

Chemical Modification of the Active Site of the Acetylcholine Receptor

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ABSTRACT The receptor for acetylcholine in the subsynaptic membrane of the electroplax of *Electrophorus electricus* is a protein with a disulfide bond in the vicinity of the active site. This disulfide can be reduced and reoxidized with concomitant inhibition and restoration of the response to acetylcholine and other monoquaternary ammonium-depolarizing agents. Conversely, the bisquaternary hexamethonium, normally a competitive inhibitor, causes depolarization, and the activity of decamethonium is increased following reduction of the disulfide. The reduced receptor can be alkylated by various maleimide derivatives and is then no longer reoxidizable. Some quaternary ammonium maleimide derivatives act as affinity labels of the reduced receptor, alkylating it at a rate three orders of magnitude faster than do uncharged maleimide derivatives. Other types of potential affinity labels also react only with the reduced receptor and the resulting covalently attached quaternary ammonium moieties interact with the active site, strongly activating the receptor. These results suggest a model for the active site and its transitions in which an activator such as acetylcholine bridges between a negative subsite and a hydrophobic subsite in the vicinity of the disulfide, causing an altered conformation around the negative subsite and a decrease of a few angstroms in the distance between the two subsites.

Acetylcholine induces changes in the ion permeability and selectivity of susceptible membranes in nerve, muscle, and electrogenic cells (for reviews, see references 1 and 2). It has long been postulated that this action is mediated by a specific receptor to which acetylcholine binds (3). It has been further suggested that this receptor is a protein and that the binding of acetylcholine is translated into a permeability change by means of a conformational change (4). I present results obtained by chemically modifying the receptor *in situ* which indicate that the receptor is a protein and which suggest a specific transition of the binding site for acetylcholine accompanying receptor activation.

THE ELECTROPLAX

The experiments described here involve modification by covalent reactions of components of single, isolated cells, electroplax of *Electrophorus electricus*. These

are large, sheetlike cells, derived from muscle, whose physiological function, the generation of transcellular current, is a consequence of their asymmetry (5, 6). The membrane on one side of the cell is innervated and electrically and chemically excitable, while that on the other side is not innervated and is inexcitable. Following neural triggering by way of a depolarizing postsynaptic

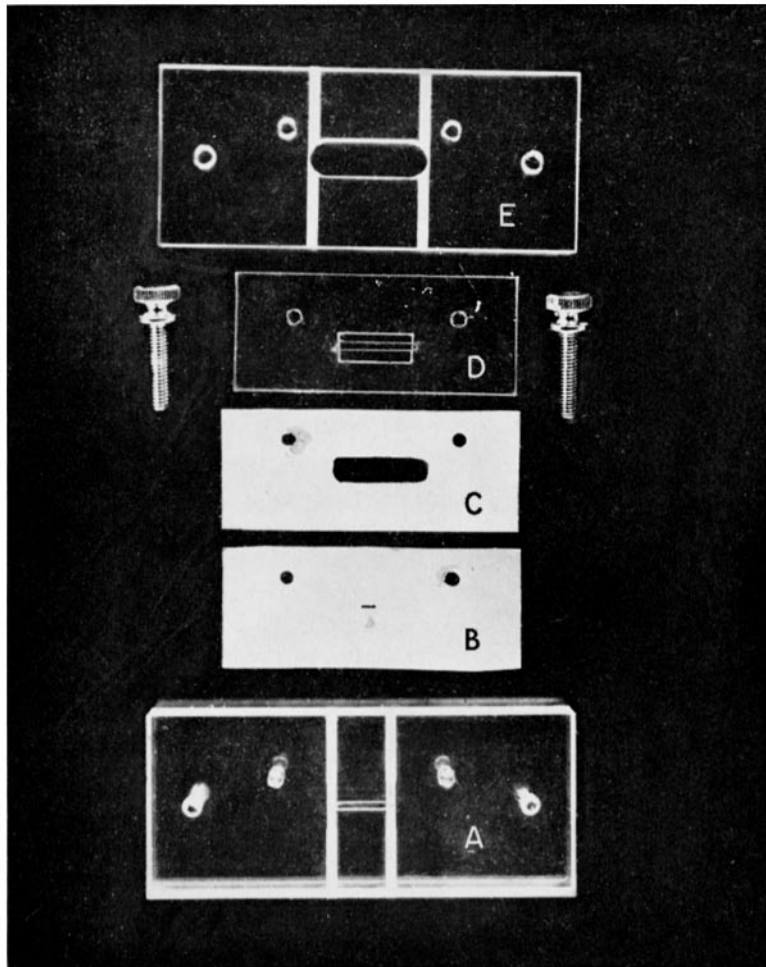


FIGURE 1. Chamber for holding electroplax. The photograph shows a disassembled chamber. The lower compartment (*A*) is formed by a trough (14×1.5 mm) in a lucite block ($9 \times 4 \times 2.5$ cm) to which are connected two inlets, one outlet, and an agar-salt bridge (via polyethylene tubing, PE 200). The Mylar window (*B*) is placed on (*A*) and the electroplax is placed, innervated side down, over the window which is lightly greased with petroleum jelly. A spacer (*C*) is placed over the window and around the electroplax, and a threaded holder (*D*) is placed over (*C*), the threads holding the electroplax against the window. The upper compartment (*E*) is placed on top of (*D*) and the whole is held together by two thumb screws.

potential, the innervated membrane generates an action potential of approximately 150 mv amplitude. The potential difference across the noninnervated membrane does not change during the change of the potential across the innervated membrane. Therefore, the amplitude of the potential change across the whole cell is approximately 150 mv. A series of several thousand cells can thereby generate a considerable electromotive force.

As previously described (7), cells are dissected from the organ of Sachs. A single cell is mounted in a chamber (Fig. 1) in which the cell separates two solutions (cf. reference 8). In this arrangement the innervated membrane faces down, pressed against a greased window, and a portion is exposed to a stream of solution flowing at 1-2 cm/sec through a narrow, shallow trough forming the lower compartment. The entire noninnervated membrane is bathed by a stationary solution in the upper chamber. The cell is impaled from above through the noninnervated membrane with a glass microelec-

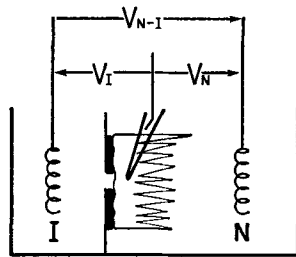


FIGURE 2. A diagrammatic representation of the electroplax mounted in the chamber and the potential differences measured. *I* refers to the innervated side and *N* to the noninnervated side of the electroplax. The assigned polarities of the potential differences are indicated by the arrows.

trode. Agar-salt solution bridges make electrical contact with the upper and lower compartments.

Three potential differences can be measured (Fig. 2): that across the innervated membrane, V_I (outside *minus* inside); that across the noninnervated membrane, V_N (outside *minus* inside); and that across the cell, $V_{N-I} = V_N - V_I$. During rest, $V_I = V_N =$ approximately 80 mv (and $V_{N-I} = 0$). Application of a depolarizing agent such as acetylcholine to the innervated membrane (for times of the order of a minute) results in a decrease in V_I and in V_N and an increase in V_{N-I} , since V_N always decreases less than V_I . These changes can be interpreted as follows (9): ΔV_N depends on a change in the intracellular concentration of sodium and potassium ions. ΔV_{N-I} depends primarily (at least initially) on the change in the relative permeability of the innervated membrane to sodium and potassium ions. ΔV_{N-I} is secondarily dependent on intracellular ion concentrations. ΔV_I , which equals $\Delta V_N - \Delta V_{N-I}$, depends on both permeability and concentration changes. The intracellular concentration change is opposed even during the application of a depolarizing agent by the action of a ouabain-sensitive active transport system, and hence the potential differences are also somewhat dependent on the

state of the active transport system. This dependence is undoubtedly due to the long duration of such depolarizations compared with the neurally evoked postsynaptic potential. The effects on the electroplax of antibiotics known to act on membrane permeability supports this interpretation of the changes in membrane potential (10). Removal of the depolarizing agent results in a rapid return of V_N , V_I , and V_{N-I} to their initial values, but the recovery of V_N and V_I is considerably retarded if the transport system is inhibited with ouabain (9).

REDUCTION OF THE RECEPTOR

The response of the electroplax to acetylcholine or to analogs such as carbamylcholine is inhibited by a brief application of a low concentration of the

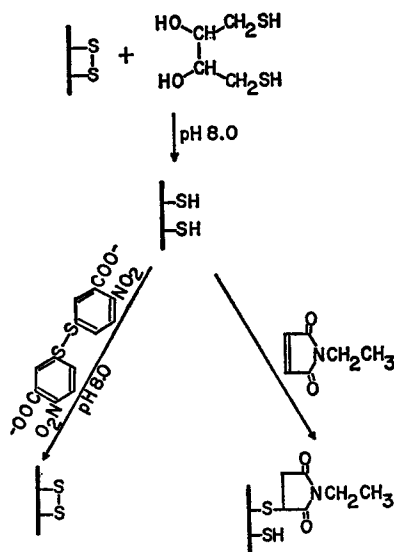


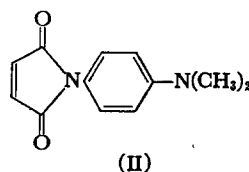
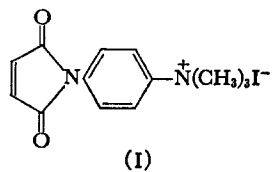
FIGURE 3. Chemical scheme for reduction by dithiothreitol and reoxidation by dithiobis-(2-nitrobenzoate) or alkylation by *N*-ethylmaleimide of a disulfide on the receptor.

reducing agent dithiothreitol to the innervated membrane. This inhibition is completely reversed by the subsequent application of an oxidizing agent such as potassium ferricyanide or 5,5'-dithiobis-(2-nitrobenzoate) (11). This action of dithiothreitol is specific in so far as the resting potential, the recovery from a depolarization, and the amplitude of the action potential are unaffected. Nor is there any effect if dithiothreitol is added to the noninnervated membrane. It was further observed that if, following dithiothreitol, *N*-ethylmaleimide is applied at a concentration otherwise without effect, the inhibition could no longer be reversed by subsequent application of an oxidizing agent. A likely interpretation of these results is that dithiothreitol is reducing a disulfide bond present in a protein intimately connected with the permeability

change; the sulfhydryls formed either can be reoxidized or can be trapped by an alkylating agent such as *N*-ethylmaleimide (Fig. 3). It should be mentioned that the response to acetylcholine and its congeners is also inhibited by application of organic mercurials such as *p*-chloromercuribenzoate and *p*-chloromercuriphenylsulfonate without prior application of a reducing agent, which is evidence for the presence of sulfhydryl groups (11). However, these sulfhydryls do not appear to be reactive towards alkylating reagents such as *N*-ethylmaleimide or iodoacetate. When applied at high enough concentrations these latter compounds appear to affect the active ion transport system as evidenced by an inhibited recovery from depolarization whereas the depolarization itself appears unaffected. The evidence for both sulfhydryl groups and disulfide linkages associated with the response to acetylcholine is evidence for the vital association of proteins with this response. Acetylcholinesterase, incidentally, appears unaffected by either dithiothreitol or the organic mercurials at concentrations effective in inhibiting the response of the electroplax to acetylcholine (12).

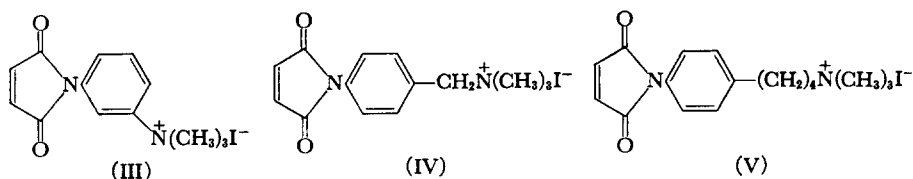
ACTIVE SITE-DIRECTED ALKYLATION OF THE REDUCED RECEPTOR

On the basis of the results cited so far it would be difficult to decide whether the susceptible disulfide is on the receptor or on some associated protein. In an attempt to decide this issue and to provide perhaps a specific label for the receptor, Winnik and I synthesized and tested some quaternary ammonium maleimide derivatives. The quaternary ammonium moiety would be expected to have an affinity for the active site (the specific binding site for acetylcholine) of the receptor. The maleimido group was shown to react with the reduced receptor (see above). These compounds are therefore potential *affinity labels* (13) of the reduced receptor. Their effectiveness would depend on the location of the sulfhydryls formed by reduction of the disulfide relative to the active site. If the reactive maleimide double bond were held in approximate juxtaposition to one of the sulfhydryls when the quaternary ammonium group was reversibly bound at the negative subsite (the presumed locus of binding of the onium group(s) common to all potent receptor activators and inhibitors), then the probability of a successful collision, and the rate of reaction, should be considerably increased over that in the absence of such affinity and positioning.



The rate of reaction of I with the reduced receptor was found to be approximately 2000-fold greater than that of its uncharged tertiary amine analog, II, or of *N*-ethylmaleimide (14). These rates were estimated on the basis of experiments in which first dithiothreitol and then the maleimide derivative and finally dithiobis-(2-nitrobenzoate) is applied to the innervated membrane of the electroplax (reference 14, Fig. 1). The assumptions are that the difference between the first response to a fixed concentration of carbamylcholine and the response after reduction is proportional to the number of receptors reduced, that the difference between the first response and the last response after reoxidation is proportional (the same proportionality constant) to the number of receptors alkylated, and that the ratio (called β) of the latter difference to the former difference is a normalized measure of the fraction of available reduced receptors that are alkylated. This ratio, β , is a function of the product of the second-order rate constant of the alkylation reaction, the concentration of maleimide derivative applied, and the duration of application. For two derivatives, therefore, the ratio of the (concentration \times time)'s corresponding to a fixed value of β should be inversely proportional to the ratio of the rate constants for alkylation of the reduced receptor. To obtain $\beta = 0.5$ (taking $-\Delta V_r$ as the response), it takes 10^{-8} M I applied 10 min, but 2×10^{-5} M II or *N*-ethylmaleimide.

A similar comparison of some related quaternary maleimide derivatives has been made (Fig. 4).¹



From the data available, it appears that the rates of reaction with the reduced receptor are in the order III < I < V < IV, with IV reacting approximately twice as fast as I. It is possible by making additional assumptions to estimate the absolute rate of these reactions. The assumptions are that one disulfide is reduced per active site and that only one of the two sulfhydryls formed is alkylated. In that case, β is a measure of the fraction of the sulfhydryls available which have been alkylated, and $1 - \beta$ is a measure of the fraction of the sulfhydryls available which have not been alkylated. Since the concentration of the maleimide derivatives is maintained constant, the reaction is pseudo-first-order and $\ln(1 - \beta) = -kmt$, where k is the second-order rate constant, m is the concentration of maleimide derivative, and t is the duration of application. In Fig. 5, $-\log_{10}(1 - \beta)$ is plotted against (mt) for the data

¹ Karlin, A., and M. Winnik. Unpublished observations.

taken from Fig. 4. For I and IV, the points are reasonably colinear for β less than 0.8; i.e., for the reaction not too close to completion. The rate constants, k_R , obtained from the slopes are shown in Table I.

In order to evaluate the effectiveness of these compounds as affinity labels, we must know what part of the differences in k_R is due to differences in the intrinsic reactivity of the maleimido group. In Table I are also shown the second-order rate constants, k_{cys} , for the reaction with cysteine in the same

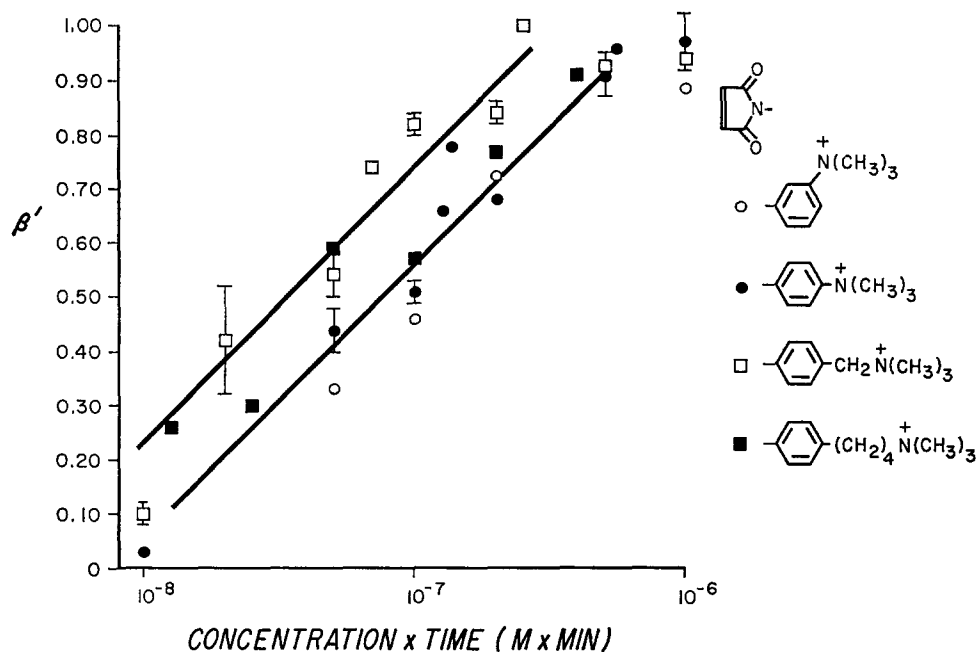


FIGURE 4. Fraction of the inhibition due to reduction which cannot be reversed by reoxidation (β') versus the product of the concentration of maleimide derivative and the duration of its application. The sequence of additions to the innervated membrane of the electroplax is carbamylcholine, dithiothreitol, carbamylcholine, maleimide derivative, carbamylcholine, dithiobis-(2-nitrobenzoate), and carbamylcholine (repeated) (14). The response is taken to be $-\Delta V_T$, 80 sec after addition of $40 \mu\text{M}$ carbamylcholine.

modified Ringer's solution as is applied to the electroplax. It is seen from the ratio of k_R/k_{cys} that whereas *N*-ethylmaleimide appears to alkylate cysteine in solution 27-fold faster and the tertiary amine analog, II, 43-fold faster than the reduced receptor, IV alkylates the reduced receptor 40-fold faster than cysteine. Taking the relative rate of alkylation by *N*-ethylmaleimide as a base, the enhancement of the rate of reaction of IV due only to its affinity for the receptor appears to be 1100-fold, that of I, 460-fold, and that of III, 270-fold (Table I). k_{cys} for V has not yet been determined.

The large enhancement factor for the rate of reaction of the quaternary

ammonium maleimides strongly suggests that they are acting as specific affinity labels of the reduced receptor. Further evidence is that these compounds all fail to react covalently with the unreduced receptor. They do bind however to the active site of the unreduced receptor, I and IV each having dissociation constants of approximately 8×10^{-5} M, determined from their competitive inhibition of carbamylcholine. The reaction of 10^{-7} M I with the reduced receptor is slowed approximately 40% in the presence of 1 mM

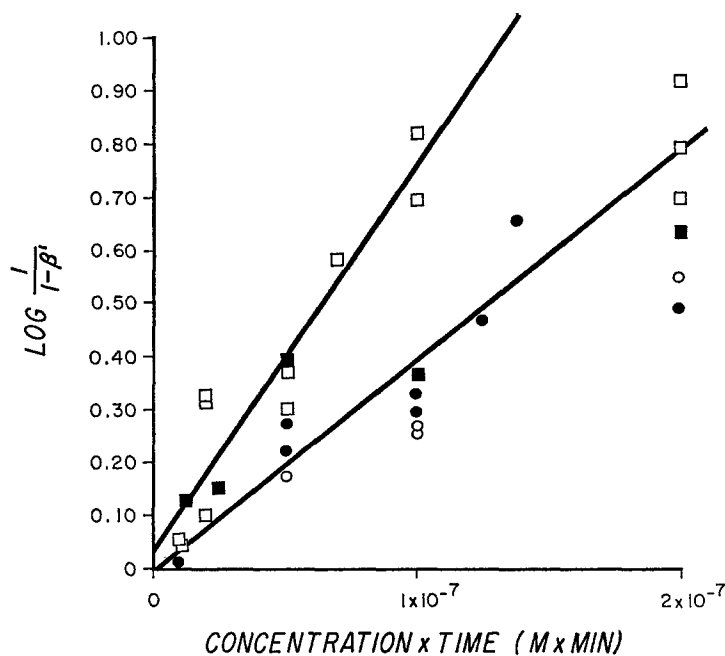


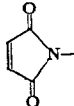
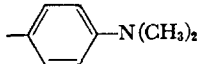
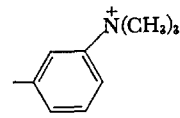
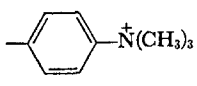
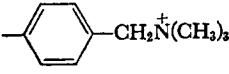
FIGURE 5. The plot of $-\log_{10}(1-\beta')$ versus concentration of maleimide derivative times its duration of application. The data are taken from Fig. 4 and the symbols are the same. Least-squares lines are shown for I and IV (disregarding points at 2×10^{-7} M \times min and greater). The rate constants are equal to $(2.303 \times \text{slope})$.

hexamethonium, another ligand of the active site (14). Hexamethonium does not appear to reduce the rate of reaction of *N*-ethylmaleimide. It appears, therefore, that competition for binding at the negative subsite is involved in the slowing of the reaction with I. On the other hand, addition of phenyltrimethylammonium ion (a potent depolarizer) together with *N*-ethylmaleimide does not *increase* its rate of alkylation of the receptor. Finally, both I and III upon reaction cause a small depolarization (Table II), which is not reversed when the unreacted reagent is removed. IV and V cause no depolarization upon reaction. The slight activation of the receptor by I and III, although not a necessary consequence of the covalent attachment of an onium group at the active site, is nevertheless evidence for such an attachment. Neither I nor

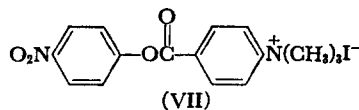
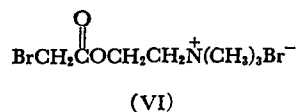
N-ethylmaleimide, following dithiothreitol, affects the activity of acetylcholinesterase, further distinguishing this enzyme from the receptor (quoted in reference 14).

TABLE I
RATE CONSTANTS FOR THE REACTION OF MALEIMIDE DERIVATIVES WITH CYSTEINE AND WITH THE REDUCED RECEPTOR

The rate constant for the reaction with cysteine, k_{cys} , was determined as described in reference 14. The apparent rate constant for the reaction with the reduced receptor, k_R , was determined as described in the text (Fig. 5). The enhancement of the rate of reaction due to binding of the reactant near the site of reaction is estimated to be k_R/k_{cys} divided by k_R/k_{cys} for *N*-ethylmaleimide.

	k_{cys}	k_R	k_R/k_{cys}	Enhancement of rate of reaction with reduced receptor due to binding near the site of reaction
	1/mole sec	1/mole sec		
$-C_2H_5$	1620 ± 130	60	0.037	1.0
 (II)	3920 ± 80	90	0.023	0.6
 (III)	9710 ± 390	$\sim 1 \times 10^5$	10	270
 (I)	8850 ± 700	1.5×10^5	17	460
 (IV)	6950 ± 340	2.8×10^5	40	1100

Silman and I have investigated two different compounds which also react covalently with the reduced receptor only.² These are bromoacetylcholine bromide (VI) and the *p*-nitrophenyl ester of (*p*-carboxyphenyl)trimethylammonium iodide (VII).

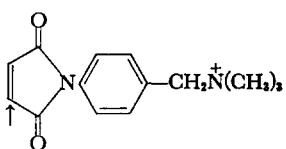
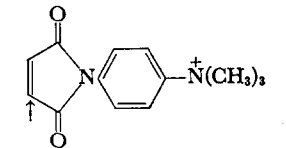
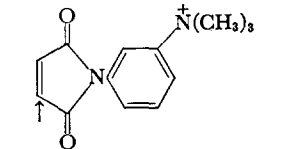
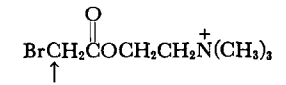
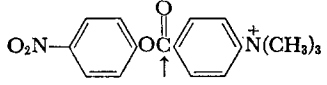


² Silman, H. I., and A. Karlin. Acetylcholine receptor: covalent attachment of depolarizing groups at active site. *Science*. In press.

Each of these compounds will react with a nucleophile such as a sulfhydryl group, VI at the α -carbon of the acetyl group, releasing bromide and VII at the carbonyl carbon, releasing *p*-nitrophenol. Acting on the unreduced receptor, VI is an activator and VII is a competitive inhibitor (with very slight

TABLE II
COMPARISON OF LENGTH, DEPolarIZING ACTIVITY, AND ENHANCEMENT OF RATE OF REACTION

The distances were measured on Corey-Pauling-Koltun models (The Ealing Corporation) and were taken from the far side of the reacting carbon atom (indicated by arrow) to the far side of the methyls on the quaternary ammonium group in the maximally extended configuration. The depolarization ($-\Delta V_T$) is that obtained following complete reaction of the affinity label with the reduced receptor. This is a steady value for the maleimide derivatives, but continues to increase for VI and VII, for which the value approximately 5 min after removing the unreacted reagent is given.¹

Compound	Maximum length of moiety added to SH	Depolarization of the innervated membrane after reaction	Enhancement of rate of reaction
	<i>A</i>	<i>mv</i>	
 (IV)	12.0	0	1100
 (I)	11.3	1	460
 (III)	10.6	2	270
 (VI)	9.4	30	
 (VII)	9.0	30	

activating properties); both effects are readily reversed by washing. Acting on the reduced receptor, the covalently attached quaternary ammonium moieties of VI and VII are strong activators (Table II). This activation cannot be reversed by washing. (No irreversible effect appears if the reduced receptor is alkylated with *N*-ethylmaleimide prior to treatment with VI and VII.) The activation can be reversed, however, by free competitive inhibitors such as tubocurarine and unreacted VII. Upon removal of the competitive inhibitor, the activation reoccurs. These results suggest a flexibility in the region of the active site permitting the covalently attached activators to be displaced from the active position by competitive inhibitors.

Since the reaction of VI and VII is followed by extensive depolarization, the method used to estimate the rate of alkylation of the reduced receptor by the maleimides cannot be applied. Not knowing k_R , we cannot determine to what extent the rates of reaction of VI and VII are enhanced by binding to the active site. There is no question however that VI and VII do interact with the active site both before and after reaction with the receptor. The effects of the quaternary ammonium maleimides and VI and VII on the reduced receptor constitute strong evidence for the location of the reducible disulfide on the receptor close to the active site.

CHANGES IN THE SPECIFICITY OF THE RECEPTOR DUE TO REDUCTION

Treatment of the electroplax with dithiothreitol results in reduction of a disulfide linkage near the active site of the receptor. The effects of this molecular alteration on the action of activators and inhibitors reflects on the nature of the interactions of these ligands with the receptor. As mentioned before, reduction inhibits the response of the electroplax to acetylcholine and to carbamylcholine. The effect of reduction on the dose-response curve of carbamylcholine has been analyzed in terms of a three-parameter representation: $z = z_{\max}/(1 + (K/a)^n)$, where z is the response and a is the concentration of activator (15). It was found, taking $-\Delta V_I$ as the response, that following reduction, K increases fourfold and n decreases approximately from 2 to 1. As a response, $-\Delta V_I$ has the drawback that it does not depend solely on the underlying changes in permeability. It is possible to calculate from ΔV_{N-I} and ΔV_N a value for the change in the ratio of sodium permeability to potassium permeability (called $\Delta\rho$) (9). If the assumptions are correct, this estimate of $\Delta\rho$ should reflect more closely than $-\Delta V_I$ the underlying permeability changes. In Fig. 6, both responses, $-\Delta V_I$ and $\Delta\rho$ are plotted versus concentration for the same cells for both the reduced and unreduced states of the receptor. $\Delta\rho$ as a function of the concentration of activator saturates less rapidly than ΔV_I , and consequently K appears larger, n smaller, and differences in z_{\max} are more apparent. Qualitatively, however, the changes following reduction are

the same for $-\Delta V_I$ and $\Delta\rho$: K increases, n decreases, and, as was not clearly recognized before, z_{\max} decreases. These changes can be neatly interpreted in terms of a two-state allosteric (16) model as all due to a decrease in the affinity of the *active* form of the receptor for the activator (15). Less theoretic-

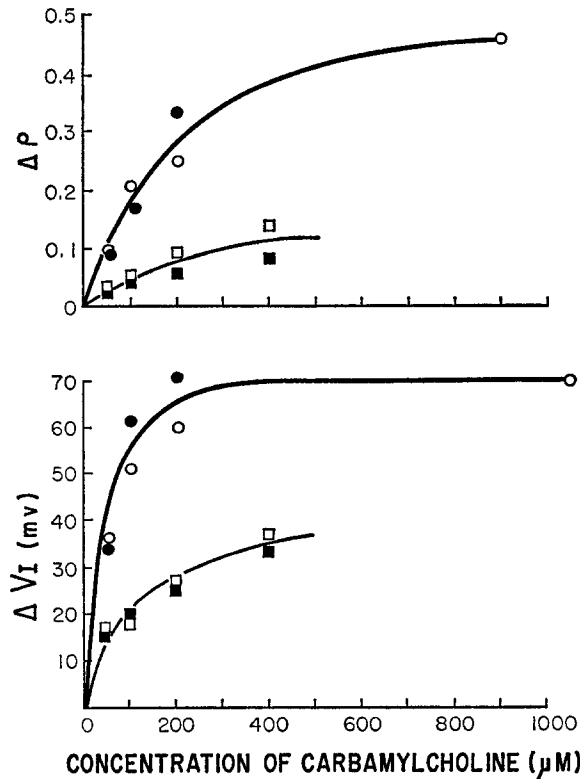


FIGURE 6. The change in potential across the innervated membrane ($-\Delta V_I$) and the change in the ratio of sodium to potassium permeability of the innervated membrane ($\Delta\rho$) in response to carbamylcholine, in the reduced (\square , \blacksquare) and the unreduced (\circ , \bullet) states of the receptor. $-\Delta V_I$ equals $(-V_N + \Delta V_{N-I})$. $\Delta\rho$ is calculated from ΔV_N and ΔV_{N-I} (9). Two electroplax were used. The first was tested for its response to three concentrations of carbamylcholine (\bullet), treated with dithiothreitol, and the response to four concentrations of carbamylcholine was determined (\blacksquare). The second was treated with dithiothreitol first, the responses determined (\square), then treated with dithiobis-(2-nitrobenzoate), and the responses redetermined (\circ).

cally, it can be said that in the reduced state the receptor binds carbamylcholine less well, that binding of carbamylcholine is translated less efficiently into a permeability change, and that an apparent positive interaction (cooperativity) between sites decreases.

Other ligands have not been looked at in as much detail as carbamylcholine, but the qualitative effects of reduction on their action are nonetheless

interesting. Applied at approximately their half-maximally effective concentration (for the unreduced state), all monoquatarnary activators tested, except tetramethylammonium, are inhibited approximately 80 % (Table III). Tetramethylammonium ion which is 10^{-8} as potent as acetylcholine, is in-

TABLE III
EFFECT OF REDUCTION AND OF ALKYLATION OF THE RECEPTOR
ON THE ACTIVITY OF REVERSIBLE LIGANDS

The length is that of the longest axis in the maximally extended configuration and taken from the far sides of the end groups. The potency is the inverse ratio of the concentration of activator to the concentration of acetylcholine, the two concentrations eliciting equal responses. The change in activity is given as the per cent of the initial control response (taken as $-\Delta V_T$). Hexamethonium was added at 1 mM, as was D-acetyl- β -methylcholine.

Compound	Maximum length	Potency relative to acetylcholine	Change in activity after:	
			DDT	DTT, NEM
	<i>A</i>		%	%
$\begin{array}{c} + \\ \text{(CH}_3\text{)}_3\text{NCH}_2\text{CH}_2\text{OCCH}_3 \\ \text{(Acetylcholine)} \end{array}$	10.6	1	-80	-96
$\begin{array}{c} + \\ \text{(CH}_3\text{)}_3\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3 \\ \text{(Butyltrimethylammonium)} \end{array}$	9.9	0.3	-75	
$\begin{array}{c} + \\ \text{(CH}_3\text{)}_3\text{NCH}_2\text{CH}_2\text{OCNH}_2 \\ \text{(Carbamylcholine)} \end{array}$	10.8	0.1	-81	-92
$\begin{array}{c} + \\ \text{(CH}_3\text{)}_4\text{N} \\ \text{(Tetramethylammonium)} \end{array}$	5.6	0.001	-55	-70
$\begin{array}{c} + \quad + \\ \text{(CH}_3\text{)}_3\text{N(CH}_2\text{)}_{10}\text{N(CH}_3\text{)}_3 \\ \text{(Decamethonium)} \end{array}$	19.6	1	+44	-94
$\begin{array}{c} + \quad + \\ \text{(CH}_3\text{)}_3\text{N(CH}_2\text{)}_6\text{N(CH}_3\text{)}_3 \\ \text{(Hexamethonium)} \end{array}$	14.4	0	depolarizes 14 mv	0 mv
$\begin{array}{c} + \\ \text{(CH}_3\text{)}_3\text{NCH}_2\text{CHOCCH}_3 \\ \\ \text{CH}_3 \\ \text{(D-Acetyl-}\beta\text{-methylcholine)} \end{array}$	10.6	0	0	

hibited on the average 55%. Alkylation of the reduced receptor by *N*-ethylmaleimide results in a further decrease in the response to these activators. Conversely, the response to the potent bisquaternary activator, decamethonium, is *increased* following reduction and *decreased* by reoxidation (Fig. 7).

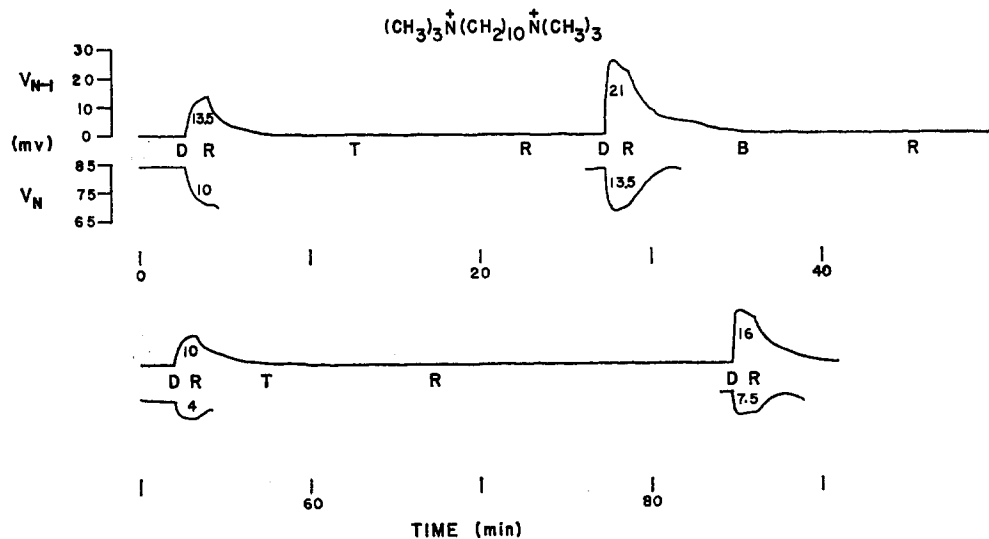


FIGURE 7. The effect of reduction and reoxidation of the receptor on the response to decamethonium. The responses to $2 \mu\text{M}$ decamethonium (*D*) in R after 80 sec are indicated. R, modified Ringer's solution (pH 7); T, 1 mM dithiothreitol (pH 8.0); B, 1 mM dithiobis-(2-nitrobenzoate) (pH 8.0). Discontinuities in the record of V_N are due to losses of impalement.

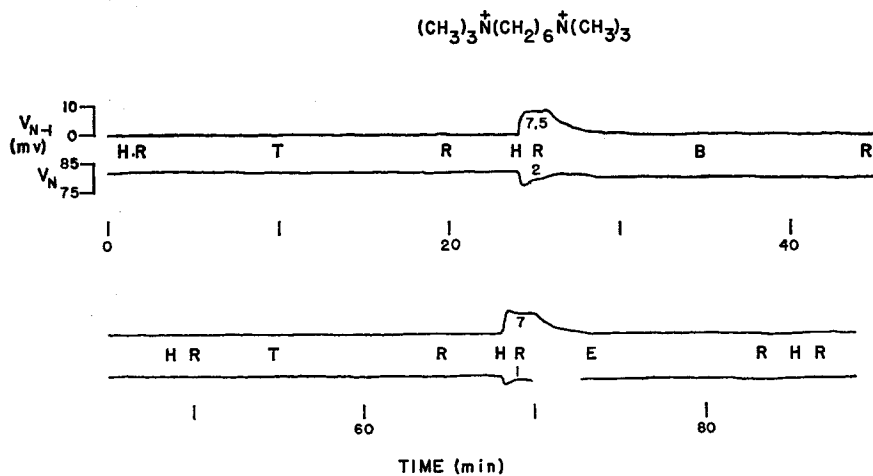


FIGURE 8. The effect of reduction and reoxidation or alkylation of the receptor on the response to hexamethonium. H, 1 mM hexamethonium; E, 10^{-4} M *N*-ethylmaleimide in R; R, T, and B as in Fig. 7.

Following reduction *and* alkylation, the response is nearly completely inhibited (Table III). Equally dramatic is the change in the action of hexamethonium, another bisquaternary ammonium compound. Normally, a competitive inhibitor, hexamethonium activates the reduced receptor (Table III and Fig. 8) (14). Reoxidation eliminates this effect, as does alkylation (Fig. 8). Clearly, reduction of the disulfide near the active site changes receptor specificity both qualitatively and quantitatively.

A MODEL OF THE ACTIVE SITE OF THE RECEPTOR

The rate of reaction of an affinity label depends on at least three factors: (*a*) the intrinsic reactivity of the reacting groups; (*b*) the affinity for the binding site; and (*c*) the proximity and orientation of the reacting groups when the label is reversibly bound. For three quaternary ammonium maleimide derivatives, (I, III, and IV) the contribution of differences in the intrinsic reactivity of the maleimido group to the differences in the rate of alkylation of the reduced receptor has been accounted for by determining their rates of alkylation of cysteine in solution. Furthermore, affinities of I and IV, and probably of III, for the unreduced receptor are not appreciably different. It is probable that the affinities of these derivatives for the reduced receptor are also similar. In that case, differences in "enhancement of rate due to binding" (Table I) are likely due to differences in proximity and orientation. Table II contains the overall distance between an ethylenic carbon of the maleimide ring and the far side of the trimethylammonium group in the configuration giving the maximum value for this distance. (There is actually little variation with possible configurational changes.) This distance can be compared with the enhancement due to binding and with the extent of depolarization after reaction. It appears that the extent of depolarization (activation) increases and the enhancement decreases with decreasing length of the moiety added to the sulfhydryl in the reduced receptor (Table II). Extending the correlation to VI and VII, for which the enhancement factor is undetermined, it appears that further decrease in the length of the covalently attached moiety increases further the extent of activation.

One possible interpretation of these correlations is that the covalently attached moieties bridge between one and the same sulfhydryl, to which they are covalently bound, and the negative subsite, to which they are reversibly bound, and that the length of the bridge determines whether the reduced receptor is in an active conformation (length approximately 9 Å) (Fig. 9 *d*) or in an inactive conformation (length approximately 12 Å or greater) (Fig. 9 *c*). In addition, among the maleimide derivatives, IV shows the greatest enhancement of rate of alkylation because the distance from the ethylenic carbons to the onium group (12 Å) corresponds most closely to the distance between the negative subsite and the reacting sulfhydryl in the energetically

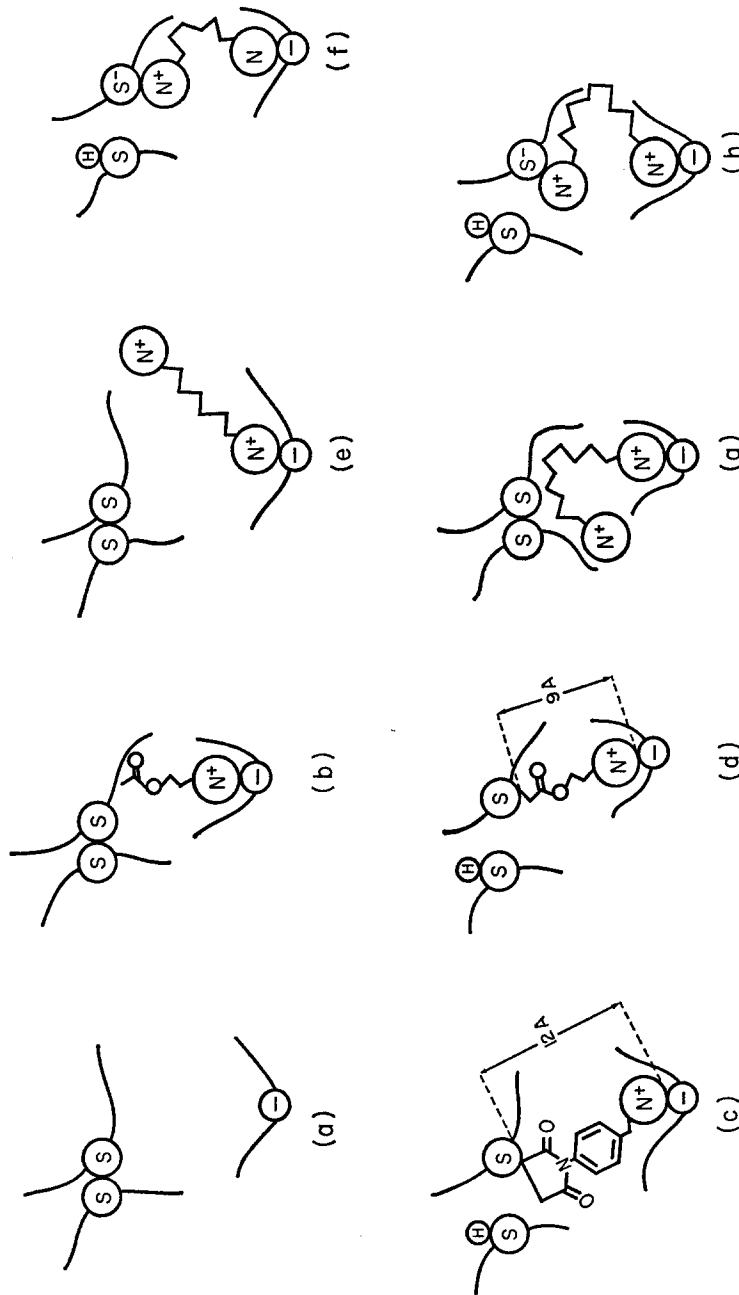


FIGURE 9. A schematic representation of the active site of the receptor. (a) Unreduced, and inactive conformation. (b) Unreduced, activated by acetylcholine. (c) Reduced, alkylated by IV, an inactive conformation. (d) Reduced, alkylated by VI, an active conformation. (e) Unreduced, binding hexamethonium, an inactive conformation. (f) Reduced, binding hexamethonium, an active conformation. (g) Unreduced, binding decamethonium, an active conformation. (h) Reduced, binding decamethonium, a more active conformation than in g. The molecular representations are not drawn to scale, but the overall distances are represented comparatively.

avored inactive conformation of the receptor. (In the presence of VI, a potent reversible activator of the receptor, the active conformation would be favored, and the enhancement of the reaction of VI (length approximately 9 Å) could be as great as that of IV.) The picture is then of the inactive state of the reduced receptor being partially characterized by a 12 Å (or greater) distance between the negative subsite and the reacting sulfhydryl, and the active state, by a distance of approximately 9 Å. There is a gradation of activity increasing as the length decreases from 11 Å to 9 Å, and even greater activity might be obtained at shorter distances.

One of the difficulties with this interpretation is the assumption that only one of the two sulfhydryls formed by reduction reacts with all the affinity labels. Conceivably, all the maleimides might react with one, and VI and VII might react with the other, and the resulting differences in orientation at the negative subsite might be the important factor determining the extent of activation. A related difficulty is the assumption that the overall length of the covalently attached moiety determines the "proximity and orientation" factor in the enhancement of the rate of alkylation, which in fact might also depend on other steric factors. Finally, the correlations are between suggested trends seen with just a few compounds. A greater range of compounds of different lengths must be tested.

Despite the present tenuousness of this interpretation, let us take it as a hypothesis subject to experimental testing and extend it to the unreduced receptor and to reversible activators. It is reasonable to suppose that noncovalently bound activators (e.g., acetylcholine) act in a similar way to the covalently bound activators, that noncovalent activators also bridge the active site between the negative subsite and a second region of positive interaction, determining the distance between these subsites, and that this distance is shorter in the active than in the inactive state (Fig. 9 *a* and *b*). The nature of the interaction at this second subsite is likely to be principally hydrophobic. This is suggested by the high depolarizing activity of acetylcholine and the inactivity of choline (17), which is also a poor competitive inhibitor (i.e., binds weakly), by the activity of butyltrimethylammonium, pentyltrimethylammonium, phenyltrimethylammonium, and benzyltrimethylammonium ions, all equipotent or nearly so with acetylcholine (18) (*p*-carboxyphenyl-trimethylammonium ion is inactive), and by the importance of hydrophobicity (and bulk) in some competitive inhibitors of the receptor (19). (The possible contribution of a hydrogen bond to the binding of acetylcholine and of carbamylcholine to the second subsite is not excluded. Such a bond, however, is neither necessary nor sufficient for activation.) The location of this second subsite is likely to be in the vicinity of the reducible disulfide, since the orientation in the active site of noncovalent and covalent activators should be nearly the same and the fully-extended lengths of the potent monoquaternary

activators (Table III) are close to the lengths of the covalent activators (Table II). Bromoacetylcholine bromide (VI), for example, is a reversible activator of the unreduced receptor and, after reaction, an irreversible activator of the reduced receptor. The lengths in these two cases are not very different. VII, on the other hand, having a bulky *p*-nitrophenyl group, is a competitive inhibitor of the reduced and of the unreduced receptor, whereas the covalently attached and shorter trimethylammoniumbenzoyl moiety is a strong activator. The hypothesis is that in both the reduced and unreduced receptor a decrease of a few angstroms in the distance between the negative subsite and a hydrophobic subsite is a characteristic of receptor activation.

Any compound which binds to one or both subsites and prevents the conformational change would be a competitive inhibitor. Hexamethonium, a competitive inhibitor ($K_i = 30 \mu\text{M}$), would fail to activate the unreduced receptor because the second trimethylammonium group would prevent positive interaction at the hydrophobic subsite (Fig. 9 *e*). Decamethonium, another bisquaternary, is a potent activator because the decamethylene chain is long enough to fold the second onium group out of the way presenting a hydrophobic region to the hydrophobic subsite at a distance of approximately 9 Å (Fig. 9 *g*). The increase in activity of decamethonium, and the activity of hexamethonium, following reduction of the receptor is likely due to the availability in the vicinity of the hydrophobic subsite in the reduced receptor of a negatively charged group to which the second onium group of each can bind (Fig. 9 *f* and *h*). Two possibilities are that either one of the two sulfhydryl groups ionizes or that an ionized carboxyl group becomes free to rotate into the site. The first possibility is favored by the fact that alkylation of the reduced receptor by *N*-ethylmaleimide eliminates the activation by hexamethonium and very much inhibits that by decamethonium (Table II). The second possibility is interesting because the effectiveness of bisquaternary competitive inhibitors such as *d*-tubocurarine suggests that there might be a second negatively charged group in the vicinity of the active site and that it is this group which is mobilized by reduction. In either case, reduction of the disulfide would decrease the hydrophobicity in the region of the postulated hydrophobic subsite, which could account for the decreased activity of monoquaternary activators as well as the increased activity of bisquaternary ligands.

The activity of tetramethylammonium ion, even though it is low, raises the question as to the necessity for an activator to bridge the active subsite. It is probably too small to interact at both the negative subsite and the hydrophobic subsite. This is perhaps reflected in the smaller inhibition of tetramethylammonium ion caused by reduction of the receptor compared with the inhibition of the more potent monoquaternary activators (Table II); i.e., reduction of the disulfide causes more disturbance of the neighboring hydropho-

bic subsite and its interactions than of the opposite negative subsite. Nevertheless, binding of tetramethylammonium ion at the negative subsite does result in activation of the receptor and, if the hypothesis is correct, in a conformational change similar to that caused by the more potent activators. It seems necessary to postulate that the *inactive* conformation is partially stabilized by intramolecular and/or solvent interactions with the negative subsite (e.g., compare with the stabilization of an ionized aspartyl residue in the interior of chymotrypsin; also note the proximity of a disulfide linkage (20)), and that formation of an ion-pair with the quaternary ammonium group disrupts this interaction and makes possible new, possibly hydrophobic interactions (cf. references 21–23) partially stabilizing the active conformation. *The transition from inactive to active state involves a conformational change around the quaternary ammonium negative subsite ion-pair and a linked decrease in the distance between the negative subsite and the hydrophobic subsite, stabilized in potent activators by a bridge of the correct length positively interacting at both subsites.*

The model for the active site of the receptor and its transitions, even if true, would not account for subtleties (e.g., the total lack of activity of acetyl- β -methylcholine on the electroplax) nor for the differences in specificity and function of acetylcholine receptors in different cells, and even in the same cell (24). Whether, in fact, the chemical modifications applicable to the receptor in the electroplax are general remains to be seen. (Reduction and reoxidation or alkylation appear to have effects similar to those on the electroplax on the response of the frog rectus abdominus muscle to carbamylcholine.)³ Even if it were true, as seems likely, that activation of acetylcholine receptors is generally accompanied by conformational changes, how such changes might be translated into changes in membrane permeability remains an open question.

I thank Dr. D. Nachmansohn for his support, and Dr. I. Silman, Dr. J. Prives, and Mr. M. Winnik for valuable discussion and help in the preparation of this manuscript. The work is supported by United States Public Health Service Grants NB-07065 and NB-03304 and by the New York Heart Association Inc. and Heart Fund. Compounds VI and VII were synthesized by Miss K. Losee and Dr. J. Bernstein of the Squibb Institute for Medical Research.

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