MOLECULAR PHARMACOLOGY, 12, 813-819

A Membrane Activation Cycle Induced by Sulfhydryl Reagents after Affinity Labeling of the Acetylcholine Receptor of Electroplax

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(Received December 15, 1975)
(Accepted May 5, 1976)

SUMMARY

BARTELS-BERNAL, EVA, ROSENBERRY, TERRONE L. & CHANG, HAI WON (1976) A membrane activation cycle induced by sulfhydryl reagents after affinity labeling of the acetylcholine receptor of electroplax. *Mol. Pharmacol.*, 12, 813-819.

Acetylcholine receptors in the innervated membrane of an electroplax cell from the electric eel *Electrophorus electricus* may be affinity-labeled, after disulfide reduction, by a variety of sulfhydryl alkylating and acylating agents. The conductance state of a cell after this receptor modification remains under receptor control but is extremely sensitive both to the structure of the affinity reagent and, frequently, to the concentration of the reagent during modification. When the affinity reagents 3-(α-bromomethyl)-3'-[α-(trimethylammonium)methyl]azobenzene bromide or 4-(4'-nitrophenoxycarbonyl)-phenyltrimethylammonium iodide are used in low concentration to label the receptor *in situ*, subsequent sequential applications of disulfides and dithiothreitol give rise to cycles of repolarization and depolarization of the innervated membrane. Since other mild oxidizing agents cannot substitute for disulfides in this activation cycle, it is concluded that the activation cycle arises from the reversible formation of a mixed disulfide on the receptor. This mixed disulfide probably involves the remaining sulfhydryl group near the acetylcholine binding site, which is formed by the initial disulfide reduction but not labeled by the affinity reagents. The membrane responses to these chemical manipulations of the receptor *in situ* suggest useful criteria for evaluating the similarity of an isolated receptor after reconstitution into black lipid membranes to the receptor in its native state.

INTRODUCTION

The importance of a disulfide bond in determining the response of the acetylcholine receptor *in situ* to applied agonists has been well established (1, 2). Investigations with the monocellular electroplax preparation from electric eel (*Electrophorus electricus*) have shown that disulfide reduction by dithiothreitol shifts the dose-response curve for depolarization by exogenous carbamylcholine to the right (1). This apparent reduction in carbamylcholine affinity is reversed by mild oxidizing agents, which presumably restore the original disulfide bonding. Application of sulfhydryl alkylating or acylating agents after disulfide reduction leads to an irreversible modification of the receptor response, and the

This investigation was supported in part by United States Public Health Service Grant NS-03304, by National Science Foundation Grants NSF-GB-40055 and 31122, and by the New York Heart Association, Inc.

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use of quaternary ammonium alkylating agents to "affinity label" both the receptor in the cell membrane and the detergent-solubilized receptor has been introduced by Karlin and his colleagues (3, 4). Affinity alkylation with 4-(*N*-maleimido)-benzyltrimethylammonium iodide occurs on a 40,000 mol wt subunit of the receptor with an over-all stoichiometry of about 1:200,000 mol wt; labeling can be blocked by specific receptor inhibitors like α-neurotoxins. Pharmacological effects of disulfide reduction and alkylation similar to those on the eel have been observed on neuromuscular junctions (5, 6). In frog neuromuscular junction it was also shown that these effects were not due to changes in the impedance of the postsynaptic membrane or in the end plate reversal potential (5); observations by these workers are consistent with a chemical modification at the agonist-binding site of the receptor.

It is of interest that the receptor response *in situ* to disulfide reduction and affinity alkylation varies among alkylating agents, with some showing irreversible receptor activation and others giving irreversible receptor inhibition (2). Two affinity alkylating agents, 4-(4'-nitrophenoxycarbonyl)phenyltrimethylammonium iodide (7) and 3-(α-bromomethyl)-3'-[α-(trimethylammonium)methyl]azobenzene bromide (8), have been reported to give irreversible partial activation only after excess free alkylating agent has been washed away. Alkylation with these two agents, while shifting the distribution of active and inactive receptor species, still permits the receptor conformational transition between the active and inactive forms. This novel feature of these two alkylating agents is exploited in our study to demonstrate reversible receptor activation of alkylated receptor by further treatment with sulfhydryl reagents.

METHODS

The technique was the same as described previously (9, 10). A single electroplax from the electric organ of *Electrophorus electricus* was mounted in a Lucite chamber and impaled with conventional micropipettes filled with 3 M KCl, and the resting potential across the innervated face was recorded on a Varian paper recorder. Concentrations of components in the eel Ringer's solution were as follows: NaCl, 160 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; NaH_2PO_4, 0.3 mM; Na_2HPO_4, 1.2 mM; and glucose, 10 mM. The pH was 7, and the temperature, 22–25°. Additional reagents, as indicated under RESULTS, were added only to the Ringer's solution bathing the innervated face. Oxidizing and reducing agents were dissolved in modified eel Ringer's solution at pH 8, where the phosphate buffer was replaced by 2 mM Tris.

QBr[2] was provided by Dr. B. Erlanger; MBTA, by Dr. A. Karlin; 2,2'-dithiobis-(ethyltrimethylammonium) dibromide, by Dr. H. Mautner; and BAC and NPTMB, by the Squibb Institute for Medical Research. Table 1 gives the structural formulae of reagents used in this study.

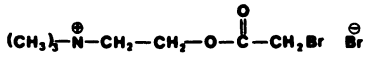
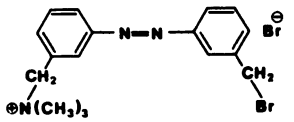
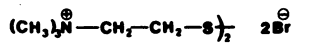
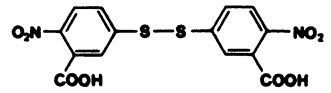
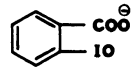
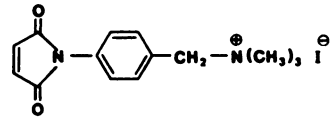
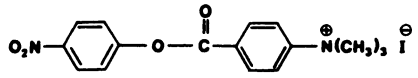
RESULTS

Reversible activation of QBr-alkylated membrane receptor by sequential treatment with certain oxidizing agents and DTT. The sequence of procedures routinely used to generate affinity-alkylated acetylcholine receptor *in situ* is shown in the upper part of Fig. 1. Treatment of the innervated face of the cell with DTT reduces the control carbamylcholine response, and exposure of the DTT-treated cell to QBr gives a small depolarization which is enhanced after the excess unreacted alkylating agent has been washed out. A similar response is observed with the alkylating agent NPTMB (7). The lower part of Fig. 1 shows the response of this affinity-alkylated cell to sequential applications of disulfides and DTT. Either 2,2'-dithiobis(ethyltrimethylammonium) dibromide or DTNB repolarizes the cell, and DTT gives a rapid, somewhat tran-

[2] The abbreviations used are: QBr, 3-(α-bromomethyl)-3'-[α-(trimethylammonium)methyl]-azobenzene bromide; MBTA, 4-(*N*-maleimido)-benzyltrimethylammonium iodide; BAC, bromoacetylcholine bromide; NPTMB, 4-(4'-nitrophenoxycarbonyl)phenyltrimethylammonium iodide; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

TABLE 1

Structural formulae of reagents used in this study

Bromoacetylcholine bromide	
3-(α-Bromomethyl)-3′-[α-(trimethylammonium)methyl]azobenzene bromide	
2,2′-Dithiobis(ethyltrimethylammonium)dibromide	
5,5′-Dithiobis(2-nitrobenzoic acid)	
o-Iodosobenzoate	
4-(*N*-Maleimido)benzyltrimethylammonium iodide	
4-(4′-Nitrophenoxycarbonyl)phenyltrimethylammonium iodide	

sient depolarization. Another disulfide compound, cystine at 10 mM, acts similarly to 2,2′-dithiobis(ethyltrimethylammonium) dibromide in repolarizing the cell after the initial affinity alkylation or the subsequent sequential application of DTT; however, depolarizations with DTT after repolarizations with these two disulfides are somewhat smaller than those after repolarizations with DTNB. After the last application of DTNB to the affinity-alkylated cell in Fig. 1, a reduced depolarization with carbamylcholine can still be obtained; this suggests that the affinity label remains covalently attached but does not completely block access of carbamylcholine to the receptor active site. An alternative explanation for this carbamylcholine response is that not all receptor sites have been affinity-alkylated; in this case the reduced depolarization would indicate the residual response of the unmodified receptors. If an experiment similar to that in Fig. 1 is carried out with NPTMB (10 μM) in place of QBr, disulfide and DTT again give cycles of repolarization and depolarization similar to that in Fig. 1. These experiments with QBr and NPTMB have been repeated many times with different cells and show high qualitative reproducibility.

When the mild oxidizing agents *o*-iodosobenzoate or potassium ferricyanide are substituted for the disulfide, the activation cycle or repolarization and depolarization shown in Fig. 1 does not occur. As shown in Fig. 2, treatment of a cell affinity-alkylated by QBr with potassium ferricyanide gives a depolarization because of the increased external potassium that is reversed in Ringer's solution. If DTT is added at this point, no depolarization is observed. However, the addition of DTNB repolarizes the cell, after which DTT will

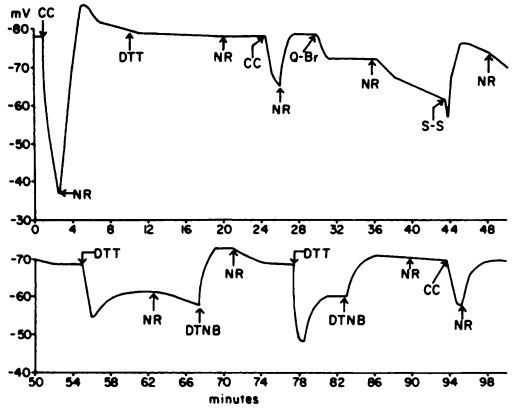


FIG. 1. *Treatment of QBr-alkylated cell with disulfides and DTT*

The membrane potential across the innervated face was recorded as outlined under METHODS. CC, carbamylcholine (40 μM); NR, Ringer's solution; SS, 2,2'-dithiobis(ethyltrimethylammonium) dibromide (0.1 mM); DTT, 1.0 mM; QBr, 1.0 μM; DTNB, 1.0 mM.

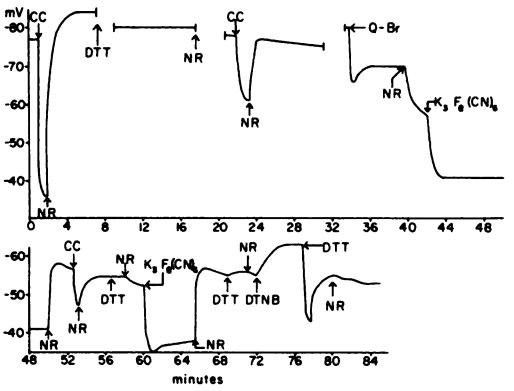


FIG. 2. *Treatment of QBr-alkylated cell with oxidizing agents and DTT*

CC, carbamylcholine (50 μM); NR, Ringer's solution; QBr, 5 μM; $K_3Fe(CN)_6$, 5 mM; DTT, 1.0 mM, DTNB, 1.0 mM. The procedure was the same as in Fig. 1.

depolarize. Thus exposure to potassium ferricyanide does not prevent the activation cycle seen with DTNB and DTT in Fig. 1. *o*-Iodosobenzoate (1 mM) gives similar results. The failure of potassium ferricyanide and *o*-iodosobenzoate to substitute for disulfides in the activation cycle contrasts with their action after DTT reduction of an unmodified cell. Both these oxidizing agents, like disulfides, reverse the pharmacological effects of an initial DTT exposure at low concentrations (potassium ferricyanide, 5 mM; *o*-iodosobenzoate, 10 μM; also see refs. 1 and 11).

If disulfides and DTT are reacting with one or more sulfhydryl groups in the QBr-alkylated cell, further exposure to alkylating agents should prevent the cycle of repolarization and depolarization seen in Fig. 1 by blocking these groups. This is in fact the case. A high concentration of QBr (1 mM) during affinity alkylation does not result in depolarization after the excess reagent has been washed out. DTNB and DTT do not affect the membrane potential in this highly alkylated cell. Furthermore, treatment of the cell with 1 mM *N*-ethylmaleimide, after affinity alkylation with low concentrations of QBr as in Fig. 1, completely blocks sequential repolarization and depolarization with DTNB and DTT.

While this observation, that high concentrations of alkylating agents block the activation cycle with DTNB and DTT, tends to confirm the involvement of a sulfhydryl group in the cell, it suggests a difficulty in quantifying the response to a given concentration of affinity label. If only "low" concentrations of affinity label lead to an activation cycle, while "high" concentrations block the cycle, an intermediate concentration range should exist in which variable effects are observed. Such an intermediate concentration appears to be 5 μM for QBr. At this concentration the activation cycle appears to be blocked in most cells (also see Fig. 4 of ref. 8) but still accessible in a few cells (see Figs. 2 and 5). This variability may depend on the metabolic state of the cell. Treatment of DTT-reduced cells with 0.5–2.0 μM QBr or 10–50 μM NPTMB usually leads to an activation cycle. At 5 μM NPTMB only a small amount of affinity labeling is indicated by the cell response, and the subsequent activation cycle is greatly reduced.

Failure of other alkylated membrane receptors to give reversible activation with disulfides and DTT. Acetylcholine receptor affinity-alkylated *in situ* either with BAC (Fig. 3) or with MBTA (Fig. 4) also may be generated, as reported previously (2). In Fig. 3, BAC depolarizes the reduced innervated membrane both before and

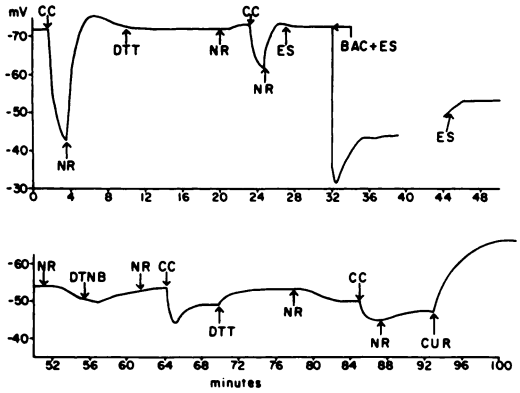


FIG. 3. *Treatment of BAC-alkylated cell with DTNB and DTT*

ES, physostigmine (50 μM); CC, carbamylcholine (50 μM); CUR, *d*-tubocurarine (0.1 mM); NR, Ringer's solution; BAC, 50 μM; DTT, 1.0 mM; DTNB, 1.0 mM. The procedure was the same as in Fig. 1. Physostigmine was added to block hydrolysis of BAC by acetylcholinesterase.

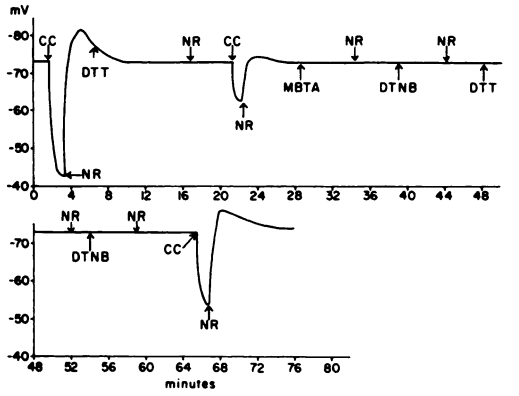


FIG. 4. *Treatment of MBTA-alkylated cell with DTNB and DTT*

CC, carbamylcholine (50 μM); NR, Ringer's solution; MBTA, 0.2 μM; DTT, 1.0 mM; DTNB, 1.0 mM. The procedure was the same as in Fig. 1.

after the excess reagent has been washed away. Subsequent application of DTNB does not repolarize the membrane, after which DTT also is without effect. The addition of *d*-tubocurarine at the end of the experiment restores the membrane potential to within 5 mV, as observed previously (7), indicating that *d*-tubocurarine can interact with the receptor to reverse the physiological effect of the covalent affinity label. In Fig. 4, MBTA does not depolarize the reduced innervated membrane either before or after washout, and sequential applications of DTNB and DTT have no effect on the membrane potential.

Effect of d-tubocurarine on reversible activation of QBr-alkylated membrane receptor by disulfides and DTT. When DTT and *d*-tubocurrarine are applied simultaneously to the QBr-alkylated innervated face subsequent to repolarization with DTNB (Fig. 5), significant inhibition of the DTT depolarization is observed. Thus *d*-tubocurarine blocks activation of the QBr-alkylated receptor as it does the activation of the BAC-alkylated receptor (Fig. 3). It is noteworthy that although *d*-tubocurarine blocks activation, it apparently does not block DTT reversal of the DTNB repolarization: DTNB must be applied again before a DTT depolarization can be obtained, even after removal of *d*-tubocurarine. These observations can be explained by competitive displacement of the quaternary ammonium group of the covalently bound labeling agent by *d*-tubocurarine acting at the acetylcholine binding site. Such action would not affect the integrity of the covalent bond. Alternatively, *d*-tubocurarine may block activation of the affinity-labeled receptors by acting allosterically at other sites on the receptor.

Figure 5 also demonstrates that the depolarizing action of DTT is seen only on the first addition of DTT; a second depolar-

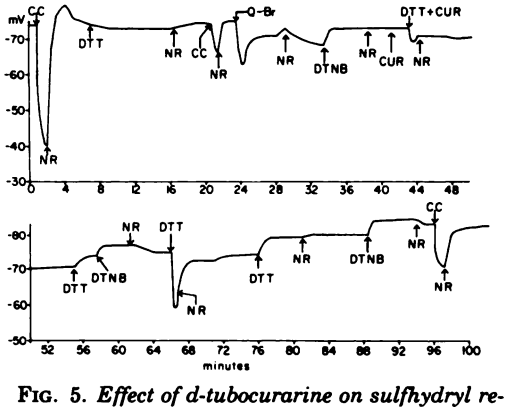


FIG. 5. *Effect of d-tubocurarine on sulfhydryl reagent-induced activation cycle of QBr-alkylated cell*

CC, carbamylcholine (40 μM); CUR, *d*-tubocurarine (10 μM); NR, Ringer's solution; QBr, 5 μM; DTT, 1.0 mM; DTNB, 1.0 mM. The procedure was the same as in Fig. 1.

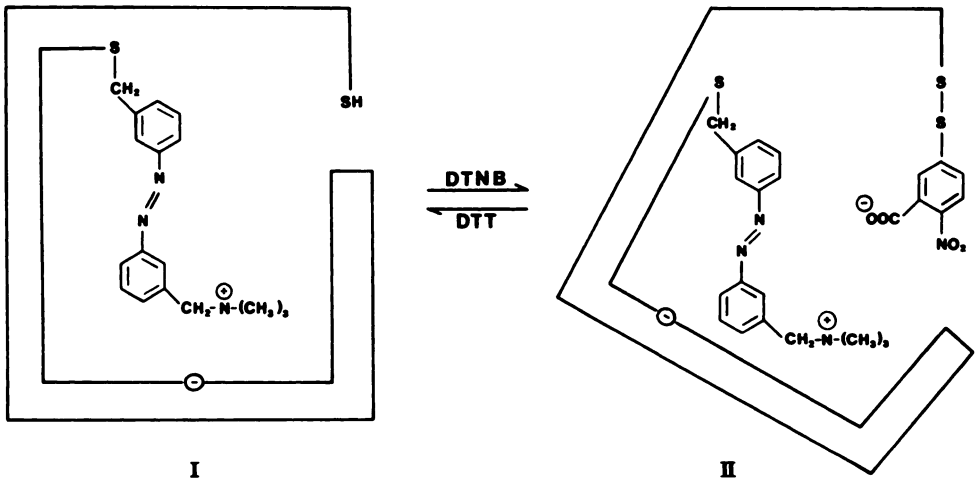
ization requires a "resetting" of the membrane by DTNB.

DISCUSSION

Affinity alkylation following disulfide reduction of the isolated electroplax cell has been carried out with [^{3}H]MBTA (3). Careful analysis of the distribution of the label by sodium dodecyl sulfate-gel electrophoresis has shown that, while not completely specific, about 10% of the label is associated with a 40,000 mol wt subunit which appears to correspond to a similar 40,000 mol wt subunit that can be affinity-labeled in the isolated acetylcholine receptor (4). No other discrete polypeptide species is labeled to this extent in the intact cell (3), and no other subunit in the isolated receptor is labeled (4). Furthermore, the over-all labeling stoichiometry of 1 MBTA/200,000 mol wt in the isolated receptor indicates no more than one labeling site per acetylcholine binding site. No direct evidence is available concerning the number of disulfide bonds in the receptor that are reduced by the initial DTT treatment prior to affinity labeling. Because our data provide no evidence for the reduction of more than one disulfide bond [see point (b) below], we assume that only a single disulfide bond in the receptor is reduced by the initial DTT treatment. Although labeling specificity studies have not been carried out with QBr and NPTMB, we shall assume that a similar specificity obtains and that our studies *in situ* reveal the effects of affinity alkylation of a single reduced disulfide bond in the acetylcholine receptor.

Our observations of a novel activation of the affinity-alkylated receptor by DTT and its reversal by disulfides occur only when QBr and NPTMB are the alkylating agents. Prior to the initial application of DTT, when no apparent covalent labeling can occur, both these agents act as reversible antagonists of receptor activation. After covalent reaction both agents act as weak agonists. In contrast, neither BAC, initially a reversible agonist, nor MBTA, initially a reversible antagonist, changes it mode of action after alkylation, and neither agent shows a reversible activation cycle with DTT and disulfides. Thus the activation cycle perhaps occurs only with affinity-alkylated receptors for which the free energy difference between the active, ion-conducting conformation and the inactive conformation is small.

A model which accounts for the activation cycle of QBr-alkylated receptors is shown in Scheme 1. We assume that only one of the two sulfhydryls generated by reduction of the native receptor with DTT can be labeled specifically with QBr (Scheme 1, structure I; also see ref. 4). The conformation of I corresponds to a partially activated receptor, i.e., one which corresponds to an increased conductance state. On addition of a disulfide the second sulfhydryl group is postulated to form a mixed disulfide II. In II the receptor is in



I II

SCHEME 1

an inactive conformation. Since the mixed disulfide species is inactive when formed either by an anionic disulfide (DTNB, II) or by a cationic disulfide (2,2'-dithiobis-(ethyltrimethylammonium) dibromide), inactivation probably occurs by a steric deformation of the active site in I rather than by an electrostatic interaction. The evidence for the conversion of I to II by disulfides is as follows. (a) Disulfides interact with a free sulfhydryl group; exposure of I to high concentrations of the alkylating agents QBr or N-ethylmaleimide blocks the conversion of I to II. (b) The sulfhydryl group which interacts with disulfides does not arise from a second disulfide bond that is generated by the initial treatment with DTT but unaffected by affinity alkylation. If such a second disulfide bond were involved, the mild oxidizing agents *o*-iodosobenzoate and potassium ferricyanide should act like disulfides in restoring this disulfide bond and in participating in the activation cycle. This expectation is based on the very low concentrations with which these agents reverse an initial DTT reduction in an unmodified cell, presumably by reoxidation to give the initial disulfide bond. Treatment of the QBr-alkylated cell with these oxidizing agents, however, does not result in subsequent activation by DTT. (c) The sulfhydryl group which interacts with disulfide is not present in the native receptor. Treatment of the cell with *N*-ethylmaleimide prior to the initial DTT reduction has no effect on the subsequent activation cycle of the QBr-labeled receptor with disulfides and DTT.

A precedent for the inhibitory effect of mixed disulfide formation in biological systems comes from studies on glycogen synthetase D by Ernest and Kim (12). These workers demonstrated enzyme inactivation on formation of mixed disulfides from oxidized glutathione and the sulfhydryl group(s) of the enzyme. The inactivated enzyme can be reactivated by reduced glutathione.

The manipulation of physiological activity by these chemical modifications of the acetylcholine receptor *in situ* may provide useful criteria for assessing model reconstitution studies involving the insertion of isolated acetylcholine receptor into artificial membranes. In particular, studies which monitor conductance changes after the insertion of putative isolated receptor into black lipid membranes (13–15) have not utilized many pharmacological criteria and may be aided by these receptor modification properties. In addition to the effects of agonists and antagonists on conductance changes, such studies could assess the similarity of the reconstituted receptor response to that *in situ* when the chemical modifications described here are carried out.

ACKNOWLEDGMENTS

We thank Dr. David Nachmansohn for his encouragement of this work. We also thank Drs. B. Erlanger, A. Karlin, and H. Mautner and the Squibb Institute for Medical Research for their generous gifts of the chemicals indicated under METHODS.

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