Preparation of Right-Side-Out, Acetylcholine Receptor Enriched Intact Vesicles from *Torpedo californica* Electroplaque Membranes†

Paul R. Hartig and Michael A. Raftery*

ABSTRACT: Intact vesicles enriched in acetylcholine receptor from *Torpedo californica* electroplaque membranes can be separated from collapsed or leaky vesicles and membrane sheets on sucrose density gradients. α-Bungarotoxin binding to intact vesicles reveals that approximately 95% of the acetylcholine receptor containing vesicles are formed outside-out (with the synaptic membrane face exposed on the vesicle exterior). The binding data also indicated that only 5% or less of the sites for α-bungarotoxin binding to synaptic membranes are located on the interior, cytoplasmic face. Intact vesicles are stable to gentle pelleting and resuspension but are easily osmotically shocked. The vesicles are impermeable to sucrose and Ficoll, but glycerol readily transverses the membrane barrier. Intact vesicles provide a sealed, oriented membrane preparation for studies of vectorial acetylcholine receptor mediated processes.

Physical disruption of membranous structures by homogenization of whole organs or cell cultures can be expected to produce several basic membrane structures: membranous sheets; intact vesicles of roughly spherical shape which are sealed to small molecules and may retain the original permeability–selectivity of the cell structure from which they were derived; and damaged vesicles which exhibit altered permeability properties but which may appear morphologically similar to intact vesicles (for a review of plasma membrane intact vesicles, see Wallach & Schmidt-Ullrich, 1977). The separation of intact vesicles from homogenates of *Torpedo* electric organ membranes would be especially useful for studies of membrane ionic permeability modulation by the acetylcholine receptor. Several research groups have utilized purified membrane preparations in their studies of acetylcholine receptor function (Cohen et al., 1972; Duguid & Raftery, 1973; Nickel & Potter, 1973; Flanagan et al., 1975) but, with the exception of recent studies on eel electroplaques (Hess & Andrews, 1977), no attempt has been made to assay or to improve the yield of intact vesicles in these preparations. Furthermore, the membrane orientation of intact vesicles has not been determined. Are they formed right-side-out with their synaptic face in contact with the bulk solution, are they inverted, inside-out vesicles with their cytoplasmic face on the outside and the synaptic face exposed to the vesicle interior, or do they form as a mixture of these species?

α-BuTx binding to electroplaque homogenates does not significantly increase following membrane disruption (Duguid & Raftery, 1973). This finding suggests that few cryptic sites exist and that most vesicles form outside-out. These binding results may, however, be dominated by membrane sheets and damaged vesicles which expose both membrane faces to the toxin. Intact vesicles must be isolated and their permeability to the probe toxin determined before their orientation can be examined. The presence of vesicular structures in electron micrographs does not by itself demonstrate vesicle intactness because a membrane defect which alters the permeability may not be morphologically obvious.

In the present study we report conditions for the isolation of intact vesicles enriched in AcChR[1] membranes from *Torpedo* electric organ. Approximately 95% of these vesicles are obtained in the outside-out orientation with their synaptic face accessible to the bulk solution. The AcChR-rich electroplaque membrane thus joins erythrocytes (Heidrich & Leutner, 1974; Steck, 1974), *Escherichia coli* (Altendorf & Staehelin, 1974; Futai, 1974), lymphocytes (Ferber et al., 1972; Walsh et al., 1976), mouse L cells (Hunt & Brown, 1975), *Dictyostelium discoideum* (Cutler & Rossomando, 1975), and ascites cells (Zachowski & Paraf, 1974) as systems from which membrane vesicles can be obtained in an oriented state.

Materials and Methods

Isolation of Intact Vesicles. Electric organs were removed from freshly sacrificed *Torpedo californica* and either used immediately or rapidly frozen in liquid nitrogen, stored at −80 °C, and thawed before use. All steps in the isolation were performed on ice or at 4 °C except as noted. The white connective tissue layer on the ventral and dorsal organ faces was removed with scissors from 150 to 200 g of organ. The organ was chopped into small (~5 g) pieces with a scissors and rinsed twice with 100 mL of isotonic NPE buffer (0.4 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4). The rinsed, chopped organ was homogenized in a 100-mL stainless steel vessel in aliquots of 45 g of organ with 45 mL of NPE buffer. The homogenization was performed on a Virtis 23 homogenizer for two periods of 15 s at full speed (19000 rpm). All homogenizations were performed under argon. The homogenates were combined and centrifuged for 10 min at 5000 rpm (4080*g*) in a Sorvall GSA rotor. The supernatant was filtered through eight layers of cheesecloth taking care to exclude the oily white tissue masses. The filtrate was centrifuged 45 min at 30000 rpm (70100*g*) in a Beckman type 35 rotor. The supernatant was poured off and the white pellets were resuspended by swirling in 25 mL of NPE buffer. The remaining dense yellow pellet was discarded. The pellet–buffer suspension was homogenized 20 s at medium speed (5000 rpm) on the Virtis 23 using the 25-mL glass vessel. The homogenate

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[1] Abbreviations used: AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; AcChE, acetylcholinesterase; AcTCh, acetylthiocholine; EDTA, ethylenediaminetetraacetate.

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was centrifuged 45 min at 30000 rpm in the Beckman 35 rotor. The pellet was resuspended by swirling in 15 mL of NPE buffer and homogenized 20 s at medium speed on the Virtis 23. The preparation at this stage is termed crude membranes.

Four to twenty percent sucrose gradients in NPE buffer were poured in Beckman SW41 cellulose nitrate tubes over cushions of 0.5 mL of 54% (w/v) sucrose in NPE buffer and 2.25-mL samples of crude membranes were applied atop each gradient. A layer of 2% sucrose is applied between the sample and gradient to prevent aggregation of membranes which can occur at the sharp sample to 4% sucrose interface. The gradients were centrifuged 1 h at 40000 rpm (195700*g*). The intact vesicle bands (see Results) were collected, combined, and diluted with an equal volume of NPE buffer. The intact vesicles were centrifuged 1–2 h at 15000 rpm (27000*g*) in a Sorvall SS-34 rotor. The resulting pellet was suspended in a small volume (typically 0.5 mL) of NPE buffer by Vortex mixing at room temperature for four periods of 5 s at full speed. Intact vesicles were stored at 4 °C and used within several hours.

α-BuTx Sites Assay of Vesicle Sidedness. Intact vesicles were incubated 30 min at room temperature with a two- to tenfold excess (2 μg/mL) of mono-α-[^{125}I]BuTx (Blanchard et al., 1979). The incubation volume was 0.25 mL in NPE buffer (0.4 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4). The vesicle solution (0.025 mL) was transferred to 0.25 mL of wash buffer (50 mM NaCl, 10 mM sodium phosphate, 0.1% Triton X-100, pH 7.4) [total sites sample] or to 0.25 mL of wash buffer containing 3.5 μg/mL of unlabeled α-BuTx [outside sites sample]. These samples were incubated 30 min and 0.1-mL aliquots of each were applied to 2.4-cm diameter Whatman DE-81 filter paper circles. Two minutes after sample application, the disks were immersed in wash buffer (~30 mL per disk) with gentle stirring for 10 min. Two subsequent 10-min washes with fresh buffer were completed and the disks were transferred to plastic tubes for counting on a Beckman Gamma 4000 γ counter. After subtraction of a buffer blank from each sample, the fraction of outside-out vesicles was determined from the ratio [outside sites counts]/[total sites counts].

Assay Methods. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard except in the case of Figure 3 where the assay of Bradford (1976) was used (solutions obtained from Bio-Rad Laboratories) in order to overcome the interfering effects of glycerol. Sucrose and glycerol concentrations were determined in gradient fractions from refractive index measurements compared with standard solutions. α-BuTx sites were determined in gradient fractions by the method of Schmidt & Raftery (1973). Acetylcholinesterase activity was determined by the method of Ellman et al. (1961).

Results

Isolation and Properties of Intact Vesicles. Intact vesicles produced by homogenization of *Torpedo* electric organ in an isotonic sodium chloride solution are expected to form with a high density membrane shell enclosing a low density interior solution. Centrifugation of a membrane homogenate on a sucrose gradient should produce a band of intact vesicles at a low equilibrium density and a high density membrane band containing membrane sheets, collapsed vesicles, and damaged vesicles, leaky to sucrose, which will equilibrate at the membrane density. Previous work has shown that acetylcholine receptor membranes equilibrate near a density of 1.17 g/mL, whereas other contaminating membranes in the homogenate equilibrate at the more common membrane

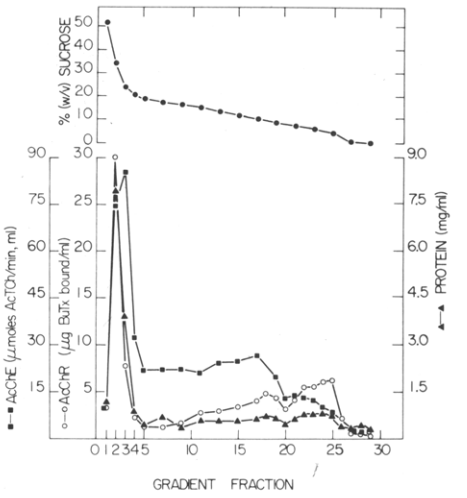

FIGURE 1: A 4–20% (w/v) linear sucrose gradient centrifugation of crude membranes (see Materials and Methods). (■) Acetylcholinesterase activity; (○) α-BuTx binding; (▲) protein; (●) sucrose concentration.

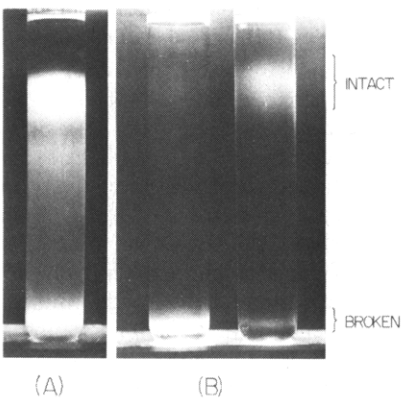

FIGURE 2: (A) A 4–20% (w/v) sucrose gradient centrifugation of crude membranes. (B) (Left) Osmotic shock of intact vesicles. Two sample volumes of distilled water was added to intact vesicles before centrifugation on the 4–20% (w/v) sucrose gradient. (Right) Isotonic dilution of intact vesicles. Two sample volumes of isotonic buffer was added to intact vesicles before centrifugation on the 4–20% (w/v) sucrose gradient.

density of 1.14 g/mL (Duguid & Raftery, 1973; Reed et al., 1975). In a 4–20% sucrose gradient which spans a density range of 1.01–1.08 g/mL, the membrane sheets, collapsed vesicles, and damaged vesicles should appear at the bottom of the gradient after centrifugation.

Figure 1 shows the α-BuTx (AcChR), AcChE, and protein profiles of electroplaque crude membranes (see Materials and Methods) centrifuged in a 4–20% sucrose gradient and Figure 2A shows a photograph of an identical gradient. The low density membrane band at the top of the gradients (fractions 20 through 26 in Figure 1) which contains the largest vesicles and is enriched in AcChR is defined as intact vesicles in these experiments. Continued centrifugation of the gradient for up to 5 h induces no change in the pattern and fails to separate additional bands from the intact vesicles.

The susceptibility of intact vesicles to osmotic shock is illustrated in Figure 2B. The gradient on the right shows intact vesicles which were isotonically diluted with 2 volumes of sample buffer and the gradient on the left shows intact vesicles osmotically shocked by addition of 2 volumes of distilled water. Addition of 0.5, 1.0, or 1.5 volumes of distilled water to intact vesicles will lyse 41, 85, and 99%, respectively, of the AcChR containing vesicles. When the shocked vesicles are centrifuged on sucrose gradients of high density, the broken vesicles move

from their equilibrium intact density of approximately 1.03 g/mL to a membrane density of 1.14 g/mL or greater. These experiments clearly show the vesicular nature of this preparation and rule out the presence of AcChR (α-BuTx binding sites) in any unusual, low density membrane. The membranes found in the middle of the 4–20% sucrose gradient, which are enriched in AcChE activity, are largely sealed vesicles because they, too, are sensitive to osmotic shock.

Although calcium ions are thought to improve the sealing of membrane vesicles and have been used in flux studies with AcChR membranes (Hazelbauer & Changeux, 1974; Michaelson & Raftery, 1974; Popot et al., 1976), we have chosen a divalent cation free isolation medium containing EDTA for this isolation method. Calcium-activated proteases and phospholipases are present in this organ which could lead to membrane structural alterations (Raftery et al., 1975, and unpublished; Chang et al., 1977). In the absence of calcium, we obtain up to 25% of the α-BuTx sites from the crude membranes as intact vesicles. The isolation medium is isotonic with *Torpedo* plasma (Brock & Eccles, 1958; Moreau & Changeux, 1976), and this high salt concentration will also serve to minimize Donnan ion equilibrium effects which could change the vesicle equilibrium density (Steck et al., 1970).

In selected organs, 10–25% of the α-BuTx binding sites of crude membranes are routinely obtained as intact vesicles. These intact vesicles bind 1–1.5 nmol of α-BuTx per mg of protein. Intact vesicle preparations of the same purity and yield may be obtained repeatedly over many months from pieces of an electric organ stored at −80 °C. The intact vesicles are somewhat unstable after isolation. When stored at 4 °C, from 2 to 20% of the vesicles, depending on the organ, will spontaneously lyse during 2 h of storage. Intact vesicles can withstand gentle pelleting (27000*g* for 1 h) and resuspension by Vortex mixing. Phase contrast microscopy shows that gentle Vortex suspension of these soft pellets produces a uniform dispersion of tiny, diffraction limited particles in rapid Brownian motion.

Homogenization of brain tissue produces synaptosomal vesicles in which pieces of postsynaptic membrane containing neurotransmitter receptors are found attached to presynaptic membrane vesicles (Whittaker, 1965). In contrast, the presynaptic nerve terminals of *Torpedo* electroplaques seem unusually fragile and are poorly preserved after organ homogenization (Sheridan et al., 1966). Recently, several laboratories have obtained synaptosomal preparations from *Torpedo* electric organ by the use of gentle homogenization (Israel et al., 1976; Michaelson & Sokolovsky, 1976, 1978) or selection of soft, juvenile tissue (Dowdall & Zimmermann, 1976, 1977). These synaptosomes display a rich array of synaptic vesicles, vacuoles, and granules inside the limiting membrane. Furthermore, these synaptosomes contain no adhering postsynaptic membrane.

Intact vesicles isolated from *Torpedo* by our methods are not synaptosomes. Intact vesicles are rich in postsynaptic AcChR and appear as predominantly monolamellar vesicles devoid of internal structure in thin section electron micrographs (C. Strader, J. P. Revel, & M. Raftery, unpublished). Intact vesicles are formed from postsynaptic membranes rather than attachment of AcChR membranes to vesicular presynaptic structures.

Intact vesicles must be impermeable to sucrose in order to equilibrate at such low densities in sucrose gradients. As expected, intact vesicles are also impermeable to Ficoll, a large sugar polymer, as demonstrated by their ability to band at the same low density in Ficoll gradients. Ficoll is not useful for the isolation of intact vesicles, however, because it induces extensive aggregation of *Torpedo* electroplaque membranes leading to poor resolution of intact vesicles from adhering membrane fragments.

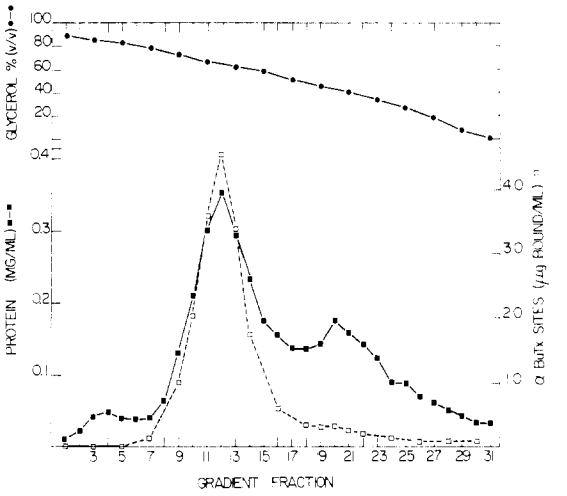

FIGURE 3: An 18–90% (v/v) glycerol gradient centrifugation of intact vesicles. (■) Protein; (□) α-BuTx binding; (●) glycerol concentration.

Figure 3 shows the protein and α-[^{125}I]BuTx binding profiles from intact vesicles centrifuged on a gradient of 18–90% (v/v) glycerol. Biological membranes are permeable to glycerol. As intact vesicles travel down the glycerol gradient, the vesicle interiors equilibrate with the changing glycerol concentration and the vesicles proceed to their membrane density. This is not an artifact arising from glycerol induced lysis of the vesicles. Membranes recovered from the density 1.19 g/mL level (fraction 12) of the glycerol gradient can be slowly dialyzed against isotonic isolation buffer and rerun on 4–20% sucrose gradients. Following dialysis these intact vesicles once again equilibrate at a density of 1.03 g/mL in the sucrose gradient. As the protein and α-BuTx binding profiles in Figure 3 demonstrate, centrifugation of intact vesicles on glycerol gradients and harvesting of the densest, AcChR enriched fractions followed by gentle dialysis provide a method for increasing the purity of AcChR containing intact vesicles.

Sidedness of Intact Vesicles. α-BuTx binds specifically and with high affinity to postsynaptic cholinergic receptors in *Torpedo* electroplaques (Lee, 1972). This toxin is a large water soluble polypeptide which will not penetrate intact membrane barriers. Its toxic action is localized to the synaptic side of postsynaptic membranes since it completely blocks neuromuscular transmission when applied to the bulk solution bathing a neuromuscular junction. Recently, the 40K subunit of *Torpedo californica* AcChR was shown to contain the binding site for this toxin (Hucho et al., 1976; Witzemann & Raftery, 1978). These properties make it an excellent marker for the synaptic face of AcChR membranes.

When added to a solution of intact vesicles, the toxin will bind only to sites on the exposed, external face of the vesicle membrane. The impermeability of intact vesicles to the much smaller sucrose molecule assures that the vesicle interior is inaccessible to the toxin. Furthermore, the toxin will uniquely select AcChR membrane vesicles from among a larger population of other vesicle types. The orientation of AcChR containing vesicles alone will be determined.

We have developed an assay for intact vesicles which measures total α-BuTx sites on both sides of the vesicle membrane and determines which fraction of the total sites is exposed on the exterior face of the vesicle. This assay utilizes nearly identical solutions for both determinations which avoids

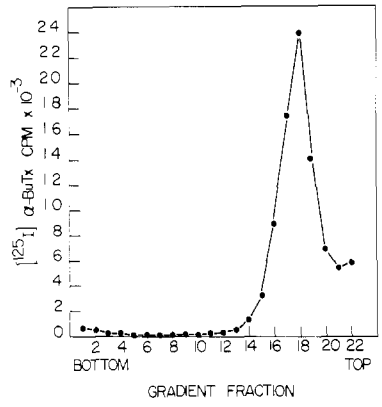

FIGURE 4: Centrifugation of intact vesicles on a 4–20% (w/v) sucrose gradient. Intact vesicles were labeled with α-[^{125}I]BuTx and centrifuged 1 h at 40000 rpm in SW 41 sucrose gradients. α-BuTx sites on the vesicles were in twofold excess over added α-[^{125}I]BuTx.

buffer artifacts during binding to DEAE-disks. [High salt concentrations and high detergent levels can inhibit AcChR binding to the disk and lead to artifactually low assay results (Schmidt & Raftery, 1973, 1974).] The method (see Materials and Methods) involves saturation of exposed sites on intact vesicles with radiolabeled mono-α-[^{125}I]BuTx followed by membrane disruption with detergents to expose sites on the vesicle interiors for binding of either ^{125}I-labeled or unlabeled α-BuTx. The irreversibility of α-BuTx binding to AcChR (Lee, 1972) prevents exchange of labeled outside sites with the newly added toxin. The sample exposed to unlabeled α-BuTx after membrane disruption contains only radiolabeled outside sites, while the sample exposed to α-[^{125}I]BuTx after membrane disruption yields the total (inside plus outside) sites. The ratio of these values yields the percentage of AcChR containing intact vesicles which are outside out (synaptic face exposed to bulk solvent). When intact vesicles are assayed by this method, we consistently find 93–97% of the AcChR vesicles are outside out with a median value near 95%.

Several control experiments were performed to test for artifacts in the assay. Figure 4 shows the α-BuTx binding profile of mono-α-[^{125}I]BuTx-labeled intact vesicles centrifuged on a 4–20% sucrose gradient. Over 98% of the α-BuTx binding was recovered at the top of the gradient in the intact vesicle band. α-BuTx binding to intact vesicles therefore does not induce vesicle lysis. Furthermore, less than 2% of the vesicles were damaged by pelleting, resuspending, and centrifuging the vesicles during the 2-h time course of this experiment.

Approximately 5% of the total sites are revealed as inside sites by addition of detergent. Increased detergent concentrations (0.5% Triton X-100) or extended incubation times do not alter the assay results. This check for saturation of α-BuTx binding is especially important if sucrose is present in the assay medium. We find that the α-BuTx association rate constant slows from 1.4×10^4 M^{-1} s^{-1} in 0.4 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4, to 3.5×10^2 M^{-1} s^{-1} when 2 M sucrose is added to this same buffer. In the presence of such binding inhibitors, it is imperative to calculate the time to completion of binding and adjust the incubations accordingly.

α-BuTx binding sites on the interior face of vesicles can be determined by an inversion of the standard assay. Outside sites are saturated with unlabeled α-BuTx and then the vesicles are disrupted by transfer to detergent solutions containing a great excess of mono-α-[^{125}I]BuTx. In this case it is important to correct for nonspecific α-[^{125}I]BuTx binding when comparing inside and total sites under the same assay conditions. After this correction we obtain good agreement between inside and outside site assays.

Conclusions

Sealed, intact vesicles enriched in acetylcholine receptor can be isolated in good yield and purity from *Torpedo* electric organ by gentle disruption and fractionation on a sucrose density gradient. Assessment of the vesicle orientation requires (1) elimination of leaky vesicles and membrane sheets from the assay; (2) use of a sidedness probe with exclusive binding to one membrane face and low nonspecific binding; and (3) determination that the sidedness probe is impermeable to the vesicle membrane. α-BuTx applied to AcChR intact vesicles fulfills these criteria and reveals that membrane vesicles from *Torpedo* electric organ spontaneously orient during formation. Approximately 95% of these intact vesicles form with the synaptic membrane face exposed on the vesicle exterior. The vesicles are stable to gentle pelleting and resuspension but are easily osmotically shocked. The vesicle membranes are impermeable to sucrose and Ficoll but glycerol readily penetrates the membrane barrier.

Simple mechanical homogenization of electric organs produces highly oriented *Torpedo* postsynaptic membrane vesicles. In erythrocytes (Steck, 1974) and mouse L cells (Hunt & Brown, 1975), specific procedures leading to endocytosis were required to create highly oriented vesicles. In lymphocytes (Walsh et al., 1976) and ascites membranes (Zachowski & Paraf, 1974), concanavalin A was used to separate oriented vesicles from a mixture of inside-out and outside-out vesicles. Nitrogen cavitation (Ferber et al., 1972), French pressure cells, and sonication (Futai, 1974; Altendorf & Staehelin, 1974) have been used to create oriented vesicles but simple homogenization does not produce highly oriented vesicles in these systems. The physical forces responsible for spontaneous *Torpedo* membrane orientation during vesicle formation remain to be determined.

From the equilibrium density of intact vesicles (1.03 g/mL) and the AcChR membrane density (1.17–1.19 g/mL), we calculate that spherical vesicles with a membrane thickness of 80 Å filled with homogenization buffer (1.015 g/mL) should have a diameter of 4800–5400 Å. Examination of intact vesicles by thin-section electron microscopy (C. Strader, J. P. Revel, & M. Raftery, unpublished) reveals a field of roughly spherical vesicles with diameters of 0.1 to 10 μm. Although the composition of the vesicle interior solution remains unknown, it must be of very low density, similar to the homogenization medium, for these values to agree.

Our binding studies with intact vesicles do not permit us to unambiguously determine the origin of the 5% α-BuTx binding increase observed after disruption of intact vesicles. Either a small population of inside-out vesicles or a small number of α-BuTx sites on the cytoplasmic face of right-side-out vesicles may contribute to the binding increase. From the extreme assumption that all vesicles are right-side out, we can conclude that no more than 5% of the α-BuTx binding sites to *Torpedo* membranes are located on the cytoplasmic face.

Hess & Andrews (1977) have isolated sealed vesicles from eel which are approximately 400-fold less pure than *Torpedo* intact vesicles as judged by α-BuTx binding per mg of protein. In the isolation of intact vesicles from eel electroplaques, Hess and Andrews used a flotation method in which intact vesicles whose interiors were filled with low density sodium chloride solution were buoyed upward in a gradient of sucrose containing cesium chloride. We have isolated intact vesicles from *Torpedo* by flotation in a sucrose gradient after applying the

membrane between the 54% sucrose cushion and the 20% sucrose layer. Under these conditions our yield is lowered by approximately 40%. This is not surprising since high density sucrose exerts considerable osmotic pressure and can be expected to extract water from the vesicle interior resulting in a higher vesicle density. This action will prevent many vesicles from rising to the top of the gradient. Since the equilibrium density of intact *Torpedo* vesicles is so low, little osmotic shrinkage is encountered as vesicles applied to the top of sucrose gradients traverse solutions of isopycnic or lower density. Thus, intact vesicles can be isolated in good yield when a *Torpedo* homogenate is applied to the top of a 4–20% sucrose gradient.

We expect these intact vesicles to provide an excellent material for in vitro flux studies with *Torpedo* acetylcholine receptor. Preliminary experiments indicate that the background cation flux signal due to leaky vesicles is greatly reduced when these intact vesicles are utilized. In addition, intact vesicles present a unique opportunity for the study of vectorial membrane processes associated with acetylcholine receptor function. Since 95% of the vesicles present only their synaptic face to the external, bulk solution and since they are sealed with interiors inaccessible to membrane impermeable reagents, these vesicles provide the opportunity to selectively examine the synaptic or cytoplasmic face of postsynaptic membranes. Studies are currently underway to assess the vectorial arrangement of acetylcholine receptor subunits in these membranes.

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