distribution differs distinctly from that reported by Hartig and Raftery [16], where a population of *Torpedo* membranes was identified that remained at a density of 1.03 g/ml after centrifugation for 5 h at 196 000 × g. When *Torpedo* electric tissue is homogenized as indicated in Methods, no post-synaptic membranes have been detected that possess the described [16] properties.

Velocity sedimentation provides a method to isolate from a suspension of the *Torpedo* post-synaptic membranes a population of vesicles that appears homogeneous in size (Fig. 6). Vesicle sizes were estimated on the basis of the observed dimensions of the circles measured by thin-section electron microscopy. A simple calculation indicates that when a sphere of radius R_0 is sectioned randomly, the mean radius ($\bar{R}$) of the sections would be equal to 0.79 R_0 and its standard deviation (S.D.) would be equal to 0.223 R_0. Hence, for a homogeneous population of spheres, the ratio S.D./$\bar{R}$ would equal 0.28, and for a heterogeneous population of spheres, the ratio (S.D./$\bar{R}$) will be greater than 0.28. For the two membrane preparations analyzed, S.D./$\bar{R}$ for the gradient pools was equal to 0.27 (Preparation I, Fig. 6B) and 0.30 (Preparation II, Fig. 6E), values close to that expected for a homogeneous population. On the other hand, for both preparations the ratios for the starting material and f 22 were 0.5 or greater (Table II), indicating the size heterogeneity of these membrane fractions.

Velocity sedimentation does not separate vesicles on the basis of their sealing to solute, and we have been unsuccessful in attempts to use dextran [27] or cesium [28] gradients to separate sealed from unsealed *Torpedo* vesicles. The membranes of the gradient pool do contain a high proportion of vesicles well-sealed to $^{22}Na^+$. Typically, about 85% of the internal $^{22}Na^+$ is associated with vesicles containing functional nicotinic receptors and about 75% is released with a half-time of 180 min (efflux rate constant, $k = 6 \cdot 10^{-5}$ s^{-1}). For a sphere of radius R_0, the permeability (P) is equal to $R_0 \cdot k/3$, and for those well-sealed *Torpedo* vesicles of 0.3 μm diameter, the calculated P_{Na} is equal to $3 \cdot 10^{-10}$ cm/s. That value may be compared with the P_{Na} for skeletal muscle ($8 \cdot 10^{-9}$ cm/s [29]) or for erythrocytes (about 10^{-10} cm/s [30]). The *Torpedo* vesicles with their high density of nicotinic receptors are apparently characterized by a low value of P_{Na}.

In the absence of cholinergic agonist, the $^{22}Na^+$ efflux rate from a vesicle will be proportional to the surface area and inversely proportional to the volume. Hence, a vesicle of radius R_0 will be characterized by a rate constant proportional to R_0^{-1}. The observed distribution of the sizes of vesicles of the gradient pool and of f 22 (Fig. 6) indicates that f 22 has a larger proportion of small vesicles. It is not clear whether the rapid phase of $^{22}Na^+$ efflux (Fig. 5, 20% in the gradient pool and 60% in f 22) is a consequence of small, sealed vesicles or alternately of 'leaky' vesicles of indeterminate size.

It is possible to compare the internal volume observed for the vesicles of the gradient pool with the volumes expected for a homogeneous population of vesicles of 0.3 μm diameter. The total internal volume of vesicles in the gradient pool is equivalent to about 1.0 μl/mg protein (Fig. 4). We present in an Appendix several different calculations of theoretical internal volumes, based upon both biochemical and

electron microscopic data, which indicate expected volumes of about 5 μl/mg protein. The origin of the discrepancy between calculated and observed internal volumes is not certain. It is significant, however, that the observed internal volumes may not represent maximal volumes, since our standard assay conditions do not include variations of osmotic pressure to maximize vesicular internal volume. In fact, preliminary results indicate that larger internal volumes can be obtained by varying the osmotic pressure of the incubation medium.

Although the alkaline treatment necessary to extract the M_r 43 000 protein results in reduced internal $^{22}Na^+$ volume [6], velocity sedimentation permits the isolation of a subpopulation of well-sealed vesicles that do not contain the M_r 43 000 protein (Fig. 8) and are characterized by large internal volumes (Table III). These experiments suggest that the M_r 43 000 protein is not essential for the maintenance of the bilayer integrity.

Appendix

Theoretical calculations of internal volume of Torpedo vesicles

An internal volume (μl/mg protein) of *Torpedo* vesicles can be calculated in several different ways from the available biochemical and ultrastructural data. We present two calculations in which the vesicle diameter is assumed to be 0.3 μm in accordance with the results obtained for the vesicles of the gradient pool (Fig. 6).

A. Derivation utilizing estimate of receptor surface density on Torpedo vesicles. Monomeric nicotinic receptor (M_r 250 000) is present in the *Torpedo* vesicles at a surface density of about 10 000/μm^2 [8,9,31], and the number and mass of receptors in a 0.3 μm vesicle are calculated to be $0.47 \cdot 10^{-20}$ mol and $1.2 \cdot 10^{-12}$ mg, respectively. Comparison of the peptide composition of the *Torpedo* vesicles before and after alkaline extraction as well as the specific α-toxin binding [4,6,7] indicates that the total amount of protein in each vesicle is twice the amount of receptor. Thus, the specific internal volume of a 0.3 μm vesicle of that mass is calculated to be 5.3 μl/mg protein.

B. Derivation utilizing estimates of the protein distribution in the Torpedo vesicles. A second calculation utilizes knowledge of the weight ratio of protein to lipid (about 2 : 1 [32,33]) and results of ultrastructural and diffraction studies [8,9,31,34,35] indicating that the protein has an overall length normal to the membrane of 110 Å with a 55 Å extension outside and about 15 Å inside the bilayer. That protein distribution implies that about 25% of the protein mass is embedded within the lipid bilayer. If the protein density is 1.3 g/ml (based on amino acid composition, see Ref. 9) and 25% of it is in the bilayer, the bilayer volume associated with 1 mg of protein is 0.19 μl from protein and 0.49 μl from lipid (density, 1.03 g/ml). For a vesicle of 0.3 μm diameter bounded by a 5 nm bilayer, the volume of the bilayer is $1.37 \cdot 10^{-21}$ m^3 and the calculated protein per vesicle is $2.0 \cdot 10^{-12}$ mg. The specific internal volume is then calculated to be 6.4 μl/mg protein. Different theoretical internal volumes can be obtained by varying the protein to lipid ratio in the vesicles as well as the percent protein contained within the bilayer (Table AI).

TABLE AI

THEORETICAL INTERNAL VOLUME OF *TORPEDO* VESICLES OF HOMOGENEOUS SIZE

Protein: phospholipid ratio (w/w)	Protein embedded in bilayer (%)	
	25	10
2 : 1	6.4	5.3
4 : 1	4.1	3.0

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