Biochimica et Biophysica Acta, 318 (1973) 69–77

BBA 76410

QUANTITATION OF ACETYLCHOLINESTERASE AND ACETYLCHOLINE-BINDING SITES IN EXCITABLE MEMBRANE FRAGMENTS FROM ELECTRIC EEL

JANUSZ B. SUSZKIW*

Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850 (U.S.A.)

(Received December 18th, 1972)

(Revised manuscript received March 13th, 1973)

SUMMARY

1. Titration of membrane-bound acetylcholinesterase with a specific active-site reversible inhibitor 3-hydroxyphenyltrimethylammonium iodide allows a quantitative determination of active site concentration of the enzyme in the membrane fragments from electroplax of the electric eel.

2. Extraction of acetylcholinesterase activity from membrane fragments by 1 M NaCl results in an enzymatically inactive preparation which is still capable of binding acetylcholine.

3. The quantity of acetylcholine binding sites is about 16–20% of the catalytic sites of the enzyme in the electroplax membrane fragments, in agreement with results obtained with monocellular electroplax preparation. The K_D for the acetylcholine-receptor complex is $1.8 \cdot 10^{-7}$ M.

INTRODUCTION

Identification and isolation of cholinergic receptor macromolecules has received considerable attention in the last few years. A severe drawback in this type of study is the difficulty in utilizing natural neurotransmitter acetylcholine as marker ligand in equilibrium binding experiments. This situation persists because of high concentrations of acetylcholinesterase in the excitable membrane preparations.

The use of stable agonists such as decamethonium or nicotine introduces a complication in the lack of absolute specificity of these compounds. This problem is well exemplified by the results of Eldefrawi *et al.*[1] who find several decamethonium binding sites present in the homogenates of electric tissue.

Changeux and co-workers[2,3] in their extensive studies attempted to resolve the problem of identification of receptor macromolecule by using neurotoxins, α-bungarotoxin from *Bungarus multicinctus*, or the α-toxin from *Naja Nigricollis*,

Abbreviation: 3-HOPTA, 3-[^{3}H]hydroxyphenyltrimethylammonium iodide.

* Present address: Biological Sciences Group, Regulatory Biology Section, U-42, University of Connecticut, Storrs, Conn. 06268, U.S.A.

which are assumed to interact specifically with the receptor; the rationale being that all of the ligand displaced by the toxin is that which specifically interacts with the acetylcholine receptor. The toxin non-displaceable decamethonium was assumed to represent the binding to the acetylcholinesterase.

An entirely different approach to identification and isolation of the receptor was taken by De Robertis and co-workers[4,5] who removed acetylcholinesterase by salt extraction and subsequently extracted a proteolipid which has acetylcholine-binding properties.

As pointed out by Hall[6] in his recent review of the subject there are a number of both qualitative and quantitative discrepancies between the findings of the various groups and a question is if all have identified the same molecule. In this paper we report a quantitation of the catalytic sites of membrane-bound acetylcholinesterase as well as the acetylcholine-binding sites which are physically separable from the enzyme. In contrast to acetylcholinesterase, the acetylcholine-binding macromolecules form a part of the lipoprotein membrane matrix. The enzyme can be characterized as an extrinsic protein, as it can be completely extracted from the membrane by 1 M NaCl. The ratio of the acetylcholine-binding sites to the esteratic sites of cholinesterase is about 0.2

MATERIALS AND METHODS

All experiments were performed in physiological eel Ringer solution, pH 7.0 and $\mu = 0.186$ (Keynes and Martins-Ferreira[7]) containing: 169 mM NaCl, 5 mM KCl_2, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4.

Eel acetylcholinesterase (EC 3.1.1.7), Grade ECHP (Worthington Biochemical Co., Freehold, N.J., U.S.A.) had specific activity of approximately 70 mmoles of acetylcholine hydrolyzed/h per mg protein.

The excitable membrane fragments were prepared from the electric organ of *Electrophorus electricus* essentially according to the method of Changeux *et al.*[8]. The electric organ was divided into 20 g sections and the tissue was finely minced in 50 ml of cold 0.2 M sucrose solution. After 1.5 min homogenization at the highest speed on the Virtis homogenizer, the preparation was sonicated for 1 min at 4–5 A setting on Branson sonicator. The preparation was then centrifuged at 4 °C for 20 min at 6500 rev./min ($8000 \times g$) in the Sorvall SS-34 rotor. The sedimented debris was discarded and the supernatant was centrifuged on a discontinuous sucrose density gradient in a Beckman SW-25.2 or SW-27 rotor for 7–10 h at an average $105000 \times g$. The sucrose gradient consisted of 5 ml of 1.5 M sucrose, 5 ml of 0.4 M sucrose and a suspension of membrane fragments in 0.2 M sucrose. The acetylcholinesterase-rich fragments which had concentrated at the interface between the 1.5 M and 0.4 M sucrose layers were collected and diluted with eel Ringer solution to bring the sucrose concentration to 0.2 M. The suspension was then centrifuged for an additional 1 h at $100000 \times g$ at 4 °C. The supernatant containing a fraction of the solubilized enzyme and other contaminating proteins was discarded. The sediment was resuspended in cold Ringer solution with a Dounce No. 23 hand homogenizer and the preparation was briefly (10 s) sonicated. This preparation was either further equilibrated against Ringer solution by dialysis or was used directly in equilibrium binding experiments. The specific activity of the membrane-bound enzyme was determined by the method of Ellman *et al.*[9] with $5 \cdot 10^{-4}$ M acetylthiocholine in 0.05 M

sodium phosphate buffer, pH 8, containing $5 \cdot 10^{-4}$ M 5,5′-dithiobis-(2-nitrobenzoic acid). Extraction of membrane-bound acetylcholinesterase was accomplished by successive washing of the membrane preparation with 1 M NaCl (ref. 10). In general from 100 to 200 mg of membrane protein was suspended in 50 ml of salt solution at 4 °C and was gently stirred from approximately 0.5 h. The suspension was then centrifuged for 1 h at $100000 \times g$ in a refrigerated ultracentrifuge. The pelleted material so obtained consisted of membrane fragments with the enzyme partially removed. Depending on the degree of enzyme extraction required the above steps were repeated, usually up to 3 times. After sufficient extraction of the enzyme activity was achieved, the membrane pellet was resuspended in a desired volume of cold Ringer solution by means of the hand homogenizer and was briefly sonicated as described previously. The preparation was then dialysed against Ringer.

For binding of acetylcholine experiments the enzyme-depleted membrane which still showed some activity was inhibited by dialysing it against 0.01 M solution of *N*-methylpyridinium-3-*O*-methanesulfonate iodide for 4 h, followed by dialysis against 3 changes of Ringer solution to remove completely the sulfonating reagent. The soluble enzyme preparation was methanesulfonated in the same way.

Equilibrium binding of 3-[^{3}H]hydroxyphenyltriemethylammonium iodide (3-HOPTA) to the soluble acetylcholinesterase and to the membrane fragments was carried out for 9 h at 25 °C when the enzyme was used and at 4 °C when the membrane fragments were used. Specially constructed microcells consisting of two 80-μl compartments separated by a Visking dialysis tubing were used. Equilibrium binding experiments using acetylcholine were carried out for 9 h using a large excess of ligand solution. A typical vessel contained 100 ml of acetylcholine in Ringer and 0.15 ml of membrane preparation enclosed in a 1 cm $\times$ 15 cm dialysis bag. For construction of binding curves 20-μl samples from both the free and protein-containing compartments were withdrawn in duplicates and were counted for radioactivity. Protein concentrations were determined at the same time by the method of Lowry *et al.*[11].

All chemicals were reagent grade, unless indicated otherwise. 3-HOPTA was prepared by methylation of *N*,*N*-dimethyl-3-hydroxyaniline (Eastman Organic Chemicals, Rochester, U.S.A.) followed by catalytic tritiation at New England Nuclear Co. (Boston, U.S.A.), as described previously[12]. The specific radioactivity of the compound was 229 Ci/mole. The radiopurity was 98% as determined by paper chromatography. Methanesulfonatepyridinium iodide was prepared according to a published procedure[13]. Acetyl-[^{3}H]choline chloride, specific radioactivity 250 Ci/mole and purity 97% was obtained from Amersham/Searle Corporation (Arlington Heights, Ill. U.S.A.). Acetylthiocholine bromide and Neostigmine bromide were from Sigma Chemical Co. (St. Louis, U.S.A.).

5,5′-Dithiobis-(2-nitrobenzoic acid) was from Calbiochem (Palo Alto, U.S.A.). Radioactivity was counted on a Packard Scintillation counter at 35–40% counting efficiency. All results were evaluated by the least squares method.

RESULTS

The number of active sites of acetylcholinesterase is usually calculated from the determination of specific activity of a preparation. Such calculations are based on two assumptions: (1) that the specific activity of pure enzyme and, (2) that its molecular

structure are well defined. However, in the case of acetylcholinesterase none of these have been established unequivocably. In addition, the determination of specific activities of membrane-bound enzyme, done at relatively high dilutions, may lead to an erroneous interpretation of actual enzyme concentration in highly concentrated membrane preparations that are normally used for equilibrium binding experiments.

Fig. 1 illustrates that the concentration of acetylcholinesterase can be determined by equilibrium binding of a specific reversible inhibitor of the active site, 3-hydroxyphenyltrimethylammonium ion (3-HOPTA). It is seen that 1 mole of 3-HOPTA binds per mole of active sites as determined by Tetram (Tetram is *O,O*-diethyl-*S*-2-diethylaminoethylphosphorothiolate) titration[14]. The binding of 3-HOPTA is completely abolished by methanesulfonation of the enzyme with *N*-methylpyridinium-3-*O*-methanesulfonate iodide which reacts irreversibly with the active site of the enzyme[15]. This indicates that 3-HOPTA binds specifically to the catalytic site of acetylcholinesterase.

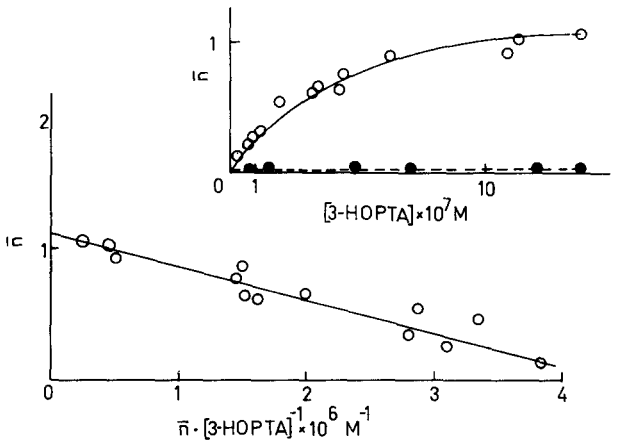

Fig. 1. Scatchard plot of binding of 3-hydroxyphenyltrimethylammonium iodide to the active and methanesulfonated, soluble acetylcholinesterase. $\bar{n}$ is the number of moles of 3-HOPTA bound per mole of active sites of the enzyme. The inset is a Langmuir adsorption isotherm for 3-HOPTA binding to the active (○) and the inhibited enzyme (●). Acetylcholinesterase (Worthington, Grade ECHP, spec. act. approx. 70 mmoles acetylcholine hydrolysed/h per mg protein) was used at concentrations from $1 \cdot 10^{-7}$ to $5 \cdot 10^{-6}$ M. The equilibrium concentrations of the ligand varied from $5 \cdot 10^{-8}$ to $5 \cdot 10^{-6}$ M. The solid lines are computed by the weighted least squares method (Wilkinson[25]). The dissociation constant, K_D, obtained from the slope, is $2.5 \cdot 10^{-7} \pm 0.2 \cdot 10^{-7}$ M, and $\bar{n} = 1.1 \pm 0.03$.

The concentration of acetylcholinesterase determined by equilibrium binding of 3-HOPTA reflects the actual number of active sites and is independent of assumptions with regard to the number of active sites per molecule of acetylcholinesterase or its specific activity. Table 1 and Fig. 2 show the results of titration of the membrane bound enzyme with 3-HOPTA. It can be seen that the amount of HOPTA titratable sites varies linearly with activity of the preparation. Removal of the enzyme by salt extraction or inhibition of the enzyme by methanesulfonation or phosphorylation results in parallel decrease in the assayable activity and total binding of 3-HOPTA. The correlation factor obtained from the slope of line in Fig. 2, is $2.9 \cdot 10^{-8} \pm 0.1 \cdot 10^{-8}$

TABLE I

TITRATION OF MEMBRANE-BOUND ACETYLCHOLINESTERASE WITH 3-HOPTA IN EEL RINGER SOLUTION, pH 7.0

Each preparation represented is from a different eel. The binding of 3-HOPTA was carried out as described in the text using a minimum of six HOPTA concentrations ranging from $6 \cdot 10^{-8}$ to $2 \cdot 10^{-6}$ M. The amount of ligand bound and the K_D values were obtained from Scatchard plots calculated by the weighted least squares method of Wilkinson[25]. The specific activity of each preparation was determined with acetylthiocholine as substrate by the method of Ellman *et al.*[9], as described in the text. The binding experiments were done in physiological eel Ringer solution, pH 7.0, at 4 °C. The k_{cat} for the membrane bound enzyme was obtained by dividing the specific activity of a preparation by the number of 3-HOPTA titratable sites.

Preparation No.	$10^{11} \times$ *moles HOPTA bound per mg protein*	$K_D \times 10^7$ *M*	*Specific activity mmoles acetylthiocholine hydrolysed per h per mg protein*	$k_{cat} \times 10^{-4}$ s^{-1}
2	12.7 ± 0.1	1.4 ± 0.02	3.85	0.9
5	4.6 ± 0.6	1.6 ± 0.2	1.6	1.0
7	2.3 ± 0.1	1.2 ± 0.2	1.3	1.6
8	9.3 ± 0.3	1.8 ± 1.0	3.7	1.1
9	10.7 ± 0.4	1.0 ± 0.1	3.4	0.9
10	9.5 ± 0.1	2.6 ± 0.7	3.2	0.9
11	5.6 ± 0.4	1.5 ± 0.3	2.1	1.0
11.2	7.1 ± 0.2	1.0 ± 0.1	2.5	1.0
17	8.7 ± 0.2	2.2 ± 0.9	3.0	1.0
15	6.4 ± 0.1	1.0 ± 0.04	2.4	1.0
14	4.4 ± 0.3	1.0 ± 0.2	1.8	1.1
19	9.1 ± 0.6	1.3 ± 0.2	3.1	1.0

h^{-1}. It can serve to calculate the actual concentration of active sites of the membrane-bound acetylcholinesterase from the specific activity of a preparation, without assumptions as to the activity of pure enzyme or its subunit structure. The calculated k_{cat} (Table I) is approximately $1 \cdot 10^4$ s^{-1} for the membrane-bound acetylcholinesterase and is somewhat lower from the value obtained with a purified commercial Worthington preparation of $1.4 \cdot 10^4$ s^{-1} (unpublished results). This could be due to the presence of nonspecific cholinesterases which do not or less efficiently hydrolyse acetylthiocholine, or to different catalytic efficiency of the membrane-bound enzyme. Scatchard plots of HOPTA binding are linear and do not reveal the presence of heterogeneous sites; nevertheless, the sensitivity of the method might not reveal the presence of sites which could contribute at most 20% to the total HOPTA-binding sites.

Fig. 3 shows that the membrane preparation devoid of cholinesterase can still bind the natural neurotransmitter acetylcholine. The amount of acetylcholine bound per mg of protein is $1.17 \cdot 10^{-11} \pm 0.04 \cdot 10^{-11}$ moles and the dissociation constant is $1.8 \cdot 10^{-7} \pm 0.2 \cdot 10^{-7}$ M. From the specific activity of the starting preparation, 2.05 mmoles/mg per h, it can be calculated that the acetylcholine-binding sites constitute about 20% of the catalytic sites of cholinesterase, without correcting for protein removed by salt extraction.

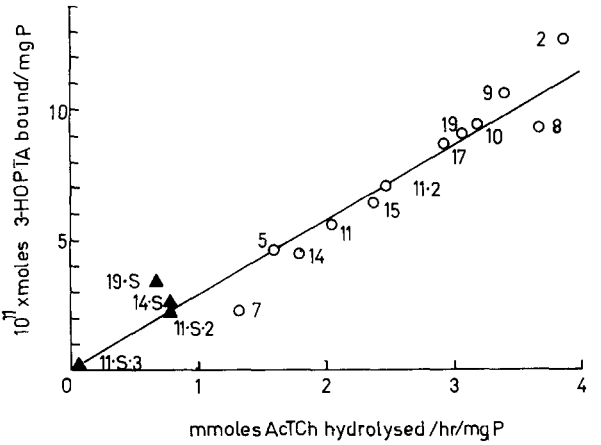

Fig. 2. Correlation curve for the HOPTA-binding sites and the specific activity of excitable membrane fragments, at 4 °C, Ringer solution, pH 7.0. The open circles (○) refer to data obtained with the membrane preparations listed in Table I. The solid symbols (▲) refer to data with membrane preparation from which the enzyme has been partially removed by extraction with 1 M NaCl. The amount of enzyme removed from the salt-extracted membranes is: 11·S·2, 70% depleted; 14·S, 50% depleted; 19·S, 80% depleted; 11·S·3, >90% depleted. The line was computed using $y=mx$ least squares program. The correlation factor which converts specific activity in mmoles acetylthiocholine hydrolysed/mg per h to moles of HOPTA bound/mg, obtained from the slope of the line is $2.9\cdot10^{-8}\pm0.1\cdot10^{-8}$ h^{-1}. The k_{cat} value calculated from the slope of the line is $1.0\cdot10^{4}\pm0.03\cdot10^{4}$ s^{-1}. The equilibrium dialysis experiments were performed as described in the text. The salt extraction of the excitable membranes is described in Materials and Methods. The amount of enzyme removed from the membranes is determined by enzyme assay using acetylthiocholine as substrate. P, protein; AcTCh, acetylthiocholine.

TABLE II

MATERIAL BALANCE OF EXTRACTION OF MEMBRANE FRAGMENTS WITH 1 M NaCl

Preparation	*Specific activity**	*Protein (mg)*	*% original*	*Enzyme activity (Specific activity × mg protein)*	*% origina*
Original membrane preparation No. 21	2.7**	86	100	234	100
Salt extracted membrane	0.8	48	56	37	16
Salt extract	14.0	11	13	160	68
Salt extracted membrane and salt extract		59	69	196	84

* The specific activity was determined with acetylthiocholine as substrate as described in the text and expressed as mM acetylthiocholine hydrolysed/mg protein per h.

** The value obtained refers to the specific activity of the original membrane in 1 M NaCl. The membrane was then separated from the supernatant as described in the text, and the specific activity of the extracted membrane fragment and the 1 M salt extract determined.

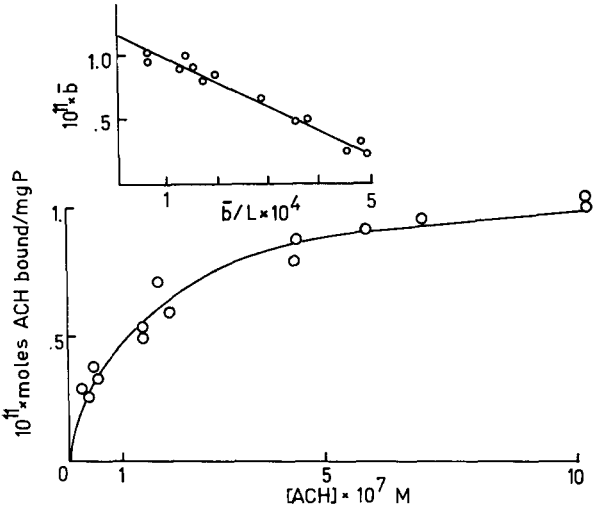

Fig. 3. Binding of acetylcholine to acetylcholinesterase-depleted membrane fragments. Acetylcholine binding to membrane preparation 11·S·3 was carried out by equilibrium dialysis in Ringer solution, pH 7.0 and 4 °C as described in the text. The membrane preparation contained $1.1 \cdot 10^{-19}$ moles of active sites per mg of membrane protein. For the binding experiments 1.1 mg (0.15 ml) of membrane preparation per dialysis bag was used, and the equilibrium dialysis medium contained $5 \cdot 10^{-6}$ M neostigmine bromide. Under these conditions less than 0.1% of the total acetylcholine was hydrolysed in 9 h of equilibrium dialysis at the highest ligand concentration used. The experimental points represent two independent experiments done at ligand concentrations ranging from $0.6 \cdot 10^{-7}$–$14 \cdot 10^{-7}$ M. The inset is a Scatchard plot for acetylcholine binding. $\bar{b}$ is moles of acetylcholine bound per mg of membrane protein; $\bar{b}/L$ is the amount bound divided by the corresponding molar concentration of free acetylcholine. The units are $l \cdot mg^{-1}$. The binding parameters calculated by the least squares method of Wilkinson[25] are: $B_{max} = 1.17 \cdot 10^{-11} \pm 0.04 \cdot 10^{-11}$ moles/mg protein; $K_D = 1.85 \cdot 10^{-7} \pm 0.23 \cdot 10^{-7}$ M. ACH, acetylcholine; P, protein.

Table II shows a representative material balance for the extraction procedure. Of the total 69% of protein recovered, 56% is recovered in the pelleted membrane fraction. This indicates that about 20% of the total protein is solubilized by salt extraction. Since complete extraction of activity is accomplished the acetylcholinesterase in the salt extract should be approx. 5-fold purified if no inactivation occurs. The purification factor indeed is 5.2-fold, indicating that no loss of activity of the enzyme takes place. The losses in total protein and activity are due to mechanical factors and can be reduced to less than 10%. Correcting for the redistribution of protein, the number of acetylcholine sites in the preparation here reported is about 16% of the catalytic sites.

DISCUSSION

By specifically titrating membrane-bound acetylcholinesterase with 3-HOPTA and the acetylcholine-binding sites with acetylcholine in the enzyme-depleted membrane preparation, it is possible to obtain the ratio of the two macromolecular components in the excitable membrane fractions from the eel. The catalytic sites of

the enzyme and the acetylcholine-binding sites are clearly separable confirming the results of the De Robertis and Fiszer de Plazas[5]. The Scatchard plot of acetylcholine binding indicates a single class of sites with which the natural neurotransmitter can form a complex with a $K_D = 1.8 \cdot 10^{-7}$ M. The dissociation constant reported here agrees with the K_D reported by De Robertis *et al.*[4] for the higher affinity site present in a proteolipid fraction of eel electroplax extract. This agreement may be fortuitous since the binding experiments of De Robertis' group were perfomed in chloroform–methanol solvent. The dissociation constant for the acetylcholine-receptor complex as reported in this paper is also comparable to one of the dissociation constants obtained by Eldefrawi *et al.*[16] from the binding of acetylcholine to the electric tissue of *Torpedo*. It is of interest that the K_D for binding of acetylcholine to membrane fragments is an order of magnitude smaller from the electrophysiologically determined K_D apparent for depolarization of electroplax by acetylcholine, which is approx. $2 \cdot 10^{-6}$ M (ref. 17). This discrepancy could be due to change in the accessibility of acetylcholine to the binding macromolecule in the fragmented membrane preparation *in vitro*, or to the possibility that in physiological experiments, even in the presence of eserine a considerable amount of acetylcholine can still be hydrolyzed in the immediate vicinity of the membrane. The latter view is supported by the findings that the K_D values for stable agonists like decamethonium and phenyltrimethylammonium ions obtained from binding experiments *in vitro*, do not differ substantially from the apparent dissociation constants obtained electrophysiologically[18]. The total amount of acetylcholine-binding sites reported in this paper is considerably less than that reported either by De Robertis and coworkers or by Eldefrawi *et al.* It can be calculated from the results here reported that there are approximately 0.009–0.016 nmole of acetylcholine binding sites/g of wet tissue (approximately 80–150 mg of membrane preparation can be obtained from 100 g of tissue by the method of Changeux). This compares favorably with 0.009–0.018 nmole/g tissue reported by Karlin *et al.*[19] and obtained from affinity labeling of acetylcholine-receptor in a single electroplax; and with 0.02–0.03 nmole/g, of tissue of high affinity sites for decamethonium and sites for muscarone and nicotine in the homogenates of eel electroplax, as reported by Eldefrawi *et al.*[1], but is an order of magnitude below the quantitity of α-bungarotoxin binding sites (0.4 nmole/g tissue) reported by Changeux *et al.*[20].

The quantity of acetylcholine receptor per g of fresh tissue as reported here and elsewhere is, at best, only an estimate of the range since it is not possible to correlate quantitatively the amount of membrane protein with the wet weight of the starting tissue. In addition it must be also stated that although methanesulfonation of the membrane did not seem to alter drastically its binding properties as tested with decamethonium (unpublished results), it is not possible to assess with confidence the degree of receptor denaturation by the combined salt extraction–methanesulfonation treatment of the membrane. Methanesulfonation of intact electroplax does not alter its response to depolarizing drugs[21]. Also, salt extraction of acetylcholinesterase from the excitable membrane fragments does not seem to affect their binding capacity for α-bungarotoxin (Dr J. Bulger, personal communication).

According to Karlin *et al.*[19], there are 4–7 times more acetylcholinesterase catalytic sites than the affinity labeled acetylcholine receptor sites in single eel electroplax. This is in agreement with the ratio of 0.2 for acetylcholine-binding sites to

catalytic sites of esterase reported in this paper, but is in disagreement with the results from α-bungarotoxin-binding experiments of Changeux *et al.*[23] where the number of receptor sites is reported to be about the same as that of acetylcholinesterase active sites. The innervated side of eel electroplax contains in addition to the post-synaptic, the presynaptic and the conducting elements, all of which exhibit uniform distribution of acetylcholinesterase activity[24] (Thomas, N., Davis, R. and Koelle G. quoted by Nachmansohn[25]). Thus, unless the acetylcholine-receptor molecules are distributed as ubiquitously as those of the enzyme, a ratio of acetylcholine receptor to acetylcholinesterase of less than 1 is not unexpected.

ACKNOWLEDGMENTS

This work was supported by a National Institutes of Health Fellowship No. 2 FO2 NS462669 from the Institute of Neurological Diseases and Stroke. The author thanks Dr G. P. Hess for the use of facilities and Dr Juian Fu for providing the least squares program.

REFERENCES

1 Eldefrawi, M. E., Eldefrawi, A. T. and O'Brief, R. D. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 1047–1050
2 Kasai, M. and Changeux, J.-P. (1971) *J. Membrane Biol.* 6, 58–80
3 Meunier, J.-C., Olsen, R. W., Menez, A., Fromageot, P., Boquet, P. and Changeux, J.-P. (1972) *Biochemistry* 11, 1200–1210
4 De Robertis, E., Lunt, G. S. and La Torre, J. L. (1971) *Mol. Pharmacol.* 7, 97–103
5 De Robertis, E. and Fiszer de Plazas (1970) *Biochim. Biophys. Acta* 219, 338–397
6 Hall, Z. W. (1972) *Annu. Rev. Biochem.* 42, 925–952
7 Keynes, R. D. and Martins-Ferreira, H. (1953) *J. Physiol. London* 119, 315–351
8 Changeux, J.-P., Gautron, J., Israel, M. and Podleski, T. (1969) *C.R. Acad. Sci. Paris* 269, 1788–1791
9 Ellman, G. L., Courtney, K. D., Andrea, Jr, V. and Featherstone, R. M. (1961) *Biochem. Pharmacol.* 7, 88–95
10 Silman, I. and Karlin, E. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 1664–1669
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
12 Taylor, P. T. and Singer, S. J. (1967) *Biochemistry* 6, 881–887
13 Ginsburg, S. (1962) *J. Med. Chem.* 5, 1364–1367
14 Suszkiw, J. (1971) *Anal. Biochem.* 44, 321–324
15 Kitz, R. and Wilson, I. B. (1962) *J. Biol. Chem.* 237, 3245–3249
16 Eldefrawi, M. E., Britten, A. G. and Eldefrawi, A. T. (1971) *Science* 173, 338–340
17 Nachmansohn, D. (1970) *Science* 168, 1059–1066
18 Changeux, J.-P., Kasai, M., Huchet, M. and Meunier, J.-C. (1970) *C.R. Acad. Sci. Paris* 270, 2864–2867
19 Karlin, A., Prives, J., Deal, W. and Winnik, M. (1971) *J. Mol. Biol.* 61, 175–188
20 Changeux, J.-P., Kasai, M. and Lee, C.-Y. (1970) *Proc. Natl. Acad. Sci. U.S.* 67, 1241–1247
21 Podleski, T. R. (1967) *Proc. Natl. Acad. Sci. U.S.* 58, 268–273
22 Changeux, J.-P., Meunier, J.-C. and Huchet, M. (1971) *Mol. Pharmacol.* 7, 538–553
23 Bloom, F. E. and Barrnett, R. J. (1966) *J. Cell Biol.* 29, 475–486
24 Nachmansohn, D. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 3170–3174
25 Wilkinson, G. N. (1961) *Biochem. J.* 80, 324–332