Isolation of Putative Acetylcholine Receptor Proteins from Housefly Brain†

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ABSTRACT: The cholinergic binding molecules in the supernatant of 100 000*g* (1 h) of housefly brain were purified 25-fold by gel filtration on Sephadex G-200. They bound an average of 1 nmol/mg protein of [^{3}H]decamethonium with K_D of 0.15 μM. Acetylcholine (ACh), in presence of 10 μM DFP (diisopropyl fluorophosphate), competitively blocked this binding with K_i of 10 μM. Both nicotinic and muscarinic drugs blocked binding of [^{3}H]decamethonium or [^{3}H]nicotine to this fraction, but α-bungarotoxin and cobra toxin did not. Affinity chromatography purified these cholinergic binding molecules further but did not increase their specific binding due to partial loss in activity during purification. However, using semipreparative electrophoresis two protein bands were isolated, and antibodies against them precipitated all the cholinergic drug binding proteins from housefly brain extracts. The data suggest that the purified housefly brain proteins may be ACh receptors, which are mixed nicotinic–muscarinic in their drug specificity. In addition α-bungarotoxin and cobrotoxin failed to bind to these putative receptors despite their partial nicotinic character. There were no antigenic similarities between these easily solubilized housefly brain putative receptors and the nicotinic ACh receptors of the electric organ of the electric ray.

The only kind of neurotransmitter receptor to be purified so far is the acetylcholine (ACh)[1] receptor of electric organs of fish (Karlsson et al., 1972; Olsen et al., 1972; Raftery et al., 1973; Klett et al., 1973; Biesecker, 1973; Eldefrawi and Eldefrawi, 1973; Chang, 1974; Ong and Brady, 1974). This feat was made possible by the high concentration of ACh receptors in this tissue and the use of affinity chromatography. The detection of high concentrations of ACh and ACh-esterase in the whole brain of the housefly, *Musca domestica* (L.) (Metcalf and March, 1950; Colhoun, 1958; Smallman and Fischer, 1961; Treherne, 1966; Lunt, 1975), pointed to the possibility that it would also be rich in ACh receptors. Our early studies demonstrated the presence of protein molecules, which bound reversibly and with high affinities several radiolabeled cholinergic ligands, leading to the suggestion that they might be ACh receptors (Eldefrawi and O'Brien, 1970; Eldefrawi et al., 1971a). The concentration of these molecules in the housefly head (1–3 nmol/g of heads) was 3-fold higher than that in the richest known source of ACh receptors, namely, the electric organ of the electric ray, *Torpedo* sp. (Eldefrawi et al., 1971b; O'Brien et al., 1972) and 50-fold more than that in mammalian brain (Hiley and Burgen, 1974; Schleifer and Eldefrawi, 1974; Yamamura et al., 1974).

A major difficulty encountered in the biochemical study of insect brain ACh receptors is the lack of electrophysiological and pharmacological data, which could guide in the in vitro identification of these molecules. This leads to a reliance on indirect evidence. The cholinergic binding molecules of housefly heads and the ACh receptor of electric organs are similar in their high affinity binding of cholinergic drugs, their proteinaceous nature, and their relative concentrations, which are 0.3–3-fold those of ACh-esterase in their respective tissues (O'Brien et al., 1970; Eldefrawi, 1976). On the other hand, there are differences between them, which cast some doubt on the identity of the housefly brain cholinergic binding molecules as ACh receptors (see Discussion below), though such differences may be due to the fact that the comparison is being made between a nicotinic neuromuscular type ACh receptor and a putative insect central nervous system ACh receptor.

The putative housefly brain ACh receptors have been partially purified by centrifugation and gel filtration (Eldefrawi and O'Brien, 1970; Donnellan et al., 1975). Extraction with chloroform–methanol has also been used in conjunction with detection of binding by Sephadex LH-20 chromatography (Cattell and Donnellan, 1972; Donnellan et al., 1975; Lunt, 1975). Although the proteolipid isolated by this method had similar localization and pharmacology to the water extracted receptor (Eldefrawi et al., 1971a; Donnellan et al., 1975), we prefer the latter approach, which avoids possible artifacts in the use of gel filtration in organic solvents as assays for binding of reversible ligands (Levinson and Keynes, 1972). In addition, Barrantes et al. (1975) have shown that there was no cross reaction between the cholinergic proteolipid extracted by chloroform and methanol from electric organ membranes and rabbit antisera produced against the detergent-extracted ACh receptor from the same tissue. Similarly there was no cross reaction between the ACh receptor protein and antiproteolipid antisera.

In this paper we report on the isolation of the putative ACh receptor proteins of housefly brain by gel filtration followed by affinity chromatography or gel electrophoresis.

Experimental Procedures

Preparation of Housefly Head Extracts. Houseflies (Wilson strain) were frozen on dry ice and their heads collected by sieving according to the method of Moorefield (1957). The heads were homogenized in ice-cold glass-distilled water, pH 7 (20% w/v), in a Sorvall Omni-mixer, filtered through four layers of cheese cloth, and centrifuged at 100 000*g*, 1 h at 4 °C. The supernatant (designated as S_1) was collected and fractionated on Sephadex G-200.

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[1] Abbreviations used: ACh, acetylcholine; α-BGT, α-bungarotoxin; PTA, (*p*-aminophenyl)trimethylammonium; DFP, diisopropyl fluorophosphate; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1 4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

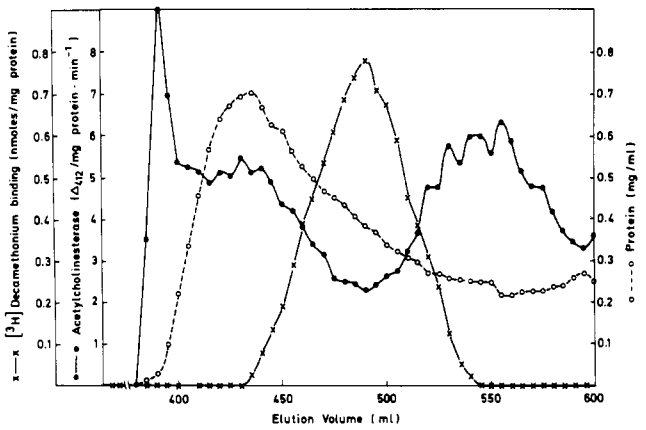

FIGURE 1: Gel filtration of the 100 000*g* of housefly brain (S_1) on Sephadex G-200. Void volume is 400 mL (and each fraction is 5 mL).

Column Chromatography. A 5 × 120 cm Kontes glass column was packed with swelled Sephadex G-200, and equilibrated with Krebs original Ringer phosphate and the flow rate adjusted to 30 mL/h. Fifty milliliters of S_1 (460 mg protein) was applied and the column eluted at 4 °C with Ringer containing 0.02% NaN_3. Five-milliliter fractions were collected and assayed for protein by the Lowry et al. (1951) method, acetylcholinesterase by the Ellman et al. (1961) spectrophotometric method, and binding of cholinergic ligands by equilibrium dialysis. The fractions containing the highest specific binding of [^{3}H]decamethonium were pooled and centrifuged at 100 000*g*, 1 h to remove molecules that aggregated during chromatography. The supernatant was designated S_2.

Equilibrium Dialysis. One milliliter of fly brain extract was placed in cellophane dialysis tubing (0.25 in., from Union Carbide) that was pretreated to eliminate contaminants (McPhie, 1971). The tubing was tied at both ends and placed into a flask containing 100 mL of Ringer solution and radioactive ligand. [^{3}H]Decamethonium (sp act. 559 mCi/mmol), [^{3}H]nicotine (sp act. 2730 mCi/mmol), and [^{3}H]-atropine (sp act. 127 mCi/mmol) were from Amersham/Searle and [*acetyl*-^{3}H]acetylcholine (sp act. 49.5 mCi/mmol) from New England Nuclear. The flask was shaken for 16 h at 4 °C and then three 0.1-mL samples were taken from bath and bag contents and counted in 10 mL of toluene–liquid scintillation solution (3.8 L of toluene, 18.05 g of PPO, 1.22 g of dimethyl-POPOP, 150 mL of BBS-3 (Beckman)) in a Packard liquid scintillation spectrometer. For each drug concentration triplicate experiments were performed. When the effect of a nonradioactive drug on binding was studied, the drug was added to the dialysis bath.

Binding of Neurotoxins. The α-neurotoxins were purified from the venoms of elapid snakes, the krait, *Bungarus multicinctus,* and the cobra, *Naja naja siamensis* (from the Miami Serpentarium), by ion-exchange chromatography on Sephadex CM-50 using an ammonium acetate buffer gradient of 0.05–1 M (pH 6.5) as previously described (Karlsson et al., 1971; Eldefrawi and Fertuck, 1974). α-BGT was radiolabeled with [^{125}I]NaI (carrier free, from New England Nuclear) using the lactoperoxidase method of David (1972) and then its biological activity was checked as previously described (Eldefrawi and Fertuck, 1974). The iodinated toxin was stored in 0.5-mL aliquots of 0.1 M ammonium acetate, pH 6.7, in polyethylene tubes at −20 °C until use. Binding of [^{125}I]-α-BGT to housefly S_2 and to purified ACh receptor of *Torpedo* electroplax was studied by ion-exchange chromatography on Sephadex CM-50 (1 × 30 cm Kontes) columns equilibrated with 10 mM Na_2HPO_4 buffer, pH 7.2. Sample volume was 0.25 mL, and elution was with same buffer at a rate of 30 mL/h.

Affinity Chromatography. Sepharose 4B was suspended in 1 M $NaCO_3$, pH 11, and then activated with CNBr–acetonitrile mixture exactly as described by March et al. (1974). The activated gel was washed with cold $NaHCO_3$ buffer, pH 9.4, then cross-linked to two units of 3,3′-diaminodipropylamine and succinic anhydride according to the procedures of Cuatrecasas (1970) and Berman and Young (1971). Finally *p*-(aminophenyl)trimethylammonium (PTA) was cross-linked on the carboxylic end of the arm. One gram of this PTA affinity gel was packed in 1 × 30 cm Kontes column and equilibrated with 10 mM Na_2HPO_4, pH 7.2. S_2 (5 mL with 1.5–2.0 mg of protein) was introduced into the column left overnight at 4 °C and then the column washed with 90 mL of 10 mM Na_2HPO_4 (pH 7.2) followed by consecutive washes (10^{-2}, 10^{-1}, and then 1 M) of either decamethonium or carbamoylcholine. Each concentration was left to equilibrate for 1 h before elution was continued. Fractions of 5 mL were collected.

Acrylamide Disc Gel Electrophoresis. Analytical acrylamide disc gel electrophoresis (Canalco) was used according to the method of Davis (1963). The gels (7% acrylamide) were stained with Coomassie Brilliant Blue R250 and destained in 7% acetic acid and then scanned in a Beckman ACTA III spectrophotometer at 570 nm. Another method was also used for staining some gels utilizing Coomassie Brilliant Blue G250 perchloric acid solution. This allows the dye to penetrate the gel fully, producing a pale orange background, while staining proteins an intense blue, and requires no destaining (Reisner et al., 1975).

For purification of cholinergic binding proteins of S_2, a Savant analytical electrophoresis apparatus with 1 × 10 cm columns was used. The gel was 5% acrylamide and 0.1% bisacrylamide, and the bath was Tris/glycine buffer, pH 8.3. The gels were prewashed for 1 h before addition of the protein sample (100 μg/gel) in 400 μL of 20% sucrose. Electrophoresis was performed at 4 °C applying 5 mV/column. After the tracking dye washed off the end of the column in about 2 h, a tapering portion of a collodion bag (VWR) filled with buffer was attached with Parafilm to the bottom end of each glass column. The desired protein bands eluted in 20–30 min afterward and were collected, concentrated in a collodion concentrator, and the protein was assayed. Purity was determined by analytical gel electrophoresis.

Production of Rabbit Antisera and Isolation of Immunoglobulins. One hundred micrograms of the purified proteins from houseflies or the *Torpedo* ACh receptor was injected with Freund's complete adjuvant (1:1 v/v) subcutaneously along the back of each rabbit. A booster shot of 100 μg was administered after 2 weeks. The Ouchterlony double diffusion reaction was used in 1% agar gel in 50 mM Tris buffer, pH 7.4. The rabbit was sacrificed after a month and the serum fraction collected and stored at −20 °C until use. Control serum was collected from a rabbit injected with Freund's complete adjuvant only.

The immunoglobulin fraction was isolated from 5 mL of rabbit antisera by ammonium sulfate precipitation and the salts were removed by dialysis against a buffered saline solution (0.01 M Na_2HPO_4–154 mM NaCl, pH 7.4). The final product contained 10 mg of protein per mL.

Results

Gel filtration of the highly pigmented, but clear, S_1 of housefly brain on Sephadex G-200 separated the pigments in fractions above 600 mL from most of the proteins (Figure 1). Although there was more than one peak of ACh-esterase, they

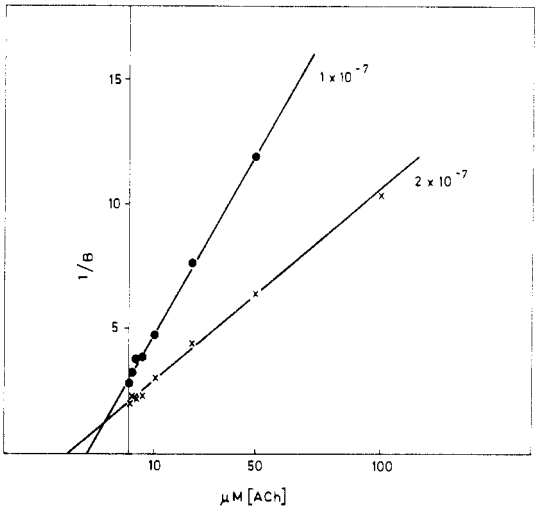

FIGURE 2: Dixon plot of the binding of 10^{-7} M (●) or 2×10^{-7} M (X) [^{3}H]decamethonium S_2 in presence of increasing concentrations of ACh-B, bir ding in nmol per mg of protein.

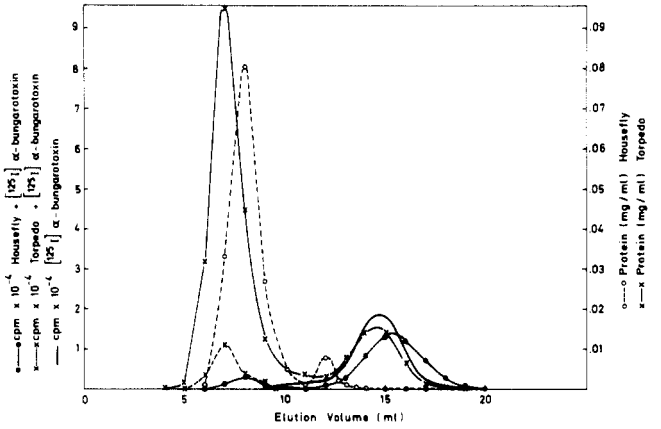

FIGURE 3: Column chromatography on CM-50 of [^{125}I]-α-BGT alone (—), after 1-h incubation with housefly S_2 (○) or purified *Torpedo* ACh receptor (X). Protein (- - - -) and radioactivity (—) are monitored. Radioactivity in the peaks on the right represents unbound [^{125}I]-α-BGT.

TABLE I: Effect of Cholinergic Drugs and Neurotoxins on the Binding of [^{3}H]Nicotine (10^{-7} M) and [^{3}H]Decamethonium (10^{-7} M) to the S_2 of Housefly Brain Measured by Equilibrium Dialysis.

Drug or toxin (10^{-5} M)	% blockade of binding[a] [^{3}H]-Decamethonium	[^{3}H]Nicotine
Acetylcholine	5.4 ± 1.2[b]	7.0 ± 1.5[b]
DFP	0	0
Acetylcholine + DFP	53.3 ± 0.7	54.6 ± 0.8
Carbamoylcholine	35.5 ± 6.0	64.8 ± 7.0
Succinylcholine	54.0 ± 8.2	
Benzoylcholine	75.2 ± 7.1	
Nicotine	50.0 ± 3.3	
Decamethonium		98.9 ± 4.4
d-Tubocurarine	40.4 ± 7.2	
PAPTA[c]	83.9 ± 3.5	
Atropine	80.3 ± 10.4	72.1 ± 4.1
Pilocarpine	83.5 ± 0.9	78.2 ± 0.5
Scopolamine	74.8 ± 5.9	
Arecoline	52.9 ± 3.1	
Isopropamide	51.5 ± 7.7	
Hexocyclium methylsulfate	90.2 ± 8.9	
Serotonin	0	
α-Bungarotoxin[c]	0	0
Cobra toxin[c]	0	0

[a] Blockade values are the means of three experiments ± standard deviations. Zero values are used when there is no significant change in binding ($p < 0.05$). [b] Since ACh-esterase is present in this S_2 preparation, in the absence of ACh-esterase inhibitor such as DFP (K_D for ACh + DFP is 10 μM), all ACh should be hydrolyzed and the low blockade observed is suggested to be due to the effect of choline. [c] All drugs are added to the dialysis bath with the radiolabeled ligand, but in the case of the neurotoxins they are added in addition to the S_2 preparation, at the final concentration stated, then after 60 min at 23 °C dialysis is started. Final concentrations of toxins used are 2.5 μM for α-BGT and 2.8 μM for the cobra toxin.

separated from the peak of the putative ACh receptors, whose binding of [^{3}H]decamethonium was used to identify them in vitro. Decamethonium was shown to be specific for the housefly brain putative ACh receptors in S_1, by binding with the highest affinity to the molecules that also bound atropine, nicotine, and muscarone (Eldefrawi et al., 1971a). The peak fraction (at 490 mL) bound an average of 1000 pmol of [^{3}H]decamethonium (at 0.1 μM) per mg of protein. When binding of [^{3}H]nicotine (0.1 μM) and [^{3}H]atropine (0.01 μM) was studied, they bound to the same fractions (435–535 mL) that bound [^{3}H]decamethonium.

Although S_1 was clear, after gel filtration turbidity appeared in the early fractions that bound cholinergic ligands. Therefore, the 10 peak fractions (at 465–510 mL) were regularly pooled and centrifuged at 100 000*g*, 1 h. The resultant supernatant (S_2) contained about 90% of the cholinergic binding molecules present before centrifugation.

Maximum binding of [^{3}H]decamethonium (at concentrations from 1 nM to 1 μM) to S_2, determined by equilibrium dialysis, and a Scatchard (1949) plot, ranged from 0.6 to 1.5 nmol/mg of protein in 35 preparations. There was a single affinity for binding and K_D was calculated to be 0.15 μM. ACh, in presence of 10 μM DFP (to inhibit ACh-esterase), competitively blocked [^{3}H]decamethonium as judged by the Dixon plot (Figure 2). Since the point of intersection of the two lines obtained with two ligand concentrations equals $-K_i$ (Dixon, 1953), then K_i for ACh was calculated to be 10 μM. Since [^{3}H]decamethonium binding to S_1 was 40 pmol/mg of protein and 1000 pmol/mg of protein to the peak fraction of the S_1 preparation, then gel filtration achieved 25-fold purification.

In order to determine the specificity of [^{3}H]decamethonium binding to S_2, the effect of a variety of drugs on this binding was studied. All cholinergic drugs tested, nicotinic or muscarinic, blocked the binding of [^{3}H]decamethonium and [^{3}H]nicotine to S_2 (Table I). This agrees with previous findings on S_1 (Eldefrawi et al., 1971a). Neither α-BGT nor cobra toxin, which bind specifically to vertebrate nicotinic neuromuscular receptors (Lee, 1972; Miledi et al., 1971), blocked the binding of [^{3}H]decamethonium or [^{3}H]nicotine to S_2. Since α-BGT might be binding to the molecule without affecting its binding of cholinergic drugs, we studied direct binding of [^{125}I]α-BGT to S_2 by gel filtration on CM-50. In this method, the proteins and protein-toxin complex were eluted in fractions 5–10 mL, while the free toxin was retained in the column and the excess eluted in 12–20 mL (Figure 3). The small amount of [^{125}I]-α-BGT that bound to S_2 proteins (Figure 3) is equivalent to 1 pmol/mg of protein, while this S_2 preparation bound 1000 pmol/mg of protein of [^{3}H]decamethonium.

In order to determine the degree of purity of S_2 and to correlate the cholinergic binding molecules with certain proteins, several fractions eluting off the G-200 column (Figure 1) were

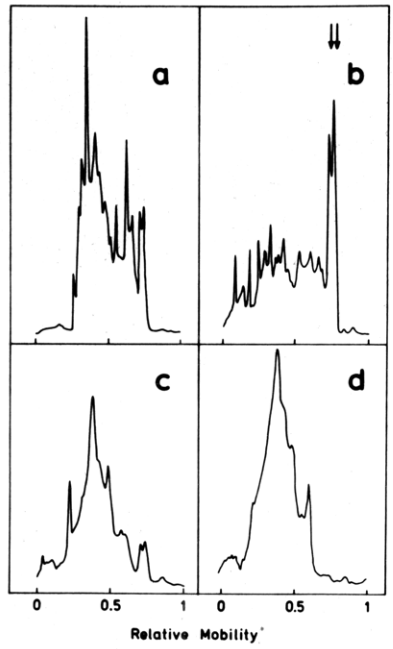

FIGURE 4: Scans of disc gel electrophoresis of (a) S_2; (b) fraction at 490 mL in Figure 1, which exhibits maximum binding of cholinergic ligands. Arrows point at the two protein bands suspected of being ACh-receptors. (c) Fraction at 525 mL in Figure 1. (d) Fraction at 550 mL in Figure 1, which does not bind cholinergic ligands.

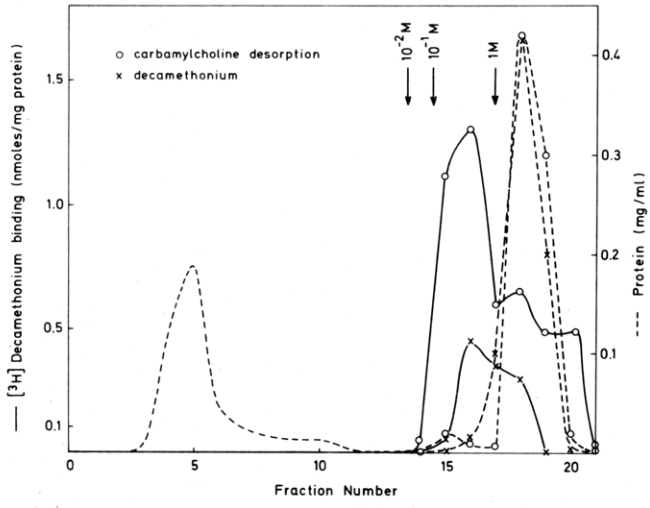

FIGURE 5: Affinity chromatography of S_2 of housefly brain on Sepharose 4B to which PAPTA is covalently attached. After adsorption of S_2 on the gel; passage of 90 mL of 10 mM Na_2HPO_4 solution (pH 7.2) elutes the protein (- - - -) peak on left, which is nonspecific. When either carbamoylcholine (O) or decamethonium (X) is added to the column at the concentrations and points indicated by the arrows, the protein peaks on the right are eluted (O - - - O and X - - - - X, respectively). When each fraction is dialyzed to remove the drug and then assayed for binding of [^{3}H] decamethonium by equilibrium dialysis (O — O and X — X, respectively), various fractions exhibit binding.

assayed by disc gel electrophoresis. S_2 contained many protein bands and was evidently impure (Figure 4a). The fraction at 490 mL, exhibiting maximum binding of [^{3}H]decamethonium, had two fast migrating bands which represented about 30% of the proteins (Figure 4b). These two bands were much smaller in the fractions at 525 mL, which exhibited low binding of [^{3}H]decamethonium (Figure 4c), and were absent in fraction 550 mL, which did not bind any (Figure 4d). This pattern suggested that the two protein bands may be the ones associated with decamethonium binding, and thus may be the putative ACh receptors.

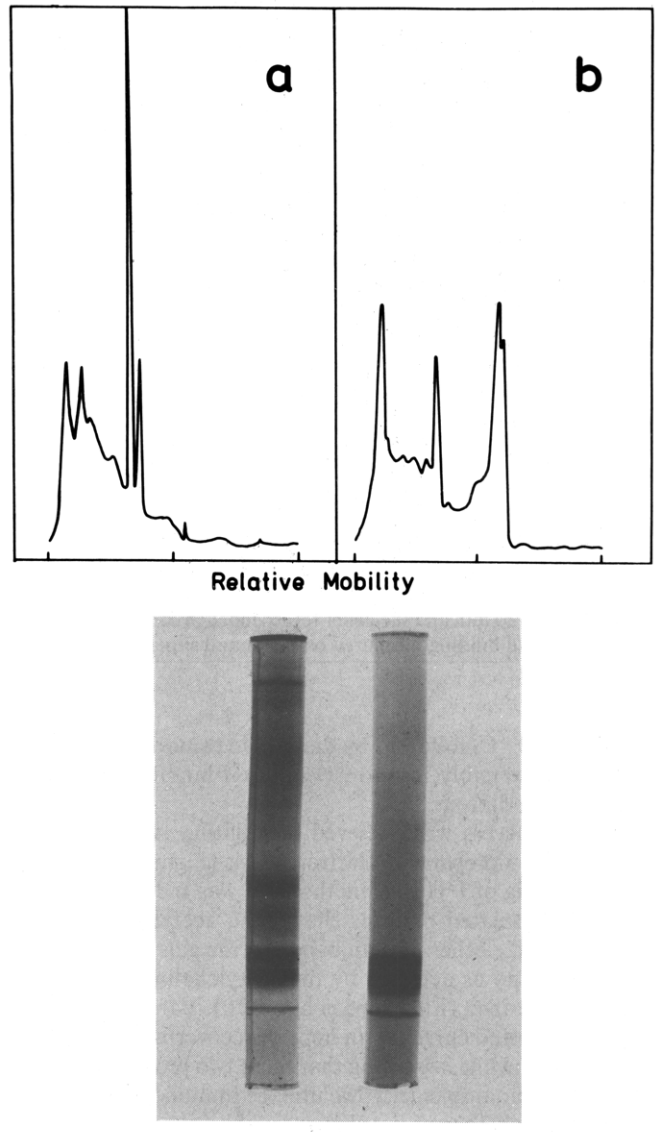

FIGURE 6: Purity of various isolated proteins. (Top) Scans of disc gel electrophoresis of fractions isolated by affinity chromatography. (a) Fraction No. 5 in Figure 5, which does not bind [^{3}H]decamethonium; (b) fraction eluted with 0.1 M decamethonium (No. 17 in Figure 5). (Bottom) Disc gel electrophoresis of S_2 (left gel) and the two housefly brain proteins isolated by semipreparative electrophoresis (right gel). Start is at top and lowest band in both gels is tracking dye.

We attempted to purify these cholinergic binding molecules with affinity chromatography. The cobra toxin affinity gel, which was successful in purifying the *Torpedo* ACh receptor, did not adsorb these molecules. Thus, we used an affinity gel where the end group was PTA. All the cholinergic binding molecules were adsorbed and washing the gel column with 10 mM phosphate buffer desorbed some nonspecific proteins (fractions 3–11 in Figure 5). A discontinuous gradient (0.01, 0.1, and 1 M) of either carbamoylcholine or decamethonium desorbed increasing amounts of protein (fractions 14–20). The highest specific binding was obtained in fractions eluted with 0.1 M solutions of either drug. Polyacrylamide gel electrophoresis revealed that affinity chromatography purified the fast migrating proteins (Figure 6, bottom, compared with Figure 4a), but there were still impurities. Nevertheless, specific binding was unchanged when decamethonium was the desorbing ligand and was increased only twofold when carbamoylcholine was used. This led to the discovery that the simple presence of a high concentration (0.1 M) of decamethonium or carbamoylcholine with the housefly brain proteins

TABLE II: Effect of Rabbit Antiserum against the Isolated Housefly Putative ACh Receptors on the Binding of [^{3}H]Atropine and [^{3}H]Decamethonium to Housefly S_2.

Sample[a]	Bound ligand (pmol/mL) [^{3}H]-Atropine (0.5 μM)	[^{3}H]-Decamethonium (0.1 μM)
Ringer	0	0
S_2 + Ringer	51.6 ± 6	279.8 ± 6
Control serum + Ringer	60.2 ± 3	20.4 ± 3
Antiserum against housefly proteins + Ringer	46.1 ± 2	14.9 ± 3
S_2 + control serum	109.2 ± 7	263.2 ± 4
S_2 + antiserum against housefly proteins[b]		
Supernatant	64.0 ± 2	15.6 ± 5
Pellet	17.2 ± 1	261.0 ± 6

[a] Incubation mixtures were kept at 4 °C for 16 h. [b] The S_2 + antiserum against housefly proteins was the only mixture with a visible precipitate at the end of the incubation period; thus it was centrifuged at 9000g for 30 min and the pellet resuspended in the original volume of Ringer and binding measured on pellet and supernatant.

of 24 h at 4 °C, followed by dialysis to remove the drug, inhibited irreversibly 70% to 90% of their binding detected by equilibrium dialysis.

Better success was achieved in isolating the two protein bands by semipreparative electrophoresis (Figure 6 lower), but their binding of [^{3}H]decamethonium was reduced by about 90%. The isolated proteins showed no acetylcholinesterase activity, while other protein bands on the gels had strong esterase activity as detected by the acetylcholine method previously described (Eldefrawi et al., 1970). Various conditions are being tested currently in hope of recovering their full activity. Meanwhile, assuming that these two protein bands had retained their antigenicity, we utilized immunological tests to determine whether or not they were the ones responsible for binding cholinergic drugs in S_2. Incubation of housefly S_2 with immunoglobulins, against the two protein bands, for 16 h at 4 °C resulted in precipitation of the cholinergic ligand binding proteins (Table II). As an index, we used binding, measured by equilibrium dialysis, of [^{3}H]decamethonium and [^{3}H]-atropine to control rabbit serum, antiserum against purified housefly proteins, Ringer solution, S_2 fraction, and mixtures (1:1, v/v) of them. Although all of the [^{3}H]decamethonium binding molecules in S_2 precipitated by the antibodies, the complex retained all of its binding. However, only about 30% of the [^{3}H]atropine binding to S_2 was detected in the precipitate, with possibly about another 30% remaining in the supernatant fraction. This fraction of atropine binding may be to S_2 proteins other than the putative ACh receptors. It is not unexpected, because atropine is less specific than decamethonium under these circumstances as seen from its high binding to sera proteins, binding that may be even higher than to S_2 proteins (Table II). Repetitive washing of the precipitated complex (S_2 + antiserum against the purified housefly proteins) did not reduce its binding of [^{3}H]decamethonium. In double diffusion tests, a sharp strong line appeared between the well containing the rabbit antisera against the two isolated housefly proteins and the wells containing either the pure housefly proteins or S_2 (Figure 7a).

The availability of antibodies against the nicotinic ACh receptor from the electric organ of *T. ocellata* allowed us to compare it with the housefly putative brain ACh receptors. In

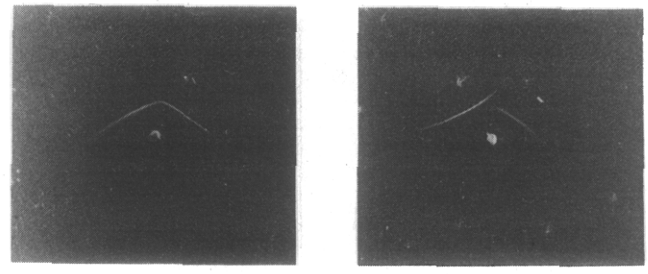

FIGURE 7: Double diffusion assay of (left) rabbit antisera for the two isolated housefly proteins (bottom well) against the two isolated housefly proteins (top left well) and S_2 (top right well); and (right) rabbit antisera for the purified *Torpedo* ACh receptor (bottom well) against the purified *Torpedo* ACh receptor (top left well) and the two isolated housefly proteins (top right well).

the double diffusion test, no lines appeared between the purified *Torpedo* ACh receptor and rabbit antisera against the purified housefly proteins, nor between the antisera to the purified *Torpedo* receptor and the purified housefly brain proteins. On the other hand, sharp lines developed between the well containing a 1:1 mixture of antisera against the pure *Torpedo* and housefly proteins and the two wells containing their respective antigens (Figure 7b). However, these lines did not connect smoothly, suggesting that they have different antigenic determinants. Incubation of antisera against the purified *Torpedo* receptor protein with housefly S_2 resulted in no precipitate and no change in [^{3}H]decamethonium binding.

Discussion

The supernatant of 100 000g of housefly heads contains protein molecules, which bind decamethonium, nicotine, muscarone, atropine, and *d*-tubocurarine with K_D in the μM range (Eldefrawi and O'Brien, 1970; Eldefrawi et al., 1971a). The K_i of 10 μM we obtained for ACh (in presence of DFP), through its inhibition of [^{3}H]decamethonium binding to the putative housefly brain ACh receptors (Figure 2), is identical with the K_D for ACh (in presence of eserine) calculated from electrophysiological studies of synaptic transmission in the sixth abdominal ganglion of the cockroach (Sattelle, 1977). These macromolecules are purified 25× by gel filtration (Figure 1), while retaining their high affinity binding of cholinergic drugs. In addition, they are also shown to bind ACh with a K_i of 10 μM (Figure 2). Reliance on binding of a single cholinergic drug as a specific label for an ACh receptor can easily be misleading, even if the binding is of a high affinity (O'Brien et al., 1972; Eldefrawi, 1976). An example is [^{14}C]dimethyl-*d*-tubocurarine, which binds to 7–10 more binding sites in the housefly brain extract than those that bind the other cholinergic drugs (Eldefrawi et al., 1971a). In the case of electric organs of fish, [^{3}H]decamethonium binds not only to the ACh receptors, but also to ACh-esterase and possibly other components of the tissue. The same is true for norepinephrine receptors (Cuatrecasas et al., 1974). Therefore, the finding that the housefly brain macromolecules bind all the above cholinergic drugs and ACh with relatively high affinities, and the inhibition of this binding by all the nicotinic and muscarinic drugs tested but not the snake venom neurotoxins (Table I), suggests that they may be ACh receptors of mixed nicotinic muscarinic pharmacology.

The data point to differences between the nicotinic ACh receptors of fish electric organs and the housefly brain putative ACh receptors. First is the apparent in vitro lower affinity of the latter for cholinergic drugs and ACh (Eldefrawi and

O'Brien, 1970; Aziz and Eldefrawi, 1973), unless we consider the hypothesis that the in vitro higher affinity binding of ACh and activators observed to electric organ ACh receptors is due to in vitro desensitization of these receptors (Weber et al., 1975). The lack of effect on [^{3}H]decamethonium or [^{3}H]-nicotine binding to the housefly S_2 fraction by α-BGT or the specific neurotoxin of *N. naja siamensis* venom (Table I), and the apparent absence of their direct binding of [^{125}I]α-BGT (Figure 3), point to additional differences. These neurotoxins bind specifically to the nicotinic neuromuscular ACh receptors and those of electric organs (Miledi et al., 1971; Lee, 1972). The possible lack of binding of α-BGT to the housefly brain proteins agrees with the findings of Donnellan et al. (1975), who could not inhibit [^{3}H]decamethonium binding to the housefly head proteolipids with α-BGT. Conversely, [^{125}I]-α-BGT binding was observed to moth antennal lobe homogenate (Sanes et al., 1977) and it was autoradiographically localized in the neuropil regions of *Drosophila* nervous system (Hall and Teng, 1976). Although the ACh receptors of electric organs have nicotinic neuromusclar-type pharmacology (Hall, 1972; O'Brien et al., 1972; Raftery et al., 1974), the putative ones of the housefly brain have nicotinic–muscarinic pharmacology (Table I). These protein molecules also appear to differ in antigenic determinants since the antisera against the purified housefly brain proteins do not recognize the *Torpedo* ACh receptor, and antisera against the latter do not recognize the housefly brain proteins (Figure 7). The housefly receptors are also more labile and lose their cholinergic binding capacity easily similar to the muscarinic cholinergic receptors of mammalian brain (Birdsall and Hulme, 1976). Since the putative ACh receptors of only three tissues of three insect species have been studied biochemically, we cannot generalize its characteristics to other insect tissues and species, nor can we guarantee that the differences observed above would extend to other nicotinic ACh receptors.

Affinity chromatography partially purified these housefly cholinergic binding molecules (Figures 5 and 6), but semi-preparative electrophoresis totally purified two protein bands as judged by disc gel electrophoresis (Figure 6, lower figure). The isolated proteins could still bind 100 pmol of [^{3}H]decamethonium per mg of protein ($\simeq$10% of their original binding capacity) but lacked acetylcholinesterase activity. This compares with 12 pmol of [^{125}I]-α-BGT bound per mg of protein for the nicotinic ACh-receptor of rat brain partially purified by affinity chromatography (Salvaterra and Mahler, 1976). The identity of the two pure proteins with the cholinergic binding molecules of S_2 is confirmed immunologically (Figure 7, left). Furthermore, the binding of [^{3}H]decamethonium to incubation mixtures of S_2 and antibodies against these two proteins is totally eliminated from solution and becomes associated with a stable antigen–antibody complex. The finding that none of the [^{3}H]decamethonium binding is inhibited in the antigen–antibody precipitate, while some of the [^{3}H]atropine binding is (Table II), raises the possibility that decamethonium and atropine bind to different sites on the putative ACh receptor. Similar observations have been made on the binding sites of agonists being different from antagonists of the ACh receptors of electric organs (Bulger et al., 1977; Fu et al., 1977; Eldefrawi and Eldefrawi, 1977).

Because of the proximity of the two protein bands, it is extremely difficult to separate them by electrophoresis. Since [^{3}H]nicotine and [^{3}H]decamethonium binding by S_2 is blocked by atropine (Table I), it is unlikely that one protein is a nicotinic receptor and the other a muscarinic one. However, we cannot discount the possibility that one protein is a by-product of the other appearing during purification, nor can we discount that only one of the two is the ACh receptor or both make one receptor molecule in the membrane. This should be resolved in future studies by separating the two bands, making antisera to each one and determining whether one or both is responsible for cholinergic drug binding. The possibility that bromophenol blue, the tracking dye used in electrophoresis, might react with the receptor during electrophoresis producing multiple forms, as was found with enzymes (Hiebert et al., 1972), was excluded because we obtained two separate bands whether or not bromophenol blue was mixed with the proteins.

Comparing the brain ACh receptors of the housefly with those of the rat, we find that the former solubilize more easily, have relatively lower affinity for cholinergic ligands (Eldefrawi and O'Brien, 1970; Schleifer and Eldefrawi, 1974) and ACh (Figure 2), and have mixed muscarinic–nicotinic pharmacology (Table I), similar to a few ACh receptors in mammalian brain stem (Bradley and Wolstencroft, 1965) and the thalamus (McCance et al., 1968). α-Bungarotoxin binding macromolecules have been detected in rat brain, and because of their nicotinic pharmacology have been identified as ACh receptors. However, there is no physiological evidence to support the hypothesis that α-bungarotoxins bind to a postsynaptic neurotransmitter receptor in mammalian central nervous system (Schmidt, 1977). The putative ACh receptors of the antennal lobes of the moth, *Manduca,* detected by α-BGT binding, appear to have mixed pharmacology (Sanes et al., 1977) similar to our present finding for the putative housefly ACh receptors. In the absence of enough electrophysiological data on the effect of drugs on insect brain ACh receptors and in view of the finding that ACh receptors of the cercal nerve giant fibre synapse of the American cockroach may be nicotinic, muscarinic, or both (Flattum and Shankland, 1971), it may be that the mixed nicotinic–muscarinic pharmacology of the housefly brain ACh receptors is characteristic of the housefly brain or possibly the insect central nervous system in general.

An important problem to resolve is how an ACh receptor, which is believed to be an intrinsic membrane protein, can be dissolved by homogenization in water. It may be that in the housefly brain it is an extrinsic membrane protein or possibly that detergent-like constituents are present in fly head tissues, which solubilize intrinsic proteins. Chromatography of S_1 on Sephadex G-200 resulted in aggregation of molecules, including some of the cholinergic binding ones, which necessitated their removal by centrifugation (see Methods). Another putative ACh receptor, also from an invertebrate, the squid, *Loligo opalescens,* has been solubilized simply by homogenization, but unlike the housefly receptor it bound α-BGT significantly (Kato and Tattrie, 1974). In one case the ACh receptor of the electric eel was solubilized without detergents by extended dialysis of the membrane against a low ionic strength buffer followed by tryptic digestion. The water-soluble and the detergent-solubilized ACh receptors shared some of their antigenic determinants, but the former aggregated to much larger molecular species (Aharonov et al., 1975).

There is a good possibility that the two proteins isolated presently are ACh receptors. If so, it would represent a first for an isolated ACh receptor in several aspects: a purified brain receptor, a muscarinic–nicotinic receptor that does not bind α-BGT, and a purified receptor that is free of added detergents.

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