On Some Structural Analogies between Acetylcholinesterase and the Macromolecular Receptor of Acetylcholine

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ABSTRACT Several properties of the enzyme acetylcholinesterase (AChE) isolated in vitro are compared with those of the membrane receptor(s) of acetylcholine expressed by the in vivo electrical response of the electroplax membrane. AChE strongly binds in vitro effectors of the electroplax: agonists e.g., decamethonium or antagonists, e.g., *d*-tubocurarine and flaxedil. It also reacts covalently with an affinity labeling reagent of the acetylcholine receptor site(s) in vivo (TDF). Two classes of sites on AChE molecule account for the binding of these quaternary nitrogen containing compounds: (1) the anionic site of the active center and (2) noncatalytic "peripheral anionic centers" located outside the active center. A disulfide bond breaking agent, dithiothreitol (DTT) alters in a parallel manner the reaction of AChE and the excitable membrane of the electroplax to TDF. The irreversibility of TDF action is lost in both cases, after exposure to DTT. Both AChE and the acetylcholine receptor thus contain disulfide bonds—they are closely related but not necessarily identical proteins.

Acetylcholine (ACh) is a general, although not necessarily unique, effector of excitable membranes (Nachmansohn, 1959, 1968). In the animal kingdom a considerable number of cells—muscular, glandular, neuronal, etc.—respond to ACh binding by a characteristic change of membrane permeability. An essential feature of the effect of ACh and its congeners is that it does not involve as a necessary step any covalent or catalytic reaction: ACh acts as a *regulatory signal* which controls membrane permeability to ions. We have proposed that ACh action on its membrane receptor is in many respects similar to the action of regulatory metabolites on allosteric proteins (Changeux, 1966; Changeux, Thiéry, Tung, and Kittel, 1967; Karlin, 1967; Podleski and Changeux, 1968).

In this paper we shall attempt to discuss some problems related to the recognition of ACh in the excitable membrane of the eel electroplax (Schof-

feniels and Nachmansohn, 1957; Higman, Podleski, and Bartels, 1964) and to the molecular structures involved in this recognition. After a first section of methodology, we shall present a number of experimental observations directly related to this question. They were performed both in vivo with the isolated electroplax and in vitro with membrane fragments and pure proteins isolated from these membranes. Some suggestions are proposed, but no definitive conclusions are yet available about the identification of the ACh receptor.

THEORY

We shall designate by *acetylcholine-macromolecular receptor* (ACh-M-receptor) the macromolecule (or macromolecules) responsible for the specific recognition of ACh and which is (or are) specifically involved in the permeability changes caused by ACh.

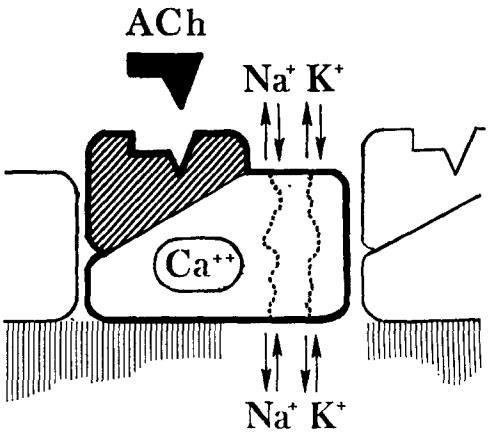

FIGURE 1. Diagrammatic representation of the acetylcholine protomer. It comprises two structural entities: the ACh-macromolecular receptor (shaded area) endowed with the capacity for specific recognition of ACh; and the ACh ionophore (clear area) involved in the selective translocation of cations promoted by ACh binding to the ACh-M-receptor. *Figure reprinted by permission from S. Jusélius Symposium, Helsinki, November 1967.* Elsevier Publishing Company, Amsterdam, London, New York. *1968. 116.*

Two classes of compounds are effectors of the ACh-M-receptor: agonists or receptor activators like acetylcholine (in the presence of eserine), decamethonium, phenyltrimethyl ammonium, etc., which promote the permeability change, and antagonists or receptor inhibitors like *d*-tubocurarine and flaxedil which block the action of receptor activators and stabilize the membrane in its resting state.

Agonists and antagonists bind to a site (or sites) on the ACh-M-receptor that we shall refer to as the ACh-receptor site (or sites) (ACh-R-site). On a given membrane or even on a given ACh-M-receptor there might exist several classes of distinct ACh-R-sites.

According to our definition, we distinguish the elements of structure in-

volved in the recognition of the agonists or antagonists, from those which account for the passive translocation or transfer of ions through the membrane or "ionophores." Ionophore is used here in a very general sense and includes any selective ion pathway, channel, or pore. The ionophores, the properties of which are under the control of the ACh-M-receptor, are referred to as the *ACh-ionophores.* The complex ACh-M-receptor plus ACh-ionophore is the *ACh-protomer.* This definition does not imply any particular structural relationship between receptor and ionophore except their coupling. They might be borne by the same macromolecule, although it is very likely that they might be distinct polypeptidic or lipoproteic entities (Fig. 1).

In this theory we propose that the translocation of ions and the binding of agonists operate through distinct sites of the ACh-protomer. Their coupling mediated by the ACh-protomer would then be an *indirect* or allosteric effect and would be analogous to the allosteric interactions observed between catalytic and regulatory sites in regulatory enzymes. These interactions would then be mediated by the ACh-protomer through some kind of reversible reorganization of its three-dimensional structure. Such a conformational transition might be analogous, although not necessarily identical, to the allosteric transition of regulatory enzymes. It should be mentioned that these mechanisms concern the early events of the membrane response to agonist binding. They might be at the origin of a sequence of secondary ionic events mediated by a variety of mechanisms including diffusible signals different from ACh.

This theory accounts for a number of properties of the so-called ACh-receptor. First of all the distinction between ACh-ionophore and ACh-M-receptor accounts for the fact that ACh promotes a variety of different ionic events on different membrane systems or even on a given membrane (reference in Grundfest, 1966). It accounts also for the fact that the same ionic events might be caused by ACh binding at sites structurally distinct; e.g., the muscarinic or nicotinic receptors.

A characteristic prediction of the theory is that ACh-M-receptor and ionophores might possibly be separated in vitro as distinct entities. The ACh-protomer should be reconstituted from the isolated units. Another prediction concerns the conformational transitions of the ACh-protomer. One should look for structural reorganization of excitable membranes in vivo and of membrane fragments isolated in vitro caused by ACh binding. The "allosteric transitions" of regulatory enzymes might offer models for such changes of structure although the structural changes which accompany ACh binding on an excitable membrane might present their own characteristics.

Any attempt to identify the ACh-M-receptor is faced with a difficult problem of methodology. One should distinguish properties which are required, or necessary, for a macromolecule to serve as a receptor from those which unequivocally show that the considered macromolecule is indeed involved in the

permeability changes caused by ACh. The capacity of *recognition* for ACh and its congeners belongs to the first group of properties. However, the structural requirements for a compound to be an effector of the ACh-M-receptor are not extremely strict. The presence of quaternary nitrogens is essential but the three-dimensional organization of their carbon chain is of secondary importance. This lack of strict specificity is an obvious difficulty for any attempt to identify the ACh-M-receptor. Since a large spectrum of ACh derivatives are known to be pharmacologically active, the apparent relative "affinities" of the compounds in vivo might fruitfully be compared with their affinity to the suspected macromolecule in vitro. However this test is ambiguous since the dissociation constants measured in vivo are not thermodynamic binding constants; in addition the dispersion in solution of the ACh-M-receptor might favor conformations of the macromolecule which are rare or even absent when the ACh-M-receptor is integrated into the membrane structure. The dissociation constants of the isolated material measured in vitro might then be different from the dissociation constants of the same material in vivo. Despite this problem a fruitful initial approach is to isolate from synaptic membranes macromolecules which possess a specificity of recognition for activators and inhibitors and whose binding constants are as close as possible to that exhibited by the excitable membrane in vivo. The most direct technique to use is equilibrium dialysis (Changeux, Leuzinger, and Huchet, 1968; O'Brien and Gilmour, 1969). Another method is affinity labeling (references in Changeux, Podleski, and Wofsy, 1967). In this technique reagents are used which (*a*) are structural analogs of ACh and thus exhibit a high affinity for the binding site of ACh in the membrane; and (*b*) possess a highly reactive group which enable them to establish a covalent bond with amino acid side-chains located within the site or in its close vicinity.

Such a reagent sould permit the labeling of the macromolecule (or macromolecules) carrying the ACh-R-site. The major limitation of this technique is the nonspecific binding of these highly reactive compounds to sites which are unrelated to the ACh-R-site.

In the course of this work we have extensively studied one of the very few proteins from the electroplax membrane which can be isolated and purified (Kremzner and Wilson, 1964; Leuzinger and Baker, 1967) and which possess, as well, the specificity of recognition of ACh and its congeners: the enzyme acetylcholinesterase. A number of similarities have been pointed out for many years between AChE and the ACh-M-receptor and the question is whether or not AChE might have a double function? Would it show both: a catalytic function; the hydrolysis of ACh at its active center and a regulatory function; the promotion of membrane permeability changes upon ACh binding at sites distinct from the active site? The direct involvement of AChE in permeability changes, i.e. its function as ACh-macromolecular receptor, is still highly con-

troversial. Striking analogies between AChE and the ACh-M-receptor will be presented in this paper. However the evidence will still not be sufficient to let us conclude that AChE is directly involved in the electrogenic action of ACh. These structural analogies might simply reflect a common capacity for recognition of ACh.

Presence of "Regulatory" Sites on AChE

As mentioned previously a necessary requirement for a macromolecule to play a role as an ACh receptor is to present a high affinity for ACh and the related neuromuscular blocking agents. Early kinetic studies with AChE from *Torpedo marmorata*, which have been largely confirmed with *E. electricus* enzyme, have shown that AChE is inhibited by a variety of curare-like agents (Changeux, 1966). These inhibitors belong to two distinct groups according to their mode of action on the enzyme. Members of the first group typified by decamethonium, a receptor activator, act as classical competitive inhibitors. To the second group belong flaxedil or *d*-tubocurarine, two typical receptor inhibitors, which only *partially* inhibit the enzyme. The interpretation we have proposed for this partial inhibition is that flaxedil and the substrate can be simultaneously bound to the enzyme; in other words their binding sites are, at least partially, distinct. In agreement with this interpretation is the observation that flaxedil and *d*-tubocurarine antagonize the inhibition by bisquaternary competitive inhibitors like decamethonium. These bisquaternary competitive inhibitors would bind both at the enzyme-active site and at sites topographically distinct from the enzyme-active center that we have named "peripheral anionic centers."

Of interest is the fact that some ligands like flaxedil assumed to bind primarily to these peripheral anionic centers potentiate the binding of other ligands, like 3-OH-phenyltrimethyl ammonium, to the active center. In other words the binding of ligands to the peripheral anionic centers might control the properties of the enzyme active center. The presence of this class of noncatalytic sites on AChE is of importance since previously when AChE was considered as a potential ACh-M-receptor, it was always assumed that the ACh-R-site was the active site of the enzyme.

Affinity Labeling of the ACh-R-Site by TDF In Vivo

Several attempts to identify the ACh-R-site with affinity labeling reagents have already been published (Takagi, Akao, and Takahashi, 1965; Gill and Rang, 1966). In our experiments we used as a label *p*-(trimethyl ammonium) benzene diazonium fluoroborate (TDF) (Changeux, Podleski, and Wofsy, 1967), a compound which gave successful labeling of the active site of anti-*p*-azotrimethyl phenylammonium antibodies (Fenton and Singer, 1965) and of bovin erythrocyte ACh-esterase (Wofsy and Michaeli, 1967). TDF is a

structural analog of phenyltrimethyl ammonium, a typical and potent receptor activator, but in addition possesses a reactive diazonium group which forms a covalent bond mainly with tyrosine, histidine, and lysine side-chains in proteins. Although it presents striking structural analogies with phenyltrimethyl ammonium, TDF is not a receptor activator. Exposure of the innervated face of the electroplax to TDF is not accompanied by any change of potential or resistance but by an irreversible loss of the sensitivity to receptor activators. TDF acts as an irreversible receptor inhibitor. Indeed, *d*-tubocurarine and flaxedil, i.e. typical receptor inhibitors, and phenyltrimethyl ammonium, a receptor activator, reduces the reaction of TDF with the membrane. This protective effect is interpreted as being due to a competition between TDF and these effectors at the level of the ACh-R-site, a further suggestion for the selective binding of TDF at the ACh-R-site.

On the other hand, it was shown that agents known to alkylate irreversibly the esteratic center of AChE such as diisopropyl phosphorofluoridate (DFP) or diisopropyl phosphorylthiocholine (phospholine) do not interfere with the action of TDF on membrane excitation. This observation indicates again that the catalytic moiety of AChE active center does not directly contribute to the recognition of ACh in the early step of the cell response. (This question shall be more extensively discussed later.)

Following the same approach, Karlin and Winnik (1968) have used, as well, a derivate of phenyltrimethyl ammonium, but their reagent possesses as a reactive group a maleimide instead of a diazonium and is consequently assumed to establish covalent bonds with sulfhydryl residues. It blocks irreversibly the response to receptor activators at concentrations several orders of magnitudes lower than TDF but exclusively after exposure of the cell to dithiothreitol, a disulfide bond–reducing agent. Both this result and the one obtained with TDF indicates that amino acid side-chains are constituents of the ACh-receptor site and strongly support the view that the membrane component which carries the site is a protein.

Finally we would like to present an experiment which shows the complexity of the problem we are dealing with. In this experiment we compare the response of the cell to two *different* receptor activators, carbamylcholine and decamethonium, after a short exposure of the membrane to TDF. Fig. 2 shows that the maximal response of these two activators is selectively and differentially altered following the pulse of TDF; in the present experimental conditions (see legend of Fig. 2) the maximal response to carbamylcholine is decreased by 50 % while the response to decamethonium is almost completely abolished.

This result supports the conclusion previously established on different grounds the conversion of shape of the dose-response curve (Changeux and Podleski, 1968), that the binding areas for decamethonium and carbamyl-

choline are, at least partially, distinct. Consequently the ACh-M-receptor has to be viewed as a complex structure. It might not simply be made up of a single polypeptide chain.

Affinity Labeling of AChE by TDF In Vitro

Since TDF is, in vivo, an affinity labeling reagent of the ACh-R-site we re-investigated with some detail the action of this compound, in vitro, on highly purified AChE (Leuzinger and Baker, 1967).

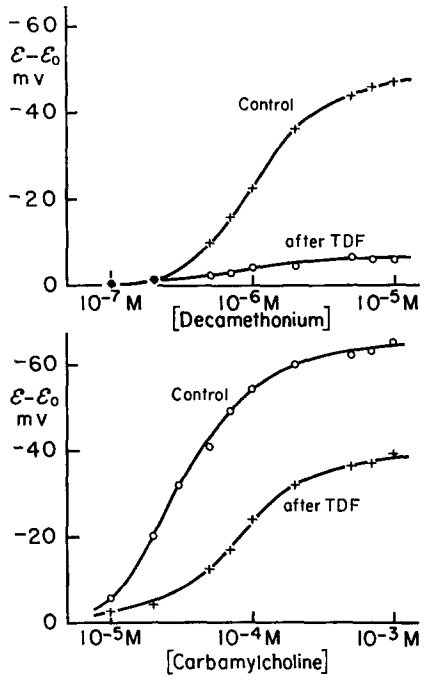

FIGURE 2. Differential inhibition of the electroplax response to two activators, carbamylcholine and decamethonium, after 2 min of exposure to 10^{-4} M *p*-(trimethyl ammonium) benzene diazonium fluoroborate (TDF).

LABELING AT THE ACTIVE SITE As first shown by Wofsy and Michaeli (1967) with AChE from bovine erythrocytes, TDF is an irreversible inhibitor of ACh-hydrolysis. We confirmed this observation with eel AChE (Meunier, 1968; Meunier and Changeux, 1969). The simplest scheme to account for AChE inhibition in the absence of substrate is the following:

$$E + TDF \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (E - TDF) \xrightarrow{k_3} IN$$

where (E—TDF) is a reversible transition complex defined by its dissociation constant $K_{TDF} = k_2/k_1$, IN an inactive apoenzyme, k_3 the rate constant of IN formation.

Under the present experimental conditions where the concentration of active sites is small as compared with the concentration of TDF and where the formation of IN is assumed to be the rate-limiting step, then, the kinetics of AChE inactivation by TDF are consistent with the proposed mechanism as long as the times of exposure are shorter than 1 min and that the substrate used in the subsequent assay is a polar substrate. As we shall see later this is no longer true for exposures of more than 1 min and when a neutral substrate is used instead of a polar one. In first approximation, the kinetic of AChE inactivation is pseudomonomolecular and is interpreted on the basis of TDF binding directly at the level of the complex enzyme-active center.

TABLE I

PROTECTION BY REVERSIBLE INHIBITORS OF AChE AGAINST THE IRREVERSIBLE INACTIVATION OF THE ENZYME BY TDF

	Initial activity
	%
No inhibitor	7.0
Decamethonium	88.5
d-Tubocurarine	46.0
Flaxedil	43.0
Hexamethonium	14.0
Phenyltrimethyl ammonium	15.0
Tetraethyl ammonium	8.0
Methylquinolinium	7.5

A sample of purified AChE is exposed for 5 min to 10^{-4} M TDF at 0°C in 5×10^{-2} M Na phosphate buffered at pH 7.0 in the presence or the absence of the indicated inhibitor, the concentration of which was always 10^{-4} M. After 5 min the mixture was diluted 200-fold and assayed for AChE activity using acetylthiocholine as the substrate (Meunier and Changeux, unpublished results).

According to Nachmansohn and Wilson (1951) AChE-catalytic site comprises a negatively charged "anionic center" and an "esteratic center." The question is then to what part of AChE-active center does TDF bind? Is it a specific labeling reagent of the anionic center? In order to answer this question we tested the action of several AChE inhibitors on the reaction of TDF with the enzyme (Meunier, 1968; Meunier and Changeux, 1969). We used two classes of inhibitors: (*a*) Competitive inhibitors specific for the anionic center, e.g. tetramethyl ammonium and others containing quaternary nitrogens; and (*b*) Inhibitors specific of the esteratic center, e.g. phospholine.

Table I shows that reversible ligands of the anionic center protect the enzyme against inactivation by TDF. The kinetic analysis of the interaction between TDF and these positively charged inhibitors have shown that, within the same limits as those previously expressed for TDF above, the interaction of

TDF and the inhibitor is competitive for the establishment of the transitory Michaelis complex.

As a test for the validity of the theory, we determined the dissociation constant of one of these inhibitors: decamethonium using both the protection against TDF inactivation and reversible competitive inhibition of the enzyme activity. Exactly the same value was found—1.7×10^{-6} M.

The sum of these results indicate that TDF binds through its quaternary nitrogen to the anionic center of the active site. The next question is then: is TDF binding at the active site restricted to the anionic center or does it involve, partially or completely, the esteratic center?

In our approach to this question we studied the binding of TDF to the enzyme after irreversible alkylation of the esteratic center by an organophosphate: 0,0-diisopropyl-phosphorylthiocholine (phospholine). The binding of TDF to the enzyme, was followed under these conditions, either indirectly through an enzyme test after reactivation of the phosphoryl enzyme by pyridine 2-aldoxime methiodide (PAM) or directly, using ^{3}-H labeled TDF.

It was shown that PAM no longer reactivates the phosphoryl enzyme after its exposure to TDF. TDF thus binds to the phosphoryl enzyme. Direct evidence in favor of this conclusion is obtained using tritiated TDF: the number of TDF molecules bound per molecule of enzyme is not changed after alkylation by phospholine. Finally, as we shall see later, after an extensive reaction with TDF, the esteratic site is still operative and splits nonpolar substrates. It is thus concluded that the inactivation of AChE by TDF is caused by the exclusive binding of TDF to the anionic center.

LABELING AT THE REGULATORY SITES In the previous paragraph the evidence was presented that TDF covalently binds at the level of the active site; we shall discuss now some experimental findings which are interpreted on the basis of TDF covalent bonding to sites distinct from the enzyme-active center (Meunier and Changeux, 1969).

The first suggestion comes from the fact that for times longer than 1 min the kinetics of inactivation of AChE no longer follow pseudo–first-order kinetics. The second is offered by the study of the effect of TDF on the hydrolysis of a neutral substrate.

In Fig. 3 are compared the effects of TDF on the catalytic activity of AChE, using either a polar substrate acetylthiocholine, ATCh, or a neutral one, indophenyl acetate. In this experiment AChE solution is supplemented at zero time with TDF at a final concentration of 10^{-4} M, and subsequently, samples are diluted at the indicated time and immediately assayed with both substrates. When ATCh is the substrate, as previously mentioned, a complete inactivation occurs. But when IPA is used, the activity only transiently decreases: a slow reactivation follows the inactivation phase. After 3 hr the activity measured is about 30% larger than the initial activity.

The early inhibition phase, observed with ATCh and IPA, is accounted for by TDF bonding at the anionic site of the active center: the access of both substrates to the esteratic site would be sterically hindered.

Several hypotheses are proposed to explain the slow recovery of the catalytic activity towards IPA hydrolysis: (*a*) a spontaneous dissociation of an AChE-TDF complex; (*b*) a slow structural reorganization of AChE, following the binding of a first TDF molecule to the active site, e.g. the intramolecular migration of bound TDF; and (*c*) the covalent bonding of additional TDF molecules to peripheral anionic sites distinct from the anionic site of the active center.

Hypothesis (*a*) does not account for the observation that the reactivated enzyme no longer hydrolyses polar substrates (i.e. ATCh). Additionally a prediction of hypothesis (*b*) is that the reactivation phase could take place in

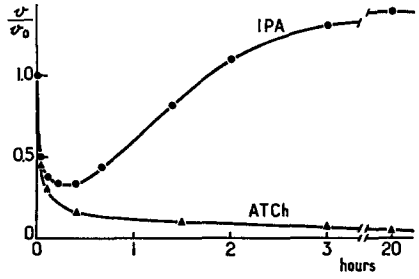

FIGURE 3. Effect of TDF on the activity of purified acetylcholinesterase. The enzyme is exposed to 10^{-4} M TDF at zero time. Subsequently the mixture is diluted, at the indicated time, and assayed on both a charged substrate, acetylthiocholine (ATCh), and a neutral substrate, indophenyl acetate (IPA). *Figure reprinted by permission from F. E. B. S. Letters, 1969, 2: 224.*

the absence of TDF. In order to test this second hypothesis unreacted TDF was, at the end of the inhibition phase (i.e. after about 10 min of incubation), either quenched by addition of an excess of histidine, or rapidly eliminated by filtration of the reaction mixture through a Sephadex G-25 column. In both cases, the reactivation rate was instantaneously decreased. Inhibition of the slow phase was also obtained by adding, under the same conditions, an excess of decamethonium (Fig. 4). In other words, the reactivation phase can be explained on the basis of a secondary slow reaction between TDF and additional sites distinct from the active site. The simplest hypothesis to account for the reactivation process would be that binding of TDF to these "additional" sites promotes a structural reorganization of the active site through indirect and thus "allosteric" interactions mediated through a conformational transition of the enzyme molecules.

After an extensive reaction with TDF, an irreversibly modified enzyme (M-AChE) is obtained, some properties of which differ markedly from those of the native enzyme (AChE). The M-AChE hydrolyzes IPA about 40 %

faster than AChE, although its ability to hydrolyze ATCh is almost completely lost. The M-AChE shows the same sedimentation coefficient (about 11 S) and the same apparent affinity for IPA ($K_M = 8.3 \times 10^{-4}$ M) as the native enzyme. But, its sensitivity to the bisquaternary ammonium inhibitor decamethonium (Deca)—$Me_{3N}^{+}(CH_2)_{10N}^{+}Me_3$—is markedly altered. As illustrated on Fig. 5, Deca inhibits the splitting of IPA by the native protein, but not by the modified enzyme.

The sum of these results is in agreement with the presence on AChE of specific sites for quaternary nitrogens, which are topographically distinct from the anionic site of the active center. This conclusion is of importance since, as we have seen it before, TDF is an affinity labeling reagent of the ACh-M-receptor, in vivo.

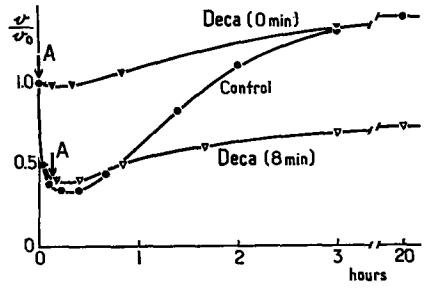

FIGURE 4. Selective blockade of the two phases of TDF action on AChE by 10^{-3} M decamethonium (Deca) (Meunier and Changeux, unpublished results).

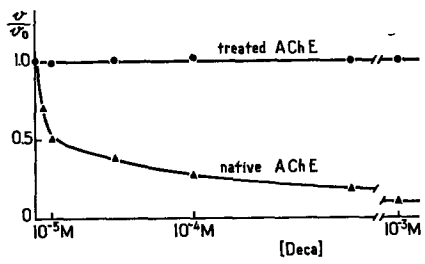

FIGURE 5. Abolition of the sensitivity of acetylcholinesterase to decamethonium (Deca) inhibition by a 3 hr exposure to 10^{-4} M TDF. The substrate was indophenylacetate. *Figure reprinted by permission from F. E. B. S. Letters, 1969, 2: 224.*

NUMBER OF TDF-BINDING SITES PER MOLECULE OF AChE AND A MODEL OF STRUCTURE OF AChE Since TDF binds covalently to sites specific for quaternary nitrogens on AChE, quantitative estimates of the number of TDF molecules bound per molecule of enzyme should give essential information on the structure of the enzyme and on its symmetry properties. We used radioactive TDF and pure AChE to count the sites (Meunier, Leuzinger, and Changeux, 1969). First of all we consider as "specific," the molecules of TDF the covalent binding of which is prevented by an excess of decamethonium. In order to identify the sites to which TDF is specifically bound we used the following graphic representation. The fractional inactivation of the enzyme, using

ATCh as a substrate, is plotted as a function of the number of TDF molecules bound. We have shown that this curve is biphasic. The first part of the curve from 0 to 90 % inactivation is almost linear and extrapolates to a number of 2.12 sites per molecule of AChE assuming a molecular weight for AChE of 2.6×10^5 daltons. The loss of activity corresponding to the remaining 10 % is obtained when, *at least*, two additional sites are filled. This shape of curve is readily interpreted on the basis of TDF binding to the two distinct classes of sites mentioned earlier. The dramatic loss of activity on a polar substrate, is interpreted as due to the binding of TDF to the active sites. The residual drop of activity on a polar substrate, corresponds, in the time scale, to the slow phase of reactivation observed with a neutral substrate: it is interpreted as caused by the binding of TDF to the "regulatory sites." There would thus be two catalytic sites plus a minimum of two regulatory sites per AChE molecule of 2.6×10^5 mol wt.

Presence of a twofold symmetry in the number of sites is further suggested by equilibrium dialysis experiment performed with decamethonium, a bisquaternary competitive inhibitor of the enzyme. Here again two main binding sites are found per AChE molecule (Meunier, Leuzinger, and Changeux, 1969) the same result is found as well with specific labeling reagents of the esteratic sites (W. Leuzinger, personal communication).

These results on the number of sites are consistent with those obtained on the molecular weight of the native enzyme and of its subunit. According to Leuzinger, Goldberg, and Cauvin (1969) AChE molecule is made up of two *different* polypeptide chains of the same molecular weight (6.5×10^4) and contains two sets of each kind. By analogy with hemoglobin it can be named an $\alpha_2\beta_2$-dimer. The question we ask now is what are the respective roles of the two different classes of subunits? One proposal is that one of the two classes of subunits is endowed with a catalytic function and carries the esteratic sites, and that the subunit of the other class, carries the regulatory sites; i.e., is a regulatory subunit. An alternative interpretation is that the two different moieties of AChE-catalytic site, e.g. the anionic center in the one hand, the esteratic site on the other hand, are borne by different chains. In other words the differentiation of the active site would reflect a differentiation of the constitutive chains of the enzyme. Discrimination between these two alternatives should readily be made by separating the chains and identifying their sites.

A virtue of the first interpretation is that it gives a molecular basis for an eventual role of AChE as an ACh-M-receptor. The two functions of the enzyme would be carried by different polypeptide chains; the regulatory subunit would be responsible for the recognition of ACh as a regulatory signal and the subsequent promotion of permeability changes, the catalytic subunit would account for the destruction of the regulatory signal and thus permit the repetitive stimulation of the synapse.

Effect of Dithiothreitol on the Reaction of TDF with AChE and with ACh Receptor

In the past few years Karlin and associates have compared the effects of a variety of sulfhydryls reagents and of disulfide bond–breaking agents on both AChE and the electroplax preparation (Karlin and Bartels, 1966; Karlin, 1967; Karlin and Winnik, 1968).

They showed for instance that exposure of the cell to dithiothreitol, dramatically alters its response to both receptor activators and receptor inhibitors but has no effect on AChE activity assayed in vitro. From these observations they concluded that AChE and the ACh-M-receptor were distinct macromolecular entities. We have reinvestigated this point with more detail using TDF as a probe (Podleski, Meunier, and Changeux, 1969).

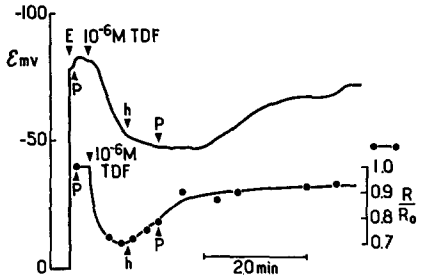

FIGURE 6. Reversible changes of potential and resistance of the electroplax membrane promoted by 10^{-6} M TDF after a 10 min exposure to 10^{-3} M dithiothreitol. E, empalement; P, Ringer's solution supplemented with 5×10^{-2} M Na phosphate pH 5.7; h, 10^{-3} M l-histidine in pH 5.7 Ringer's solution to quench unreacted TDF. E, potential in millivolt, R, resistance measured following the technique described by Podleski and Changeux, 1969 *b*. *Figure reprinted by permission from Proc. Nat. Acad. Sci. U.S.A., 1969, in press.*

Fig. 6 shows that a treatment of the innervated face of the electroplax by DTT is accompanied by a remarkable modification of its reaction with TDF. Although an irreversible inhibitor in the normal cell, TDF becomes a reversible activator in the DTT-treated cell. Moreover in vitro exposure of pure AChE to DTT is also accompanied by a change of its reactivity to TDF. TDF no longer acts as an irreversible inhibitor of DTT-treated AChE: it becomes a reversible competitive inhibitor of the enzyme. Under these conditions, the Ki for TDF as a reversible inhibitor is found the same (2×10^{-5} M) as the dissociation constant measured for the establishment of the reversible Michaelis complex in the first step of the affinity labeling of the native enzyme. DTT

treatment thus impairs the establishment of the covalent bond between TDF and some residue of the enzyme-active site. DTT treatment modifies *both* the sensitivity of the electroplax membrane in vivo and the reactivity of AChE in vitro. Instead of supporting the distinction between AChE and the ACh M-receptor, the present experiments with DTT suggest their similarity. It should be emphasized however that their identity cannot be established on this basis.

Binding of ACh to AChE in the Presence of Eserine

The ACh receptor binds ACh. But AChE is present in large amounts in the electric organs and splits ACh at an extremely fast rate. It thus seems hardly possible to demonstrate ACh binding in vivo, as well as in vitro under such circumstances. It is an old pharmacological observation however that ACh sensitivity is preserved and more often potentiated in the presence of specific inhibitors of AChE like eserine, diisopropyl phosphorofluoridate and various organophosphates such as paraoxon, phospholine, methane sulfonyl fluoride, etc. This fact is of importance since it shows that the irreversible alkylation of AChE-esteratic site does not block the response of the membrane to cholinergic agents and consequently that the esteratic site of the enzyme is not part of the ACh-R-site. This proposition has been well documented with the electroplax preparation (Podleski, 1967) for a variety of activators, in particular for 3-OH phenyltrimethyl ammonium a compound known to interact with AChE-esteratic center through its 3-OH residue. Neither the shape nor the position of the dose response curve to the activators tested are significantly modified by the irreversible acylation of AChE-esteratic site.

Should we conclude from these observations that the protein AChE is not the ACh-R-macromolecule? This would be the case if we were able to show that once the AChE-esteratic site is blocked by eserine or DFP then the enzyme no longer binds ACh and its derivative. First of all, as already mentioned, TDF, an affinity labeling reagent of the ACh-R-site, does bind to AChE when the seryl group of its esteratic site is alkylated by a diisopropyl phosphoryl residue. Moreover as we shall see now ACh itself, the physiological transmitter, does interact with AChE in the presence of eserine (Changeux, Leuzinger, and Huchet, 1968).

This was shown using the simple technique of equilibrium dialysis which let Gilbert and Müller-Hill (1966) identify the genetic repressor of the Lac-operon. We started using membrane fragments purified from the innervated face of the electroplax (Changeux, Israel, Gautheron, and Podleski, 1969) and ^{14}C acetylcholine labeled on its acetyl group. In the presence of 10^{-5} M eserine or 10^{-6} M phospholine, the radioactivity accumulates inside the bags con-

taining the membrane fragments. Although an unambiguous physicochemical interpretation for this accumulation has not yet been proposed we shall say that ACh "binds" reversibly to the membrane fragments. The component which accounts for this binding is thermostable, resists to pancreatic lipase, trypsin, and chymotrypsin but is digested by pronase. It is a protein. In order to identify this protein we centrifuged membrane fragments dissolved in 1 % deoxycholate on top of a sucrose gradient established in 10^{-3} M Na phosphate pH 7.0 supplemented with 1 % deoxycholate. Both ACh binding and AChE activity were assayed in each fraction collected after

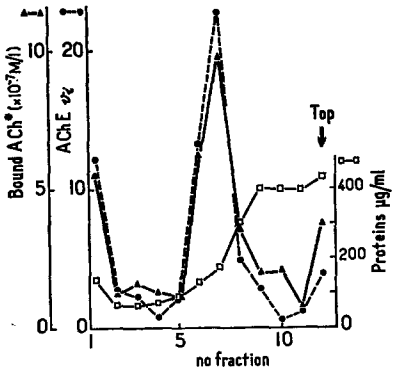

FIGURE 7. Sedimentation in sucrose gradient of membrane fragments treated with 1% deoxycholate. Sodium deoxycholate is first added at room temperature for a few minutes to the suspension of membrane fragments in sucrose containing 9.2 mg protein per milliliter. The mixture is then dialyzed against 1% deoxycholate in 10^{-3} M Na phosphate, pH 7.0, for 1 hr and added on top of a sucrose gradient stabilized in 10^{-3} M Na phosphate, pH 7.0, supplemented with 1% deoxycholate. The gradients are centrifuged for 4 hr at 50,000 rpm in a SW 65 rotor in a Beckman Spinco preparative ultracentrifuge. AChE and ACh binding in the presence of 10^{-5} M eserine are measured in each fraction. Initial rates of acetylthiocholine hydrolysis (v_i) are expressed in millimoles per minute per microliter of fraction. *Figure reprinted by permission from F. E. B. S. Letters, 1968, 2: 77.*

centrifugation. As shown on Fig. 7 both the ACh-binding protein and AChE sediment at exactly the same velocity.

We therefore tested the binding of ACh to pure AChE prepared from electric tissue and found, indeed, that the pure protein binds ACh in the presence of 10^{-5}M eserine.

A striking quantitative feature of ACh binding to AChE is that, under the present experimental conditions the binding curve does not follow a simple Langmuir isotherm: there is no tendency for saturation at high ACh con-

centration; above 10^{-6}M-free ACh the amount of bound ACh increases almost linearly with the concentrations of free ACh; the Scatchard plot of the binding data is not linear and does not extrapolate to a finite number of sites at infinite ligand concentration (Fig. 8).

At high ACh concentrations the number of ACh molecules bound per molecule of AChE becomes remarkably large. Values up to several hundreds are counted. The magnitude of the number of ACh molecules bound in

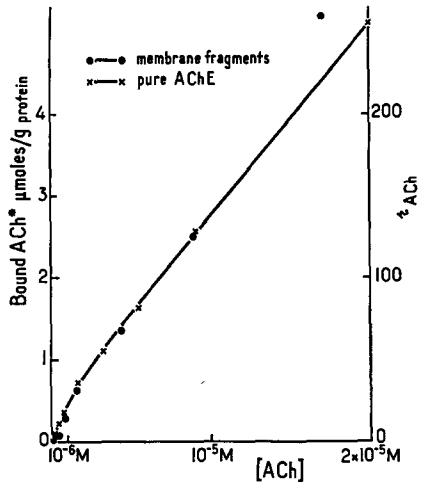

FIGURE 8. Binding of ACh to membrane fragments and pure AChE both from electric tissue. ACh bound per gram protein is plotted as a function of free ACh concentrations (membrane fragments). r_{ACH} is the number of ACh molecules bound per molecule of pure AChE (mol wt = 2.6×10^5) (Leuzinger, Goldberg, and Cauvin, 1969), and [ACh] is the free concentration of ACh expressed in moles per liter. AChE concentrations are estimated spectrophotometrically (ϵ = 16.1 (Leuzinger, Baker, and Cauvin, 1968)), chemically (Folin reaction, and Biuret), and by enzyme assay. *Figure reprinted by permission from F. E. B. S. Letters, 1968, 2: 77.*

these conditions seems somewhat paradoxal in connection with our present knowledge of AChE quaternary structure and binding sites. This unique property of AChE has not yet received a reasonable physicochemical interpretation.[1]

Of importance is the observation that this unusual capacity for ACh binding appears to be a specific property of AChE. First of all, the amount of ACh bound by the membrane fragments is integrally accounted for by their content in AChE estimated through an enzyme test (about 5 % w/w of the

[1] *Note Added in Proof.* Recent experiments have shown that much less displacement of radioactivity occurs when acetylcholine labeled on the methyl group is used instead of ^{14}C-acetylcholine. The process seems to be complex and might involve a covalent reaction in addition to reversible binding.

total protein). Furthermore four unrelated proteins tested under the same conditions, serum albumin, β-galactosidase, ribonuclease, and catalase, do not show any significant binding of ACh. Another important fact is that ACh bound to AChE is itself displaced by compounds pharmacologically active on the electroplax preparation such as decamethonium, *d*-tubocurarine, and flaxedil. ACh binding is also considerably reduced in the presence of high salt concentrations (10^{-1} M NaCl) or negatively charged detergents (1 % Na deoxycholate). The binding of ACh in the presence of eserine seems thus to involve a special class of sites, distinct from AChE-catalytic sites, which present a high affinity for curare-like agents and the positive charge of ACh molecule seems essential for the interaction of ACh with these sites.

In order to identify these ACh-binding sites we studied the effect of TDF

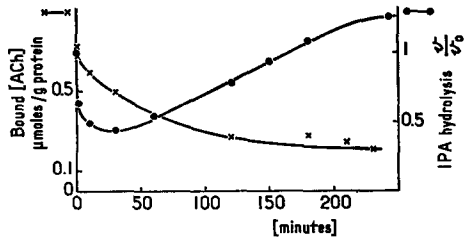

FIGURE 9. Comparative effects of TDF on AChE activity assayed on a neutral substrate (indophenylacetate) ●-●, and ACh binding in the presence of eserine measured by equilibrium dialysis, ×-×. At zero time a solution of AChE in 5×10^{-2} M Na phosphate buffer pH 7.0 is supplemented with 10^{-4} M TDF; at the indicated times samples of the mixture are made 10^{-3} M with l-histidine to block unreacted TDF. Then IPA hydrolysis and ACh binding are assayed as described previously.

on ACh binding to AChE in the presence of eserine. The reaction with TDF was followed by IPA hydrolysis. It was of interest to test (*a*) if the capacity to bind ACh was abolished after exposure to TDF, and (*b*) in the positive case if the loss of ACh binding corresponds to the fast inactivation phase or to the slow reactivation.

FIG. 9 shows that exposure to TDF is accompanied by a dramatic loss of its ability to bind ACh and that the kinetics of this decay does not follow the first rapid phase of enzyme inactivation. The irreversible inhibition of ACh-binding sites is thus unrelated to the binding of TDF to AChE active center. On the other hand this kinetic follows grossly the slow reactivation phase that we interpreted as due to the binding of TDF to "regulatory sites" distinct from AChE-active sites. This result is in agreement with our initial hypothesis that there exist AChE-binding sites for quaternary ligands distinct from the anionic site of the active center.

DISCUSSION

In this paper we have compared a few properties of AChE isolated in vitro with those of the ACh-receptor expressed by the in vivo response of the electroplax membrane. Aware of the fact that a number of theoretical and practical difficulties are inherent in such a comparison, we may summarize the main conclusions as follows:

1. Blockage of the esteratic center of AChE does not alter the response of the cell to receptor activators. Consequently the esteratic center of AChE is not involved in the early recognition step which is at the origin of the permeability changes caused by ACh and its congeners.

2. AChE strongly binds compounds which are known to be potent effectors of the electroplax membrane; e.g., phenyltrimethyl ammonium, decamethonium, as receptor activators, *d*-tubocurarine, and flaxedil as receptor inhibitors, or TDF an affinity labeling reagent of the ACh-R–site in vivo. It is concluded that there exist on AChE molecule binding sites for quaternary ligands: peripheral anionic centers, or regulatory sites, which are topographically distinct from the anionic center of the active site.

3. A disulfide bond–breaking agent, dithiothreitol (DTT), alters in a parallel manner the reaction of AChE and the excitable membrane of the electroplax with TDF. The irreversibility of action of TDF is lost, in both cases, after exposure to DTT. Both AChE and the ACh-M-receptor thus contain disulfide bonds.

4. In the presence of inhibitors of its esteratic center such as eserine or phospholine, AChE binds, in vitro, the physiological transmitter: acetylcholine. This binding primarily involves the regulatory sites located outside the active site.

From these observations no definitive conclusion concerning the nature of the ACh-macromolecular receptor can be proposed. An interesting hypothesis is that the regulatory sites present on AChE molecules might play a physiological role as ACh-R-sites. This hypothesis is supported by the observation that AChE contains two classes of subunits and that one of them might be a noncatalytic subunit. The noncatalytic subunit would then represent a "regulatory" subunit specialized in the electrogenic action of ACh. We wish to emphasize however that this hypothesis does not exclude the possibility that proteins different from AChE-catalytic or -regulatory subunits might be, or contribute to, the ACh-macromolecular receptors. Accounting for the evidence that there might be several classes of ACh-R-sites even in a system as simple as the electroplax, the question remains whether or not the "regulatory" sites present on AChE might be one of them?

In any case, it is clear that the ACh-M-receptor and AChE are closely re-

lated proteins. The distinction between both classes of structures will thus require a careful physicochemical analysis and possibly a comparative sequence analysis of the suspected polypeptide chains.

The observed analogies between AChE and the ACh-M-receptor might be unrelated to the electrogenic function of ACh and simply due to the fact that they both show a common specificity of recognition for ACh. Even in this case, this approach will have been fruitful since it already let us find some original properties of AChE and the ACh-receptor.

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