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Purification of *Torpedo californica* Post-synaptic Membranes and Fractionation of their Constituent Proteins

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A rapid method for preparation of membrane fractions highly enriched in nicotinic acetylcholine receptor from *Torpedo californica* electroplax is described. The major step in this purification involves sucrose-density-gradient centrifugation in a reorienting rotor. Further purification of these membranes can be achieved by selective extraction of proteins by use of alkaline pH or by treatment with solutions of lithium di-iodosalicylate. The alkali-treated membranes retain functional characteristics of the untreated membranes and in addition contain essentially only the four polypeptides (mol.wts. 40000, 50000, 60000 and 65000) characteristic of the receptor purified by affinity chromatography. Dissolution of the purified membranes or of the alkali-treated purified membranes in sodium cholate solution followed by sucrose-density-gradient centrifugation in the same detergent solution yields solubilized receptor preparations comparable with the most highly purified protein obtained by affinity-chromatographic procedures.

Electric organs of *Torpedo californica* have been used as an excellent source of postsynaptic membranes containing nicotinic acetylcholine receptor (Duguid & Raftery, 1973; Reed *et al.*, 1975). ACh receptor isolated from *T. californica* and purified by affinity-chromatographic procedures (Schmidt & Raftery, 1972) consists of four types of polypeptide subunits having mol.wts. 40000, 50000, 60000 and 65000 as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Raftery *et al.*, 1974; Weill *et al.*, 1974; Vandlen *et al.*, 1976). The agonist-binding site has been shown by affinity labelling to be located on the mol.wt.-40000 subunit (Moore & Raftery, 1979). In addition to the four ACh-receptor subunits the other major polypeptides found in ACh-receptor-enriched membrane fragments have mol.wts. 43000 and 90000 on sodium dodecyl sulphate/polyacrylamide gels. Eldefrawi *et al.* (1977) reported that in 1% Triton X-100 extracts of *T. ocellata* membrane fragments the agonist-binding site could be separated from an H_{12}-HTX-binding component by gel filtration. Sobel *et al.* (1978) suggested that a 43000-mol.wt. polypeptide contains the binding site for histrionicotoxin and local anaesthetics in membranes isolated from *T. marmorata*. In contrast, Neubig *et al.* (1979) prepared Ach-receptor-enriched membranes from *T. nobiliana* and *T. californica* lacking a 43000-mol.wt. component by virtue of alkali treatment at pH 11 and measured specific binding of a ^{14}C-labelled local anaesthetic analogue to these membranes. In addition, we have recently shown by using a different approach that [^{3}H]H_{12}-HTX appears to bind to one or more of the Ach-receptor polypeptides from *T. californica* rather than to the mol.wt.-43000 component (Elliott & Raftery, 1979). It seems unlikely that these contrasting results are due to differences in the subunit structures of ACh receptors from the various *Torpedo* species. Claudio & Raftery (1977) showed that antibodies raised against each of the *T. californica* ACh-receptor subunits cross-reacted only with the corresponding subunit from *T. marmorata*, *T. nobiliana* and *Narcine brasiliensis*, and thus conservation of subunit antigenic determinants was demonstrated. The separable H_{12}-HTX-binding component observed by Eldefrawi *et al.* (1977) was probably due to non-specific binding to lipid-detergent micelles, since in this work no correction for the considerable non-specific binding component was made. Elliott and Raftery showed that very low concentrations of Triton X-100 (<0.1%) inhibited specific H_{12}-HTX binding to

Abbreviations used: ACh, acetylcholine; α-BuTx, α-bungarotoxin; H_{12}-HTX, perhydrohistrionicotoxin.

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detergent extracts of *T. californica* membranes. The results of Sobel *et al.* (1978) indicating that the mol.wt.-43000 polypeptide contains the histrionicotoxin-binding site were obtained by using a different methodology. Unlike Neubig *et al.* (1979), who measured direct binding to membranes of the local anaesthetic analogue with unchanged K_d and stoichiometry in the presence and absence of a polypeptide of mol.wt. 43000, Sobel *et al.* (1978) observed an apparent histrionicotoxin-induced decrease in quinacrine fluorescence. This decrease amounted to approx. 10% of the total fluorescence and had a very slow time course. This fluorescence quenching may not have reflected the primary, specific effect of histrionicotoxin.

Several methods have been published for the preparation of ACh-receptor enriched membrane fragments from *T. californica* (Duguid & Raftery, 1973; Reed *et al.*, 1975) and *Torpedo marmorata* (Cohen *et al.*, 1972; Sobel *et al.*, 1977) employing sucrose gradients in swinging-bucket and zonal rotors. The separation is a result of the unusually high density of the ACh-receptor-containing membranes [sp. gravity ~1.18 compared with 1.14 for the average density of membranes from *Torpedo* electroplax (Hartig & Raftery, 1979)]. These separations have been quite time-consuming. For example, the large-scale zonal method involved an overnight sucrose-density-gradient centrifugation. We report in the present paper a rapid and reproducible method for the preparation of highly enriched ACh-receptor membranes by using a reorienting sucrose step gradient. After this separation a highly purified preparation of solubilized ACh-receptor can be prepared from these membranes by use of an additional sucrose-gradient step in sodium cholate solution. In addition, selective extraction of polypeptides other than the ACh-receptor subunits from the enriched membranes can be achieved by use of high pH or by lithium di-iodosalicylate. Solubilization of membranes treated by these methods in sodium cholate solutions followed by centrifugation in sucrose gradients containing the detergent yields a preparation of purified ACh receptor comparable in purity with that previously obtained by affinity-chromatographic procedures (Raftery *et al.*, 1975; Vandlen *et al.*, 1976, 1979). These membrane preparations are shown to have unaltered functional properties: the rate constant for ^{125}I-labelled-α-BuTx–ACh-receptor complex formation is not affected; they can be shown to undergo a change of affinity (low to high) induced by carbamoylcholine with a half-time identical with that observed for untreated membranes; they bind [^{3}H]H_{12}-HTX with unaltered K_d and stoichiometry compared with α-BuTx-binding sites, in addition to retaining the same kinetic mechanism for binding and, finally, they respond to carbamoylcholine by rapid efflux of vesicle-entrapped $^{22}Na^+$.

Materials and Methods

Assays

The concentration of ^{125}I-labelled α-BuTx-binding sites was determined by the DEAE-cellulose disc assay of Schmidt & Raftery (1973) by using ^{125}I-labelled α-BuTx prepared from *Bungarus multicinctus* venom (Sigma Chemical Co.) as described by Blanchard *et al.* (1979). The ability of the membrane-bound ACh receptor to undergo the transition from a low- to a high-affinity state for agonists was determined from the difference in the inhibition of ^{125}I-labelled-α-BuTx binding caused by carbamoylcholine observed with and without preincubation (Lee *et al.*, 1977; Quast *et al.*, 1978). Protein concentrations were determined by the method of Lowry *et al.* (1951), by Fluram assay (Pierce Chemical Co.), by amino acid analysis or by a combination of these methods. [^{3}H]H_{12}-HTX binding was measured by centrifugation assay or equilibrium dialysis as previously described (Elliott & Raftery, 1977). The kinetics of H_{12}-HTX binding to the membranes were determined as described by Schimerlik *et al.* (1979), with ethidium bromide as an extrinsic fluorescence probe (Schimerlik & Raftery, 1976). The agonist-induced efflux of $^{22}Na^+$ from ACh-receptor-containing vesicles was measured by the method of Miller *et al.* (1978).

Preparation of membrane fragments

T. californica electroplax organs obtained from freshly killed animals were either used immediately or frozen in liquid N_2 and stored at −90°C until use. After connective tissue was dissected away the organ was minced into small pieces and an equal volume of cold buffer [10mM-sodium phosphate (pH 7.8), 400mM-NaCl, 5mM-EDTA, 0.02% NaN_3, 5mM-iodoacetamide, 10mM-phenylmethanesulphonyl fluoride] was added. After a 2min initial grind at high speed in a commercial Waring blender the homogenate was reground in small portions for four periods of 30s at 30000rev./min in a Virtis 60. Connective tissue and other large particles were pelleted by centrifugation of the homogenate at 5000rev./min for 10min in a Sorvall GSA rotor. The supernatant was passed through two layers of cheesecloth and centrifuged at 16000rev./min for 1h in a Beckman Type 35 rotor at 2°C. The pellet was resuspended for two periods of 30s at 30000rev./min in the Virtis homogenizer in 10mM-sodium phosphate (pH 7.4)/1mM-EDTA/0.02% NaN_3. Sucrose and NaCl were then added to final concentrations of 30% (w/w) and 400mM respectively.

Of this sample 10ml/tube was layered on discontinuous sucrose gradients consisting of 5ml of 50% (w/w), 5ml of 39% (w/w) and 12ml of 35% (w/w) sucrose in 0.4M-NaCl/10mM-sodium phosphate

(pH 7.4)/1 mM-EDTA/0.02% NaN_3. The samples were overlaid with the above buffer containing no sucrose to fill the tubes to 34 ml. The tubes were centrifuged at 45000 rev./min for 1 h in a Beckman model L-5 centrifuge with a Beckman VTi50 vertical rotor with slow acceleration and deceleration in the range of 0–1000 rev./min. Identical results were obtained by using a Sorvall TV-850 rotor. After centrifugation three light-scattering bands were observed: (heavy bands at the top and middle of the gradients and a lighter one near the bottom). The bands were collected by aspiration, diluted 2-fold into buffer containing no sucrose and centrifuged for 1 h at 30000 rev./min in a Beckman Type 35 rotor. The membranes were resuspended in the Virtis homogenizer and assayed for ^{125}I-labelled α-BuTx binding and protein concentration.

Treatment of the membranes with protein perturbants

Alkali treatment of the membranes was performed essentially as described by Steck & Yu (1973) for erythrocyte 'ghosts'. For routine preparations membranes containing approx. 60 mg of protein were pelleted and then resuspended in 32 ml of ice-cold water. The pH was adjusted to 11.0 with NaOH, and the membranes were immediately pelleted for 30 min at 30000 rev./min in a type 35 rotor. The pellet was resuspended in buffer at pH 7.4.

The treatment of membranes with lithium di-iodosalicylate was performed essentially as described by Steck & Yu (1973) for erythrocyte 'ghosts', with some modifications. Membranes containing approx. 60 mg of protein were spun down and resuspended in 4 ml 10 mM-sodium phosphate, pH 8.0. Aliquots containing approx. 8 mg of protein were diluted 6-fold into appropriate volumes of 10 mM-sodium phosphate (pH 8.0) and 60 mM-lithium di-iodosalicylate in the same buffer to reach the desired final lithium di-iodosalicylate concentrations. The membranes were incubated on ice for 30 min and then centrifuged at 18000 rev./min in a Sorvall SS-34 rotor for 45 min. The pellets were resuspended in phosphate buffer at pH 7.4.

Fractionation of the protein components of enriched membranes

The membranes were dissolved in 2% sodium cholate containing 10 mM-sodium phosphate (pH 7.4)/400 mM-NaCl/1 mM-EDTA/0.02% NaN_3 by stirring on ice for 30 min, followed by centrifugation at 100000 *g* for 1 h at 0°C. A portion (1 ml) of the supernatant containing 10 mg of protein was applied to a 13 ml 5–20% (w/v) sucrose gradient containing 2% cholate. The gradients were centrifuged for 18.5 h at 40000 rev./min in a Beckman SW 41 rotor at 0°C. Approx. 13 fractions (1 ml) were collected and assayed for protein and ^{125}I-labelled α-BuTx-binding sites.

Gel electrophoresis

Sodium dodecyl sulphate/8.75% polyacrylamide gels were run in the buffer system of Laemmli (1970). The gels were stained for protein with 0.05% (w/v) Coomassie Brilliant Blue in 10% acetic acid/25% methanol and destained in the same solution without the dye. Densitometer scans were made at 550 nm with a Gilford 240 with linear-transport accessory.

Electron microscopy

For negative staining, membrane fragments were diluted to a protein concentration of 0.5–1.2 mg/ml. One drop of this suspension was incubated on a carbon-coated copper grid for 1 min and then removed with filter paper. Grids were stained with one drop of 2% phosphotungstic acid (pH 7.4) for 5 min, blotted dry and examined on a Phillips 201 electron microscope.

Results and Discussion

Crude homogenates

The yield of ACh-receptor-rich membranes as determined by the concentration of ^{125}I-labelled-α-BuTx-binding sites remaining in the supernatant after centrifugation in a GSA rotor at 5000 rev./min was found to depend largely on homogenization techniques. The use of a tissue/buffer ratio of 1:1 (w/v) was found to result in release of approximately twice the toxin-binding activity observed for a 2:1 (w/v) ratio. The additional homogenization with the Virtis apparatus after the initial grinding also increased the yield. This was due to an increased efficiency of homogenization, since pieces of unground organ that were observed when only the Waring blender was used were not present after the Virtis step. Careful dissection of all connective tissue also increased yield by improving the efficiency of homogenization.

Reorienting gradient centrifugation

Iodoacetamide treatment. Addition of iodoacetamide to the buffer during the initial homogenization of the electroplax organ resulted in increased specific activity of the ACh-receptor-enriched membrane fragments obtained after reorienting sucrose-gradient centrifugation. An increase of 2-fold was consistently observed. Iodoacetamide treatment was found not to interfere with the following membrane activities: (i) the rate of ^{125}I-labelled α-BuTx-binding ($\sim 2 \times 10^4 M^{-1} \cdot s^{-1}$); (ii) the K_d and the stoichiometry of H_{12}-HTX binding [as previously reported (Elliott & Raftery, 1977) the K_d was approx. 0.5 μM

with 1 H_{12}-HTX molecule bound/4 α-BuTx-binding sites]; (iii) the ability to undergo transition from a low-affinity state to a high-affinity state for agonists; (iv) agonist-induced cation flux as measured by carbamoylcholine-induced release of $^{22}Na^+$, which could be blocked by α-BuTx (Miller *et al.*, 1978). The cation-flux experiments were done by using crude homogenates rather than material purified by sucrose-gradient centrifugation.

Separation of membrane populations by sucrose-gradient centrifugation. Fig. 1 shows a typical gradient profile. The majority of the α-BuTx-binding activity was found in the middle band, which was aspirated, diluted and pelleted. The resuspended pellet was used in further experiments as described below. Typical specific activities were of the order of 1.2–2 nmol of ^{125}I-labelled α-BuTx-binding sites/mg of protein. A typical preparation yielded 100–150 nmol of ^{125}I-labelled α-BuTx-binding sites in the middle band/g wet wt. of electric organ.

The osmotic-shock step preceding the sucrose-gradient-centrifugation step improved the yield, but not the specific activity, of the middle band of membrane fragments. This was not unexpected, since the sucrose concentration around this band agrees well with the previously reported value for the equilibrium density of ACh-receptor-enriched membrane fragments from *T. californica* (Hartig & Raftery, 1979).

Since the centrifugation was only 1 h in duration, denaturation of the protein components during prolonged exposure to high concentrations of sucrose was minimized. Although the specific activities varied from preparation to preparation the ratio of the specific activities for the three light-scattering bands was highly reproducible (see Table 1). Polyacrylamide/sodium dodecyl sulphate-gel electrophoresis of protein from the middle fraction showed prominent bands corresponding to the four ACh-receptor subunits as well as heavily staining components of apparent mol.wts. 43000 and 90000. The top fraction showed heavy staining at mol.wt. 90000 with lesser staining of bands of apparent mol.wt. 40000, 50000, 60000, 65000 and 43000 (see e.g. Fig. 5*b*). The gel profile of the bottom fraction resembled that of the top fraction, and this band was probably due to aggregation of membrane fragments.

Effects of modification of the gradient on separation of membrane fragment populations. The separation achieved by reorienting sucrose-density-gradient centrifugation was only slightly affected by

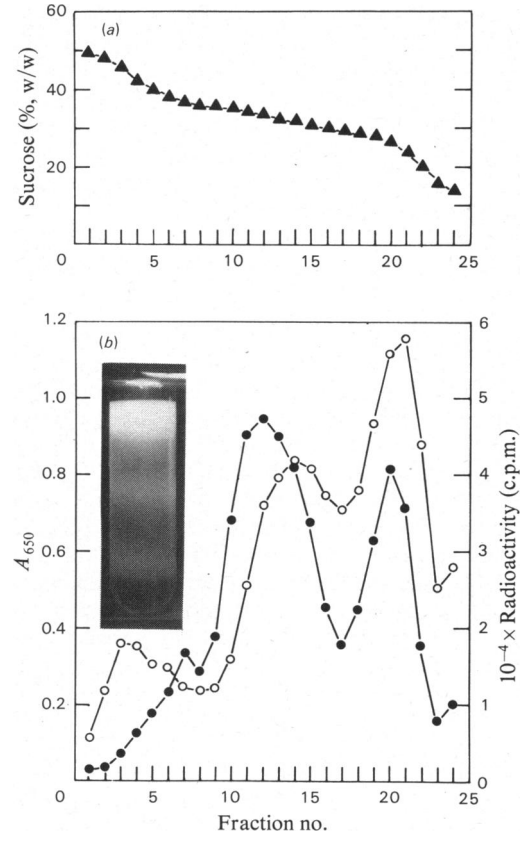


Fig. 1. *Profiles of ^{125}I-labelled α-BuTx binding* (●), *protein concentration measured by the method of Lowry et al.* (1951) (○) *and sucrose concentrations* (▲) *of a typical vertical-rotor fractionation of membranes*
The insert shows a photograph of the gradient before fractionation.

Table 1. *Distribution of ^{125}I-labelled-α-BuTx binding and protein after reorienting sucrose-gradient centrifugation of Torpedo membranes*

Fraction	Protein (mg)	^{125}I-labelled-α-BuTx-binding sites (nmol)	Specific activity (nmoles of ^{125}I-labelled-α-BuTx-binding sites/mg of protein)	Material recovered (% of starting material)	
				Protein	Toxin binding
Starting material	434	291	0.67		
Top	189	60	0.32	43.5	20.6
Middle	80	108	1.35	18.4	37.1
Bottom	74	36	0.49	17.1	12.4

small changes in the relative volumes of the 35% (w/w) and the sample [30% (w/w)] layers. For instance, when the volume of the 35% (w/w) layer was decreased from 12ml to 10ml, with a corresponding increase in the volume of the sample from 10ml to 12ml, three membrane fragment bands were still observed. The relative specific activities of these bands were unchanged, but their positions in the tube relative to each other were slightly shifted. If, however, the 39% (w/w) layer was omitted and the volume of the 30% (w/w) (sample) layer increased by a corresponding amount from 10ml to 15ml, a slightly different pattern was observed after centrifugation. A new light-scattering band appeared between the top and middle bands (Fig. 2).

The peaks for protein and toxin binding of the middle band were observed to be slightly skewed in the original gradient (see Fig. 1), but this was not observed by using the shallow gradient. In this

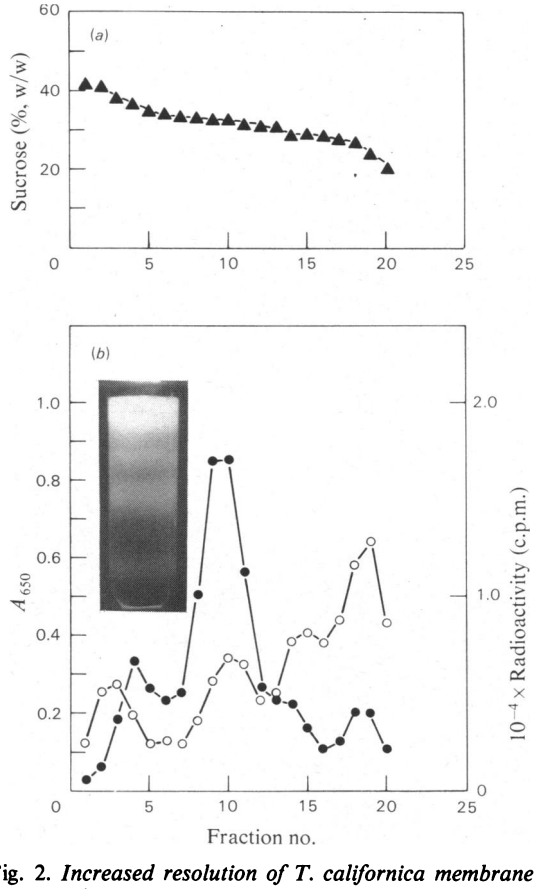


Fig. 2. *Increased resolution of T. californica membrane fragments on sucrose density gradients*

After centrifugation on the modified gradient [39% (w/w) sucrose replaced by 35% (w/w)] as described in the text, a single tube was fractionated and the individual fractions were assayed for: (*a*) sucrose concentration by refractometry and (*b*) ^{125}I-labelled α-BuTx binding (●) and protein (○) by the method of Lowry *et al.* (1951). The insert shows the separation of the membrane fragments after centrifugation. The four light-scattering bands correspond to the four protein peaks observed in the profile.

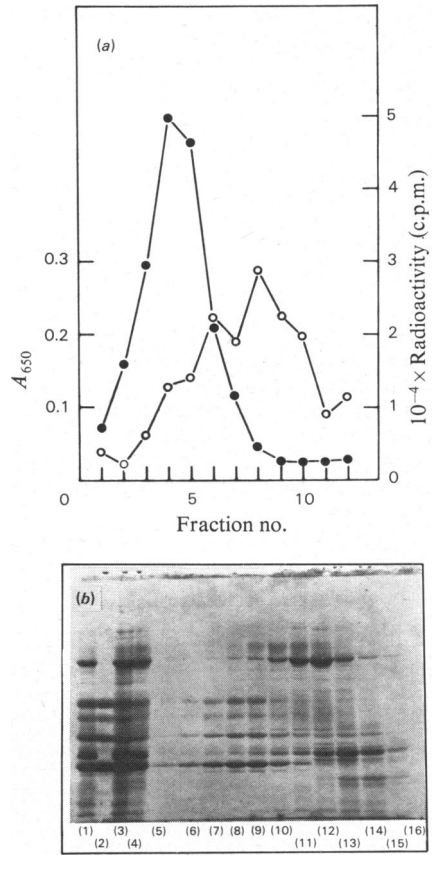


Fig. 3. *Fractionation of a 2% cholate extract*

(*a*) shows profiles of ^{125}I-labelled α-BuTx binding (●) and protein measured by the method of Lowry *et al.* (1951) (○) of a 5–20% (w/v) sucrose-gradient fractionation of a 2% cholate extract made from the middle band from the membrane fractionation. The buffer was 10mM-sodium phosphate (pH 7.4)/400mM-NaCl/1mM-EDTA/2% sodium cholate/0.02% NaN_3. (*b*) shows sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fractions resulting from sucrose-gradient fractionation of the cholate extract. Gels (5)–(16) are for the fractions from the sucrose gradient, starting at the bottom of the gradient with fraction 1, gels (1) and (3) are for membrane fragments from the middle band, gel (2) is for ACh receptor purified by affinity chromatography and gel (4) is for the cholate extract applied to the gradient.

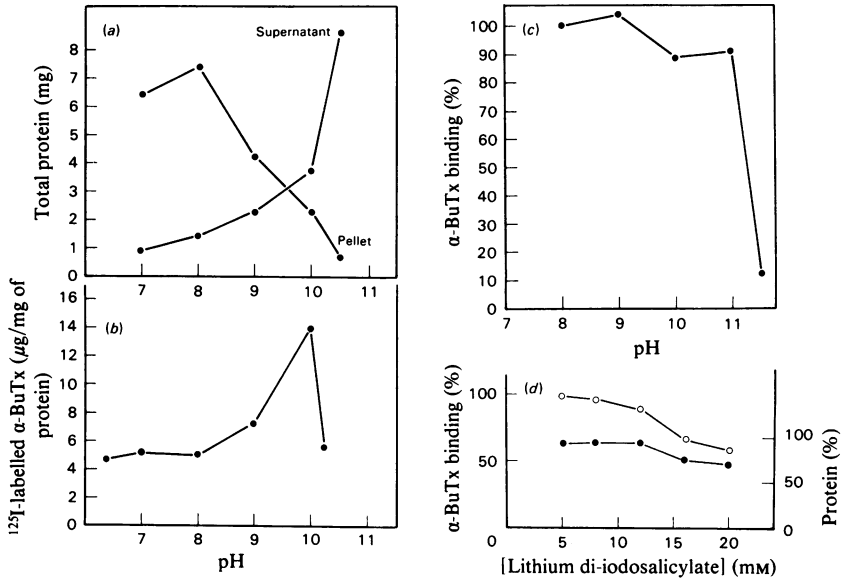


Fig. 4. *Treatment of membranes by alkali and lithium di-iodosalicylate*

(*a*) shows protein recovery for membranes treated at various pH values as follows: 6.4 ml samples of middle-band membranes suspended in water, containing 8.5 mg of protein and 33 nmol of ^{125}I-labelled α-BuTx-binding sites, were adjusted to various final pH values with NaOH. (*b*) shows the specific activity of these membranes after pH treatment. (*c*) shows ^{125}I-labelled α-BuTx-binding activity remaining in the membranes after pH treatment. (*d*) shows the recovery of ^{125}I-labelled α-BuTx binding and protein in membranes treated in various lithium di-iodosalicylate concentrations. Symbols: ●, protein content; ○, α-BuTx binding.

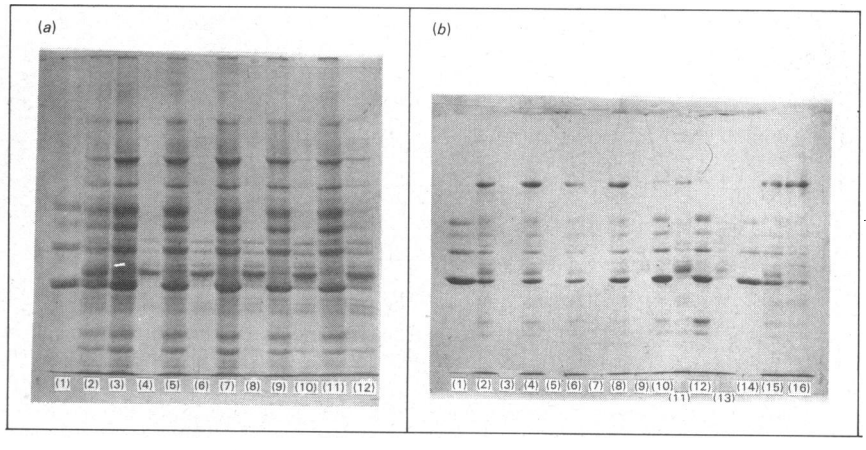


Fig. 5. *Electrophoresis of treated membranes*

(*a*) shows sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of lithium di-iodosalicylate-treated membranes. (1), ACh receptor; (2), membranes before treatment; pairs of gels (3) and (4), (5) and (6), (7) and (8), (9) and (10), (11) and (12) are polypeptides in treated membranes and in the supernatants at 5, 8, 12, 16 and 20 mM-lithium di-iodosalicylate in 10 mM-sodium phosphate (pH 7.4) respectively. The membranes before treatment contained 1 nmol of α-BuTx-binding sites/mg of protein. (*b*) shows sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of membranes treated at various pH values as described in Fig. 4. Gels (1) and (14) show ACh receptor purified by affinity chromatography, pairs of gels (2) and (3), (4) and (5), (6) and (7), (8) and (9), (10) and (11), (12) and (13) show membranes and supernatants after treatment at pH 7.4, 8.0, 9.0, 10.0, 11.0 and 11.5 respectively. Gel (15) shows membranes from the middle band of the vertical rotor preparation; gel (16) shows membranes from the top band.

shallow gradient this band was resolved into two fractions with the denser fraction having the higher specific activity.

Further purification of the membranes and membrane components

Purification of ACh receptor from cholate-solubilized fractionated membranes. When 2% cholate extracts of the membranes banding in the middle of sucrose density gradients were run on 5–20% sucrose gradients in 2% cholate, the ACh receptor migrated as the heaviest protein. Fig. 3(*a*) shows a profile of a typical gradient, and Figure 3(*b*) shows sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the resulting fractions; the tubes near the bottom of the gradient contained nearly pure ACh receptor by this criterion. The mol.wt.-90000 and -43000 bands peaked relatively close to one another, but they were reasonably well separated from the ACh receptor. These proteins migrated with apparent sedimentation coefficients of 8.2S and 6.5S respectively. For comparison, the ACh receptor sedimented as one peak having a value of 13.2S, which compares favourably with values previously obtained for the dimeric form (Raftery *et al.*, 1972; Reynolds & Karlin, 1978). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of this purified ACh-receptor preparation showed that the predominant polypeptide species present were the four ACh-receptor subunits (Fig. 3*b*). The small band migrating slightly faster than the mol.wt.-40000 band is thought to be a degradation product; it is found in some but not all preparations of membranes and purified ACh receptor (C. D. Strader & M. A. Raftery, unpublished work). The separation of the ACh receptor from other proteins in this case was dependent on the predomination of the dimeric form (see Raftery *et al.*, 1972; Suarez-Isla & Hucho,

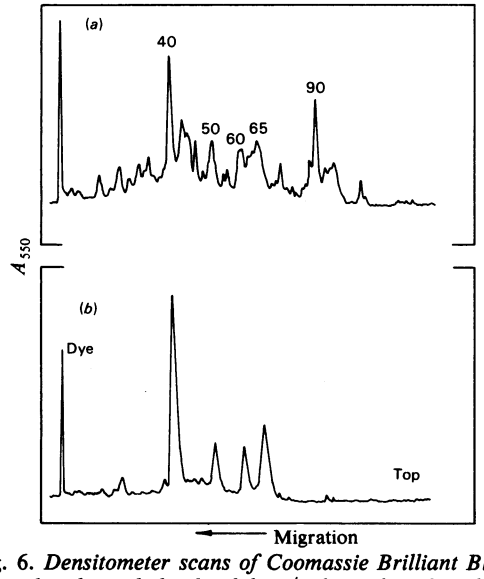


Fig. 6. *Densitometer scans of Coomassie Brilliant Blue-stained sodium dodecyl sulphate/polyacrylamide gels of membranes before (a) and after (b) treatment at pH 11*
The 40000-, 50000-, 60000-, 65000- and 90000-mol.wt. polypeptides are indicated in (*a*).

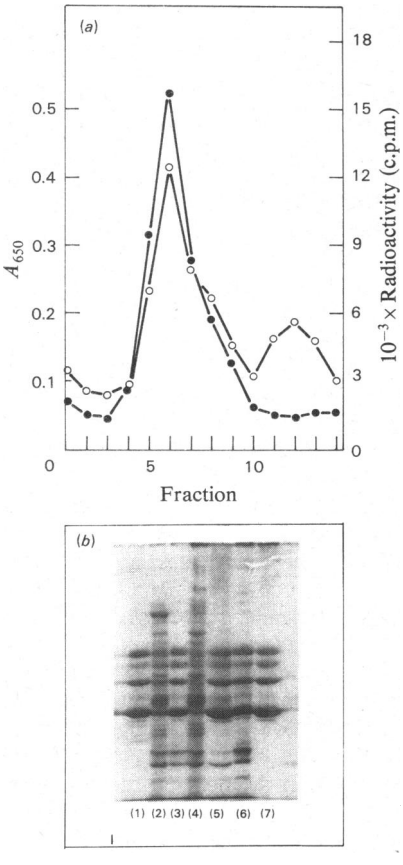


Fig. 7. *Fractionation of a 2% cholate extract of alkali-treated membranes*
(*a*) shows profiles of ^{125}I-labelled α-BuTx binding (●) and protein measured by the method of Lowry *et al.* (1951) (○) of a 5–20% (w/v) sucrose-gradient fractionation of a 2% cholate extract of pH 11-treated membrane fragments. The buffer is the same as in Fig. 2. (*b*) shows sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of: (1) ACh receptor purified by affinity chromatography; (2) the membrane fragments used in this experiment; (3) the membrane fragments after treatment at pH 11; (4) the supernatant containing polypeptides extracted at pH 11; (5) the 2% cholate extract of the pH 11-treated membranes; (6) the pellet containing material not solubilized by 2% cholate; (7) fraction (6) (the peak tube) from the sucrose-gradient centrifugation.

1977; Chang & Bock, 1977; Hamilton *et al.*, 1977; Witzemann & Raftery, 1978*a*) that resulted from the iodoacetamide treatment. The 9S form, which is the predominant species found without iodoacetamide treatment, would not be well separated from the other components.

Alkali treatment of fractionated membranes. Membranes from the middle band of the preparation made in the vertical rotor were treated with increasing pH and low ionic strength (Fig. 4). At pH 11 essentially all of the mol.wt.-43000 polypeptide and most of the mol.wt.-90000 polypeptide were found in the supernatant fraction, along with several minor bands (Figure 5*b*). The membranes contained the four polypeptides identified as subunits of the ACh receptor (Raftery *et al.*, 1974, 1975; Weill *et al.*, 1974; Karlin *et al.*, 1975; Vandlen *et al.*, 1976; Witzemann & Raftery, 1978*b*), having mol.wts. 40000, 50000, 60000 and 65000 as well as minor bands of molecular weight significantly less than 40000 and a band at mol.wt. 90000. Scans at 550nm of Coomassie Brilliant Blue-stained gels of membranes before and after treatment at pH 11 are shown in Fig. 6. Below pH 11, extraction of protein was not efficient, and above this pH ^{125}I-labelled α-BuTx-binding activity decreased rapidly (Fig. 4). In the experiment described in Figs. 4(*a*)–4(*c*), 5(*b*) and 6(*a*) and 6(*b*), protein recovery in the pH 11 pellet was 38% and recovery of ^{125}I-labelled α-BuTx-binding sites was 91%. A typical experiment yielded 75% recovery of toxin sites, although lower recoveries were also obtained in some preparations.

At the electron-microscope level, the alkali-treated membrane fragments were virtually indistinguishable from the untreated ones. Both preparations consisted primarily of spherical vesicles ranging in diameter from 100 to 1000nm, many of which were covered with the 6–7nm-diameter rosettes characteristic of ACh-receptor containing membranes (see Plate 1) (Nickel & Potter, 1973; Cartaud *et al.*, 1973; Raftery *et al.*, 1974).

Purification of ACh receptor from alkali-treated membranes after cholate solubilization. Cholate extraction of alkali-treated membranes results in a slight purification of the solubilized material (Fig. 7*b*), indicating that some of the lower-molecular-weight impurities are more resistant to cholate solubilization. When a cholate extract of the pH 11-treated membrane preparation was centrifuged on a 5–20% (w/v) sucrose gradient containing 2% cholate, a small amount of protein separated from the ^{125}I-labelled α-BuTx-binding peak. A profile of a gradient is shown in Fig. 7(*a*). Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the peak tube from the region of ^{125}I-labelled α-BuTx-binding activity showed that it contained only the four ACh-receptor polypeptides (Fig. 7*b*). This preparation of solubilized ACh receptor is as pure on the basis of this criterion as the most highly purified ACh receptor obtained by affinity-chromatographic procedures (Raftery *et al.*, 1975; Vandlen *et al.*, 1976).

Lithium di-iodosalicylate treatment of fractionated membranes. Membranes from the middle band of the vertical rotor preparation were also treated with lithium di-iodosalicylate, an agent used to effectively dissociate several erythrocyte membrane polypeptides (Steck & Yu, 1973). Gel electrophoresis (Figs. 5*a* and 8) of membranes before and after treatment with lithium di-iodosalicylate (up to 220mM) revealed that most of the mol.wt.-43000 polypeptide, along with several minor bands, was removed. The mol.wt.-90000 polypeptide, however, was essentially retained. The recovery of protein and ^{125}I-labelled α-BuTx-binding sites at several lithium di-iodosalicylate concentrations is plotted in Fig. 4(*d*). It can be seen that more than 94% of toxin-binding sites were recovered on treatment with solutions less than 12mM in lithium di-iodosalicylate.

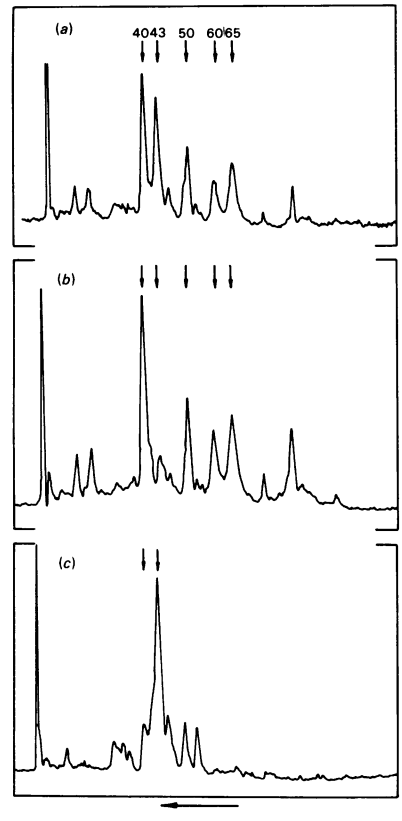
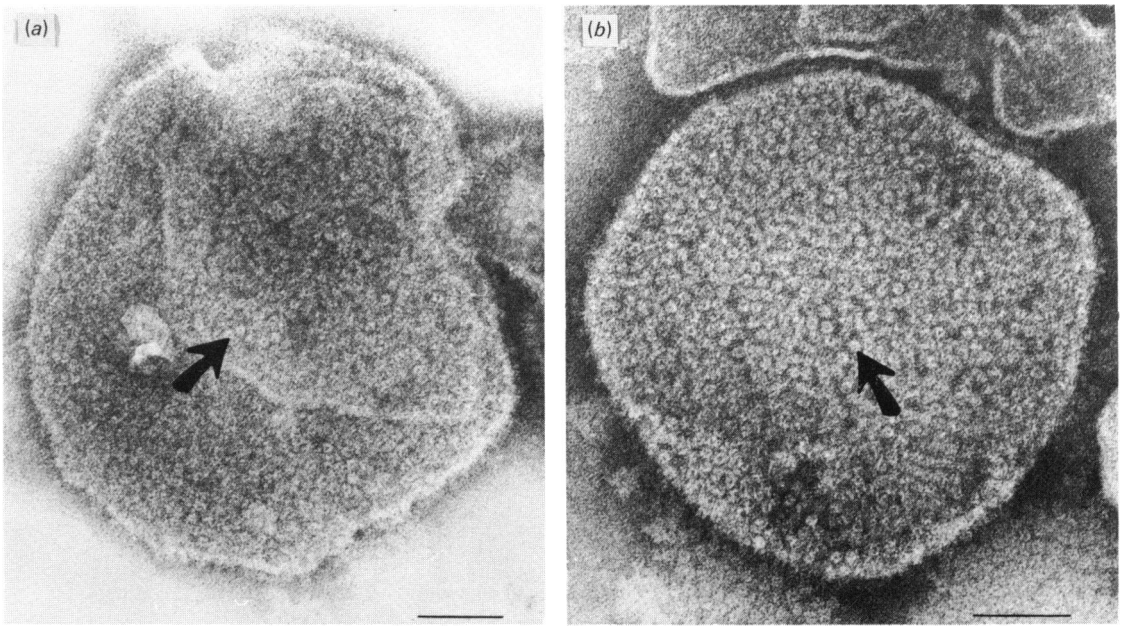


Fig. 8. *Densitometer scan of sodium dodecyl sulphate/polyacrylamide gels of membranes before treatment (a), after treatment in 20mM-lithium di-iodosalicylate (b) and of polypeptides removed by the lithium di-iodosalicylate treatment (c)*
Absorbance was measured at 550nm.



EXPLANATION OF PLATE 1

Negative staining of purified and pH-treated membranes

Arrows point to the rosettes corresponding to ACh-receptor molecules. The bar indicates 50 nm. (*a*) shows a membrane vesicle prepared from the middle band of the sucrose gradient, as described in the text. (*b*) shows a membrane vesicle from the same preparation after treatment at pH 11.

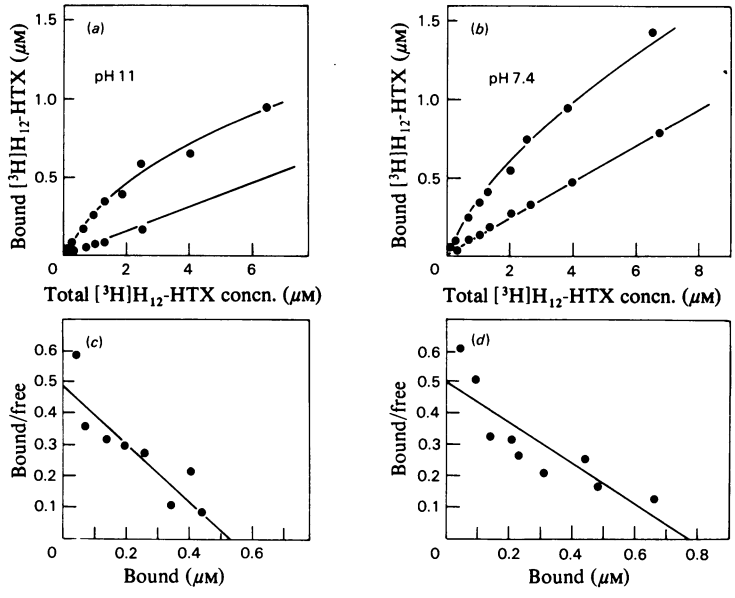


Fig. 9. *[^{3}H]H_{12}-HTX bound to alkali-treated (b and d) and untreated (a and c) ACh-receptor enriched membranes*
In both bases the concentration of ^{125}I-labelled α-BuTx-binding sites was 1.4 μM and the buffer was *Torpedo* Ringers solution {20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/250 mM-NaCl/5 mM-KCl/4 mM-$CaCl_2$/2 mM-$MgCl_2$ (pH 7.4)}. In (*a*) and (*b*) the upper trace shows total [^{3}H]H_{12}-HTX bound and the lower trace shows [^{3}H]H_{12}-HTX non-specifically bound in the presence of unlabelled 40 μM-H_{12}-HTX. (*c*) and (*d*) are Scatchard plots of specific [^{3}H]H_{12}-HTX binding, calculated by a weighted linear least-squares fit of the data points.

Table 2. *Functional properties of alkali-treated ACh-receptor-enriched membrane fragments from T. californica*
The rate of α-BuTx binding was determined by DEAE disc assay with [ACh-receptor sites] = 0.84 μM and [^{125}I-labelled α-BuTx] = 62.5 nM.

Parameter	Control	After treatment
Rate of ^{125}I-labelled-α-BuTx binding	$(2.9 \pm 0.3) \times 10^4$ M^{-1}·s^{-1}	$(2.6 \pm 0.3) \times 10^4$ M^{-1}·s^{-1}
Half-time for carbamoylcholine-induced affinity change*	74 ± 22 s	73 ± 5 s
[^{3}H]H_{12}-HTX† K_d	1.13 ± 0.24 μM	1.09 ± 0.02 μM
N	0.35 ± 0.12	0.33 ± 0.07
Kinetics of H_{12}-HTX binding‡		
k_1	$(1.8 \pm 0.1) \times 10^{-2}$ s^{-1}	$(1.5 \pm 0.1) \times 10^{-2}$ s^{-1}
K	4.6 ± 1.0 μM	3.0 ± 0.7 μM

* The rate of the change from low to high affinity for agonists was determined under conditions of excess receptor sites over ^{125}I-labelled-α-BuTx as described by Lee *et al.* (1977) and Quast *et al.* (1978). The concentration of carbamoylcholine was 5 μM and concentrations of ACh-receptor sites and toxin were the same as those given in the legend above.

† [^{3}H]H_{12}-HTX K_d and N (ratio of [^{3}H]H_{12}-HTX sites to ^{125}I-labelled-α-BuTx sites) were determined as previously described (Elliott & Raftery, 1977). The control values are the average per five experiments; the 'after treatment' values are the means for two experiments.

‡ Data from stopped-flow experiments of the effect of H_{12}-HTX concentration on the kinetics of binding to ACh-receptor by using the fluorescent probe ethidium and procedures described by Schimerlik *et al.* (1979). Apparent rate constants ($k_{app.}$) were measured under pseudo-first-order conditions in which the final concentration of ACh-receptor was 0.3 μM (in α-BuTx-binding sites) and the concentration of H_{12}-HTX was varied from 1 μM to 15 μM. The observed binding kinetics were consistent with the two-state model (Schimerlik *et al.*, 1979):

$$R + L \underset{}{\overset{K}{\rightleftharpoons}} RL \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RL^*$$

The kinetic parameters k_{+1} and K were obtained from a non-linear regression fit to the equation $k_{app.} = k_{-1} + k_{+1}L/(L + K)$, by using an estimated value of k_{-1} equal to 1.5×10^{-3} s^{-1}. Variation of k_{-1} between 0 and 3×10^{-3} s^{-1} did not significantly affect the values of k_1 and K.

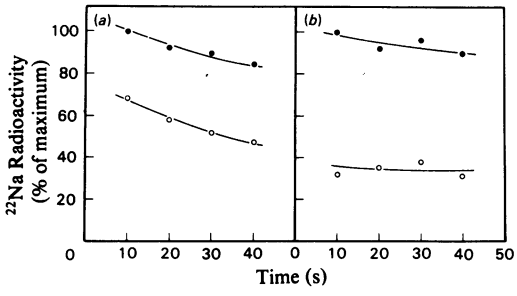


Fig. 10. *Carbamoylcholine-induced $^{22}Na^+$ efflux from re-orienting gradient membranes before (a) and after (b) pH 11 treatment*

A membrane preparation loaded with $^{22}Na^+$ was diluted 20-fold into 10mM-Tris/HCl (pH 7.4) (●), or 10mM-Tris/HCl (pH 7.4) plus 100μM-carbamoylcholine (○) and the vesicle-entrapped radioactivity was assayed by the Millipore-filter method (Miller *et al.*, 1978). Symbols: ●, iso-osmotic dilution (20-fold) of membranes into 10mM-Tris/HCl (pH 7.4); ○, dilution into 10mM-Tris/HCl (pH 7.4), containing 100μM-carbamoylcholine. The maximal entrapped radioactivity was approx. 4300c.p.m. for the untreated membranes and approx. 1300c.p.m. for the alkaline-extracted membranes. The difference may result from alteration of vesicle interior volume during the alkali treatment.

Functional properties of alkali-treated membranes. The highly purified alkali-treated membranes, which lacked the mol.wt.-43000 polypeptide, retained the ability to bind [^{3}H]H_{12}-HTX with unchanged stoichiometry and K_d (Table 2; Fig. 9). Agonist-induced rapid $^{22}Na^+$ efflux was also retained after pH 11 treatment (Fig. 10). The following properties also remained unchanged: ^{125}I-labelled α-BuTx association rate, the rate of the change from low to high affinity for agonists and the mechanism of H_{12}-HTX binding as determined by stopped-flow experiments with ethidium as indicator. Those results are shown in Table 2.

Conclusions

Several features of the preparations described in the present paper deserve further comment. The methods described permit rapid purification of both membrane-bound and solubilized purified ACh receptor, thus minimizing the possibility of degradation from proteinases and phospholipases present in membrane fractions (H.-P. Moore, W. Wu & M. A. Raftery, unpublished work). By use of a simple two-step procedure, namely rapid centrifugation in a sucrose gradient formed in a reorienting rotor, followed by a brief exposure to pH 11.0, a membrane fraction can be obtained that contains essentially only the four polypeptides characteristic of purified ACh receptor. This fraction retains all the functional properties of the system so far investigated *in vitro*. One such property, the direct and specific binding of H_{12}-HTX, has clearly eliminated the notion that this toxin binds specifically to a polypeptide of mol.wt. 43000 that is associated with the membranes before alkali treatment and that binds the fluorescent local anaesthetic and anti-giardiasis agent quinacrine hydrochloride (Sobel *et al.*, 1978). The purity of this preparation is also close to that of solubilized purified ACh receptor of the highest specific activity prepared by affinity-chromatographic methods (Raftery *et al.*, 1975). Purified ACh receptor can also be obtained from these membranes by a simple centrifugation step after dissolution into sodium cholate. The ease of removal of this detergent makes it ideal for studies of reconstitution of functionality, such as recovery of [^{3}H]H_{12}-HTX binding.

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