*COMPARED EFFECTS OF DITHIOTHREITOL ON THE INTERACTION OF AN AFFINITY-LABELING REAGENT WITH ACETYLCHOLINESTERASE AND THE EXCITABLE MEMBRANE OF THE ELECTROPLAX**

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Abstract.—*p*-(trimethyl ammonium) benzene diazonium difluoroborate (TDF), an affinity-labeling reagent of the acetylcholine receptor site(s), which in the normal cell acts as an irreversible inhibitor becomes a reversible activator after *in vivo* exposure of the electroplax to dithiothreitol (DTT), a disulfide bond reducing agent. After *in vitro* exposure of acetylcholinesterase to DTT, TDF becomes a reversible competitive inhibitor of the enzyme, using indophenyl acetate as the substrate. Both acetylcholinesterase and the macromolecular receptor of acetylcholine thus contain disulfide bonds. Additional experiments with DTT suggest that there might exist several different classes of receptor sites for cholinergic agents in the excitable membrane of the electroplax.

In a preceding paper we have shown that *p*-(trimethyl ammonium) benzene diazonium difluoroborate (TDF) may be used as an affinity-labeling reagent for the site (or sites) which are involved in the electrogenic action of acetylcholine (ACh) on the electroplax membrane.[1] On the other hand we have studied the affinity labeling by TDF of acetylcholinesterase (AChE), an essential component of the electroplax membrane,[2, 3] and have shown that TDF covalently binds to at least two classes of sites on the enzyme.[4] One of them is constituted by the anionic site of the active center and the other by a group of noncatalytic, anionic, sites located outside the active site: the "peripheral anionic centers."[5] The question then is raised: Do any of these TDF binding sites carried by AChE correspond, in part at least, to the physiological receptor site(s) of ACh? The present experimental evidence is still too fragmentary to allow a definitive answer to this question. We shall only present some experimental data which indicate that the reactivity toward TDF of both AChE and the macromolecular receptor(s) of ACh present striking similarities.

Karlin and associates[6–8] have compared the effects of a disulfide bond reducing agent, dithiothreitol, *in vivo* on the electroplax preparation and *in vitro* on purified AChE.[3] They have shown that DTT treatment perturbs the electroplax response to a variety of cholinergic agents. For instance, the response to a typical receptor activator, carbamylcholine, is reduced while hexamethonium, a compound reported to be a receptor inhibitor in the normal cell, becomes a receptor *activator* in the DTT-treated cell (DTT-cell). On the other hand, DTT has no effect on AChE activity *in vitro*, and Karlin concluded from these experiments that AChE and the ACh-macromolecular receptor were distinct macromolecular entities.[7]

We have reinvestigated this point in some detail, using TDF as a probe. It was found that TDF, an irreversible receptor inhibitor of the normal cell, becomes a *reversible* receptor *activator* of the DTT-cell. Furthermore, after extensive exposure of AChE to DTT *in vitro*, TDF becomes a *reversible* competitive inhibitor of AChE using indophenylacetate as the substrate. These results suggest that both AChE and the macromolecular receptor for ACh possess disulfide bonds whose rupture leads to parallel alterations of the properties of both components.

(1) *In vivo Effect of DTT on the Reaction of TDF with the Electroplax.*—As shown previously, exposure of the innervated membrane of the electroplax to TDF does not result in any change of membrane potential or resistance, while, on the other hand, the cell response to receptor activators such as carbamylcholine (Carb) or decamethonium (Deca) is reduced. TDF behaves as an irreversible receptor inhibitor. Dramatic alterations occur following a ten-minute treatment of the innervated membrane of the electroplax with 10^{-3} *M* DTT in Ringer's solution buffered at pH 8.0 with 2×10^{-3} *M* Tris. At 5×10^{-7} *M* TDF, and at higher TDF concentrations a depolarization and a decrease of membrane resistance are recorded: in other words, TDF acts as an *activator* of the DTT-cell (Fig. 1*b*).

Continuous perfusion with TDF gives, in these conditions, steady-state depolarizations like a typical activator in an untreated cell. For concentration of TDF above 10^{-5} *M*, steady states are no longer observed, and an initial depolarization is rapidly followed by a repolarization (Fig. 1*a*).

In order to reduce the nonspecific binding of TDF to the membrane, experi-

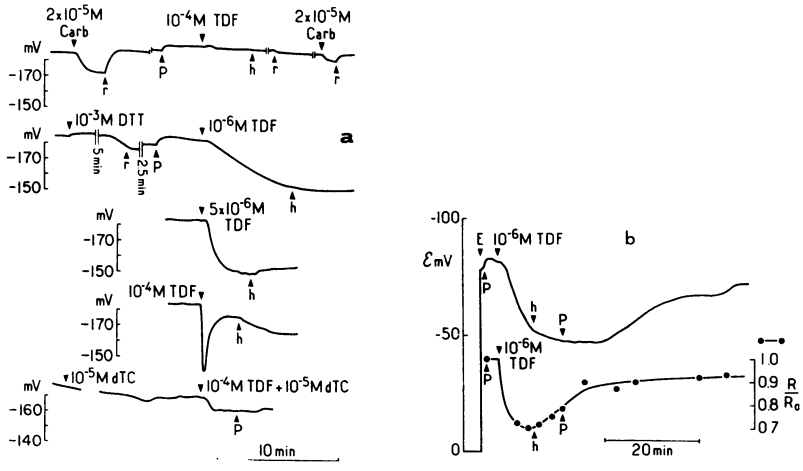

FIG. 1.—Reversible depolarization of the electroplax membrane by TDF after a 10-min exposure to 10^{-3} *M* DTT. (*a*) Effect of various concentrations of TDF and blockade by *d*-tubocurarine. *First line:* control; *second, third, fourth, and fifth lines:* the cell was exposed to 10^{-3} *M* DTT in Ringer's solution buffered with 2×10^{-3} *M* Tris pH 8.0. (*b*) Reversibility of the effect of TDF on membrane resistance and potential. *Abbreviations:* *r*, Ringer's solution; *P*, Ringer's solution supplemented with 5×10^{-2} *M* Na phosphate pH 5.7; *h*, 10^{-3} *M* L-histidine in *P*, to quench unreacted TDF; *Carb*, carbamylcholine chloride; *dTC*, *d*-tubocurarine chloride.

ments were performed at acidic pH (close to pH 5.7) where the reactivity of the diazonium group is decreased. At this pH the response of the normal cell to Carb is reduced by a factor of 2. It is remarkable that in the same conditions the response of the DTT-cell to TDF is large. TDF following DTT treatment thus acts as one of the most powerful activators known of the electroplax.

One of the most striking features of the response of the DTT-cell to TDF concerns the reversibility of TDF action. As shown on Figure 1*b*, both the depolarization and the decrease of membrane resistance produced by TDF are reversed by perfusion with Ringer's solution supplemented with 5×10^{-2} *M* sodium phosphate pH 5.7: TDF acts as a *reversible* receptor activator. The rate of repolarization, measured in these conditions, are much slower than those recorded with the normal cell using activators such as Carb or Deca, but at all TDF concentrations tested, the reversion of TDF effect is complete in less than 20 minutes. Moreover the DTT-cell still responds to Carb after TDF exposure.

In a previous work[9, 10] we have presented experimental evidence that the binding sites for Deca and Carb might be topographically distinct. It was then of interest to reinvestigate the effect of DTT on the cell response to both Carb and Deca. We confirmed the observation of Karlin and Bartels[6] that the response to Carb is decreased after DTT treatment, but in addition we found that the response to Deca is potentiated instead of being reduced (such an observation has also been made independently by Karlin[11]). Furthermore we observed that the amplitude of the response to Deca is not stable with time (Fig. 2): About 20 minutes after DTT exposure the amplitude of the response decreases by 50 per cent, while in the case of the normal cell repetitive exposures to 4×10^{-6} *M* Deca (four times the concentration used in Fig. 2) always give the same response. Of interest is the fact that the response to TDF following

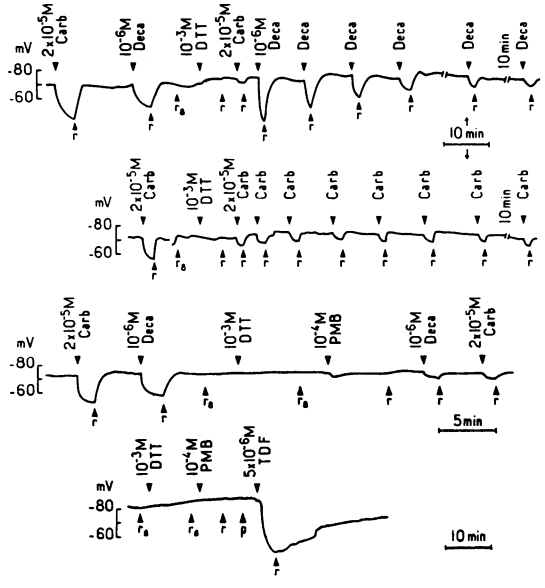

FIG. 2.—Differential effects of DTT on the electroplax response to decamethonium, carbamylcholine, and TDF. The cell was exposed to 10^{-3} *M* DTT in Tris Ringer pH 8 (r_8) for 5 min. *First line:* the amplitude of the potentiated response to 10^{-6} *M* decamethonium (*Deca*) decreases as a function of time. *Second line:* the amplitude of the reduced response to 2×10^{-5} *M* Carb (*Carb*) is stable with time. *Third and fourth lines:* effect of PMB on the DTT-cell *P* (see Fig. 1).

DTT also decreases with time, while in the same conditions the response to Carb remains stable.*

A variety of sulfhydryl group reagents was then tested following DTT treatment. 10^{-3} M dithiobisdinitrobenzoic acid (DTNB) was found to reverse the effect of DTT completely, whereas after 10^{-4} M N-ethyl maleimide (NEM) both the potentiation of the Deca response and the evoked depolarization by TDF were nearly abolished. On the other hand, 10^{-4} M p-hydroxymercuribenzoate (PBM) markedly decreased the potentiation of the response to Deca without having any effect on the responses to TDF or Carb (Fig. 2). Control experiments showed that these reagents, at the concentration used, have no effect on the response of the normal cell to receptor activators.

This group of results suggests that the receptor mechanism involved in the evoked response to TDF, the potentiated response to Deca, and the reduced response to Carb following DTT exposure are different. This conclusion is in agreement with the suggestion made earlier, on different grounds, that the receptor sites for Carb and Deca are at least partially distinct.[9, 10]

(2) *Effect of DTT on the Reaction of TDF with Acetylcholinesterase.*—We studied in parallel the *in vitro* effect of TDF on purified acetylcholinesterase prepared from electric tissue.[3] We first confirmed the observation of Karlin that neither the affinity nor the maximal velocity of AChE is modified after DTT exposure, with both neutral and polar substrates. However, as illustrated in Figure 3, after several hours' exposure of AChE to 10^{-2} M DTT (DTT-AChE), TDF no longer inactivates AChE irreversibly. It still inhibits AChE assayed on a neutral substrate, but *reversibly*. The kinetics of inhibition follows the straightforward Michaelis and Menten laws for competitive inhibition.

With native AChE the covalent binding of TDF at the active site is assumed to involve, at first, the establishment of a reversible transitory complex:

$$E + TDF \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (E \cdot TDF) \underset{}{\overset{}{\rightarrow}} E \cdot I.$$

The question is: Which of the two steps is altered after exposure of AChE to DTT? In order to answer this question we first determined $K_T = k_2/k_1$ of the first step by graphic analysis of the kinetic data obtained with native AChE (Fig. 4) and then measured the K_i of TDF for the competitive inhibition of DTT-AChE.

It was found that the values measured for both constants are identical: $K_T = K_i = 2.5 \times 10^{-5}$ M. DTT treatment thus appears to impair only the covalent attachment of TDF to the enzyme, presumably by decreasing the reactivity of the amino acid side chain located in the neighborhood of the active site which bonds to the diazonium group.

We then tried to reverse the effect of DTT by subsequent incubation of DTT-AChE with various thiol-group reagents. Three of them were tested: PMB, NEM, and DTNB; only PMB showed an effect. After PMB exposure, TDF again irreversibly inhibits AChE, but the kinetics of inhibition differ from those observed with the native protein. With indophenylacetate as the substrate, only the first phase of inactivation occurs: it is no longer followed by a

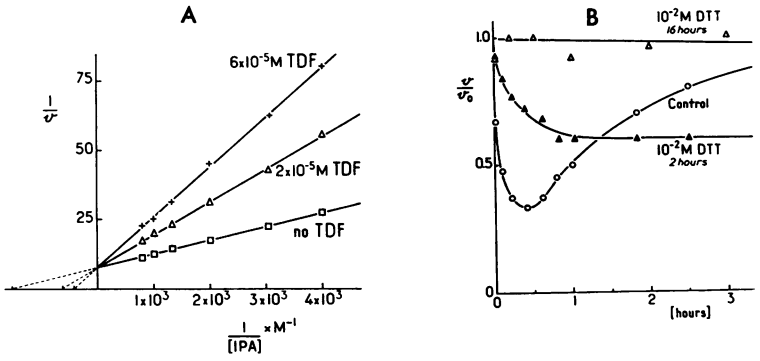

FIG. 3.—*In vitro* effect of DTT on AChE. (*a*) Abolition of the irreversible inhibition of AChE by TDF after exposure to 10^{-2} *M* DTT. A solution of AChE from electric eel (specific activity: 160 μmoles IPA hydrolyzed/ mg protein) containing 0.7 mg protein/ml made in 5×10^{-2} *M* Na phosphate, pH 7.0, was incubated with 10^{-2} *M* DTT for the indicated period of time at 4°C; the reaction was stopped by rapid filtration of the mixture on a G-25 Sephadex column. The fraction having the highest activity which contained around 0.35 mg protein/ml was made 10^{-4} *M* with TDF. The mixture was then diluted 200-fold at the indicated time and AChE activity assayed on IPA following the method of Kramer and Gamson.[15]

AChE was a gift of Dr. W. Leuzinger from Columbia University and TDF was from Drs. H. Mautner, Yale University, and M. Julia, Institut Pasteur.

(*b*) Competitive inhibition of IPA hydrolysis by TDF after 16 hr exposure of AChE to 10^{-2} *M* DTT. DTT-AChE was prepared according to the method described in the legend of (*a*). The concentration of protein in the assay medium (5×10^{-2} *M* sodium phosphate pH 7.0) was 1.75 μg/ml.

reactivation phase. We have interpreted this second phase as due to the covalent binding of TDF to peripheral anionic sites distinct from the anionic site of the active center: in the DTT-PMB enzyme either TDF no longer reacts with these sites or the "indirect" interaction between these sites and the enzyme-active center no longer occurs; in other words, in the terminology used with regulatory enzymes, the enzyme is "desensitized." Experiments are presently in progress to distinguish between these two alternatives.

Discussion.—The experiments presented in this paper indicate that DTT, a compound known to reduce disulfide bonds in proteins, alters in a parallel manner the reactivity of both AChE and ACh receptors toward TDF, an affinity-label-

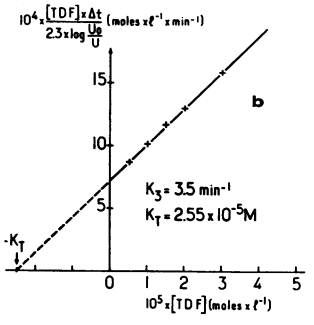

FIG. 4.—Determination of the dissociation constant of the transient reversible complex (AChE·TDF) established as the first step of the affinity labeling of native AChE by TDF. A solution of purified AChE (Sigma Chemical Co.) in 5×10^{-2} *M* Na phosphate buffered at pH 7.0 containing 1.5 μg of protein/ml is supplemented at zero time with TDF at the indicated concentration (the temperature was 23°C). At time Δt a sample is diluted 100-fold in the assay medium containing 5×10^{-2} *M* Na phosphate pH 7.0, 5.0×10^{-4} *M* 5,5′-dithiobis-2-nitrobenzoic acid, and 5.0×10^{-4} *M* acetylthiocholine, and the enzymatic activity *U* is measured. U_0 is the activity measured in the absence of TDF. In this experiment Δt was 1 min.

ing reagent. The most striking feature is that in both cases, after DTT treatment, TDF still exhibits a high affinity for both macromolecules but its effects seem to be reversible. We must emphasize, however, that the comparison of the reactivity of both ACh binding proteins can hardly be rigorously done in the same conditions. First of all, with the native electroplax, the kinetic constants of the irreversible binding of TDF to the membrane site are not accessible since the product of the reaction of TDF with the membrane is not yet measured quantitatively. In addition, we used different ranges of DTT concentration with AChE and the electroplax. With the electroplax a five-minute exposure to 10^{-3} *M* DTT is sufficient to bring about a profound alteration of the cell response. With AChE a treatment of several hours with 10^{-2} *M* DTT is required for an observable effect. However, such a difference should not be overemphasized since the reactivity of proteins integrated in a membrane and dispersed in solution might be widely different. Finally, the most important fact is that the concentration of TDF which promotes a reversible depolarization of the DTT-cell are much lower than those that we used, with similar exposure time, to obtain an irreversible inhibition of the native cell. However, as previously mentioned, the response of the DTT-cell to TDF is maximal around 5×10^{-6} *M;* in these conditions it can be reasonably assumed that the majority of TDF binding sites which are involved in the depolarization process are occupied by TDF, and for exposure times identical to those used for the affinity labeling of the native receptor, no irreversible effects are observed. A reasonable interpretation is that the affinity of TDF for the membrane increases after DTT treatment and that TDF no longer reacts covalently with these high-affinity receptor sites.

In the case of AChE the situation is much simpler and we have shown that the K_i for the reversible inhibition of DTT-AChE by TDF is almost identical to the dissociation constant of the first reversible complex TDF-AChE established in the course of the affinity labeling of the active site of native AChE. Only the *rate* of the covalent attachment of TDF to AChE active center is decreased. Thus the number, nature, and position of the amino acid side chains which account for the recognition of TDF at the AChE active center have not been drastically altered after DTT treatment. DTT promotes a minor but characteristic rearrangement of AChE which decreases the reactivity of an amino acid side chain (presumably a tyrosyl) which reacts with the diazonium group. A similar interpretation can be proposed for the reversibility of TDF action on the membrane, although it cannot be ruled out that the TDF binding sites which account for the depolarization of the DTT cell do not derive from the class of site which reacts with TDF in the normal cell, but correspond to an entirely new class unmasked by DTT treatment.

In the course of these studies we have compared the effects of DTT on the electroplax response to a variety of cholinergic agents. Several results tend to suggest that *distinct sites* account for the permeability changes caused by decamethonium and carbamylcholine. (1) After DTT treatment the response to Deca is potentiated while the response to Carb is decreased. In addition, the potentiation of the response to Deca tends to decay with time, while the inactivation of Carb response remains stable. PMB following DTT treatment blocks

the potentiated response to Deca but does not affect the reduced response to Carb. (2) In a normal cell, as previously mentioned, the dose-response curve to Carb is converted from a sigmoid into an hyperbola in the presence of Deca.[9] (3) When the responses to Carb and Deca are compared after a brief exposure of the normal cell to TDF, the response to Deca is found to be more extensively decreased than the response to Carb.[10]

The experimental data, however, are still too fragmentary to let us conclude whether or not these different sites are carried by the same macromolecular complex, although the experiment referred to in section (2) strongly supports this conclusion.

The possibility that the AChE molecule would be the macromolecular receptor for at least one class of these binding sites still remains. In connection with this problem it should be mentioned that (*a*) AChE presents a high affinity for Deca in high salt media (the K_D of the complex AChE-Deca, measured by equilibrium dialysis, is 1.65×10^{-6} *M* in 5×10^{-2} *M* Na-phosphate buffered at pH 7.0);[12] (*b*) Deca binding to AChE is completely abolished after exposure of AChE to TDF.[12] It is suggested that if the AChE molecule were involved in some electrogenic process as a macromolecular receptor, it would be likely to carry the receptor site for Deca.

An important feature of the consequence of DTT treatment is that TDF, an inhibitor of the normal cell, becomes an activator, a result which parallels the observations made by Karlin and Winnik with hexamethonium.[8] Following our general hypothesis that the molecular mechanisms which account for membrane excitation resemble in many respects the regulatory interaction mediated by allosteric proteins, we wish to point out that this effect of DTT is highly reminiscent of some properties observed several years ago with a bacterial regulatory enzyme, the biosynthetic L-threonine deaminase. Indeed some mutation in the structural gene of L-threonine deaminase leads to an inversion of its response to the feedback inhibitor, L-isoleucine; L-isoleucine acts as an activator of the mutant protein.[13] These effects can be interpreted in terms of the theory that we have extensively discussed previously to account for allosteric interaction in regulatory enzymes and excitable membranes.[14]

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* The Hill coefficient (n_H) of the response curve to Deca immediately following DTT treatment is close to 2 as in the control, whereas the n_H of the response to Carb is reduced to 1. However, 30 min after DTT treatment the n_H of the residual response to Deca decreases as well, to a value close to 1. The apparent depolarizing effect of TDF is due to TDF and not to a product of the reaction of TDF with DTT: if the native electroplax is exposed to a mixture of TDF and DTT, no depolarization occurs.

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