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The Effect of Electrical Stimulation on the Action of Sulfhydryl Reagents in the Giant Axon of Squid; Suggested Mechanisms for the Role of Thiol and Disulfide Groups in Electrically-Induced Conformational Changes

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Summary. Conduction block by thiol reagents is potentiated by repeated, brief electrical stimulation. These studies have been quantitated with N-ethylmaleimide and mercurochrome showing a nonlinear relationship between dose and number of stimuli required to produce inexcitability. *p*-Chloromercuribenzoate, mercurochrome and fluorescein mercuric acetate block conduction and are reversible with β-mercaptoethanol and exhibit the "stimulation effect." N-Ethylmaleimide, Ellman's reagent (DTNB), and 2-dimethylaminoethyl selenolbenzoate exhibit the "stimulation effect", but blockade is irreversible. In a series of local anesthetics, procaine, 2-diethylaminoethyl thiolbenzoate, and 2-dimethylaminoethyl selenolbenzoate, only the selenolester reacts with SH groups and shows a "stimulation effect". Iodoacetate and iodoacetamide block nerve conduction without a stimulation effect. Possible interpretations of this effect include: altered permeability, unmasking of buried SH groups in the membrane, or electrolytic reduction of disulfides.

Extensive work has been conducted in recent years to show that conformational changes occur in nerve membranes during the conduction of the nerve impulse [4, 30, 31]. It was proposed in 1952 that alterations in ion permeability in electrically excitable membranes involve a conformational change triggered by the attachment of acetylcholine to a protein, the "acetylcholine receptor" [22]. Since then, many laboratories have attempted to isolate the "acetylcholine-receptor" biopolymers from a variety of sources [14] and have provided evidence that proteins are major components of the receptor molecules in all cases studied, although it must be assumed that other biopolymers are involved in the functions of the intact system.

In view of the essential nature of proteins in excitable membranes, it is not surprising that their functions in both axonal and synaptic preparations are modified by sulfhydryl reagents. Smith [27] and Huneeus-Cox, Fernandez and Smith [16] have demonstrated conduction block in nerve fibers of frog, lobster and squid with mercuric chloride and organic mercury derivatives as well as with 5,5′-dithiobis (2-nitro benzoic acid) (Ellman's Reagent, DTNB), a thiol oxidizing agent.

Similarly, it was observed in synaptic preparations such as the electroplax from *Electrophorus electricus* [19] and the frog neuromuscular junction (E. M. Landau, *personal communication*) that electrical excitability could be blocked by sulfhydryl reagents. Furthermore, Karlin and Bartels [19] demonstrated that treatment of the electroplax preparation with *p*-chloromercuribenzoate (PCMB) or dithiothreitol (DTT) inhibits the carbamylcholine depolarization reversibly. N-Ethylmaleimide (NEM), on the other hand, inhibits only repolarization, suggesting that sulfhydryl groups are available for reaction with NEM only in the depolarized membrane. NEM binds only to the reduced electroplax preparation, abolishing the reversibility of the inhibition by DTT and PCMB.

These observations with excitable membranes and the demonstrated susceptibility of several membrane systems to organic and inorganic mercury compounds [24, 33, 34] suggested that it might be interesting to study the effects of electrical stimulation on the reactivity of excitable membranes with sulfhydryl reagents. Preliminary experiments by Hillman and Mautner [15] on squid giant axons showed that conduction block by PCMB and NEM was potentiated by repeated, brief electrical stimulation. It was the purpose of the present work to extend and quantitate these observations.

Materials and Methods

Giant axons of the squid *Loligo pealii* obtained at the Marine Biological Laboratories, Woods Hole, Massachusetts, were dissected and most of the adjacent small nerve fibers were removed. In those experiments where microelectrode impalements were employed, an effort was made to remove all of the small fibers adhering to the giant axons in the region of impalement.

Solutions of the various compounds used were freshly prepared using artificial seawater (423 mM NaCl, 9 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 2.15 mM $NaHCO_3$) buffered at pH 7.6 to 8.0 with 1 mM Tris. This concentration of Tris has been shown to have no effect on spike height over a period of several hours [26].

For external recordings the nerve was placed on five Ag–AgCl electrodes in a 25-ml Lucite chamber: two recording electrodes, two stimulating and one ground. Every 5 or 10 min the seawater was removed for recording of the axonal action potentials. The nerve was stimulated a few times with pulses of 0.4-msec duration and of controlled intensity. At the minimal voltage required to stimulate the giant axon, the small nerve

fibers did not respond. A Grass S4 stimulator and a Grass SIU 4678 stimulus isolation unit were used. Responses were recorded on a Tekronix Type 564 storage oscilloscope via a Tektronix Type 3A3 dual-trace differential amplifier and photographed using a Tektronix Type C-27 camera.

To test whether a drug exhibits a "stimulation effect", that is, whether the effect of a drug is increased by electrical stimulation of the nerve fiber, the compound was applied to the axon in a concentration which produced a 10 to 20% decrease of action potential amplitude. The time of exposure is concentration-dependent. The chamber was drained. Then the fiber, kept moist with artificial seawater, was stimulated at threshold levels 1 to 6 times/sec for up to 240 sec or until blockade occurred. Controls demonstrated that the repeated draining of fluid with the extracellular technique does not introduce significant variations in the parameters measured. Controls also demonstrated the ability of the axons to survive extended periods (over 60 min) of repeated electrical stimulation at this frequency. It is necessary to block conduction partially before stimulation can produce complete block. The number of stimuli required to produce inexcitability was stored and monitored on the oscilloscope screen. Each axon was used for only one complete experiment (block and reversal of conduction block).

For recordings with intracellular electrodes, a Lucite chamber of 5-ml capacity was mounted on a transilluminated stage. Seawater or test solutions entered one end of the chamber from a reservoir. Solutions were continually removed from the other end of the chamber by the use of suction. Stainless steel clamps covered with polyethylene secured the fiber in the chamber. Square-wave pulses from a Grass S4 and a Grass SIU4678 were applied to the fiber through fine silver wires. Glass microelectrodes filled with 3 M KCl and having a tip resistance between 10 and 15 MΩ were used.

To monitor the membrane potentials a strip of Ag–AgCl wire which made contact with the microelectrode was connected to a Bioelectric Instruments NF-1 preamplifier unit. The signal was led into a Tektronix Type 564 oscilloscope and the action potentials photographed. Variations in resting membrane potential were followed on a Varian G-14A strip chart recorder.

2-Dimethylaminoethyl selenolbenzoate and 2-diethylaminoethyl thiolbenzoate were synthesized according to literature methods [13]. Iodoacetate was recrystallized from petroleum ether. All other reagents were of analytical grade.

All experiments were carried out at 18 to 20 °C.

Results

The "stimulation effect," i.e., acceleration of conduction block by repeated, brief electrical stimulation of the nerve fiber after exposure to the test drug, was shown by the following compounds: mercuric chloride, PCMB, mercurochrome, fluorescein mercuric acetate, NEM, DTNB and 2-dimethylaminoethyl selenolbenzoate. For instance, a nerve which shows a decrease in action potential amplitude of 10 to 20% after exposure to 5×10^{-6} M PCMB for 10 min becomes completely inexcitable after stimulation at 6 pulses per sec for about 15 sec, even though, without stimulation, no further inhibition would be seen even after an hour or more. Measurements with 5×10^{-6} M PCMB and 1 mM NEM demonstrated no notable effect of these compounds on the resting membrane potential for the incu-

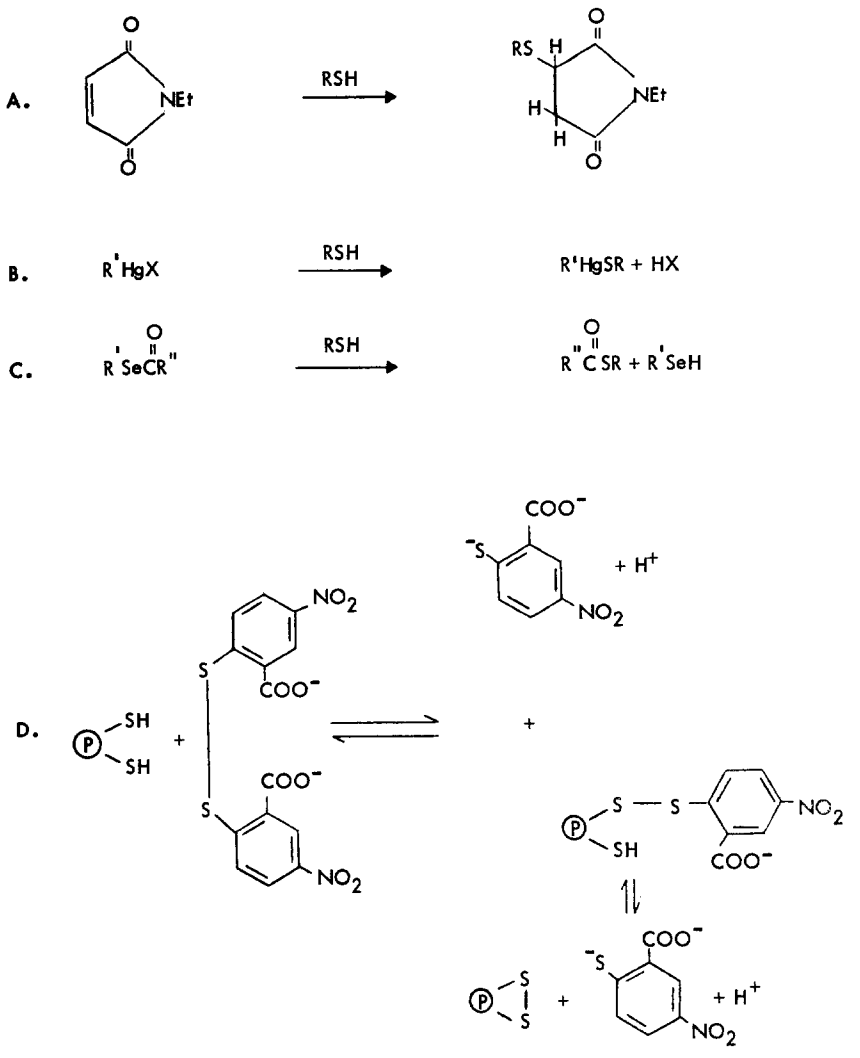

Fig. 1. Thiol reactions of (*A*) NEM, (*B*) organic mercurials, (*C*) selenolesters and (*D*) DTNB

bation times studied. The inhibitory action of the mercury derivatives is largely reversible with 10 mM 2-mercaptoethanol, while the effects of NEM and the selenolester are irreversible. Dithiothreitol (DTT) applied externally in concentrations as high as 20 mM does not affect conduction. Iodoacetate and iodoacetamide (10 mM) block conduction irreversibly but do not exhibit the "stimulation effect."

So far, of all the molecules investigated, only those capable of reacting with thiols induce inhibitory effects which are potentiated by electrical

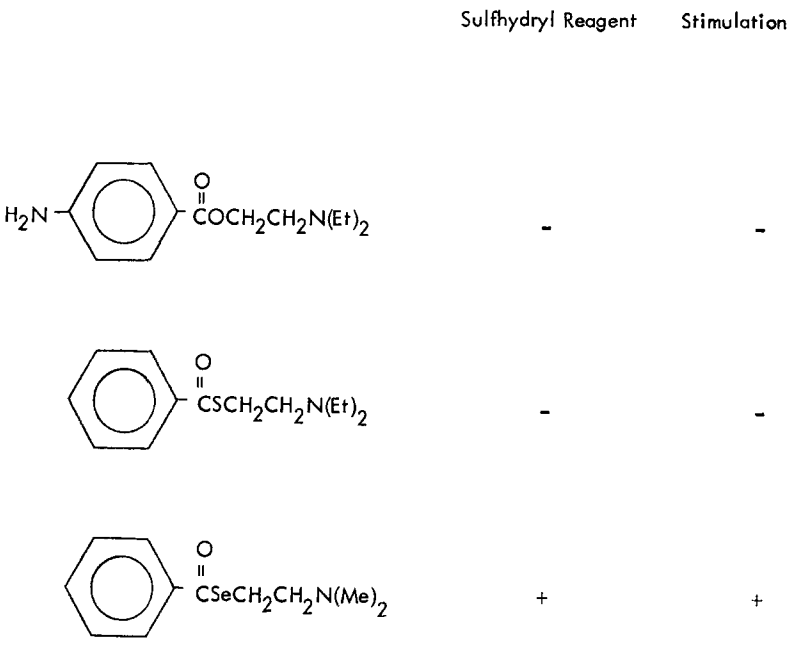

Fig. 2. Local anesthetics used in this study. Local anesthetic activity of the above thiol ester and selenol ester exceeds that of procaine (Rosenberg, P., Mautner, H. G. 1967. Acetylcholine receptors: similarity in axons and functions. *Science* **155**:1569). Note the parallelism between the ability of a compound to react with thiols and its increased effect on axonal conduction following electrical stimulation

stimulation. Their mechanisms of action are summarized in Fig. 1. It is worth noting that of the three local anesthetics shown in Fig. 2, only the selenolester exhibits the "stimulation effect," since among such isologs only selenolesters react efficiently with thiols [21].

Ellman's reagent, DTNB, causes the oxidation of thiols to disulfides, a reaction involving the disproportionation of mixed disulfides. DTNB, in addition to exhibiting the "stimulation effect," blocked conduction irreversibly. At a concentration too low to cause blockade (5×10^{-5} M), DTNB produced an increase in the duration of the action potential (*see* Fig. 3). In a total of five experiments, 5×10^{-5} M DTNB increased the duration of the externally-recorded action potential by a mean value of 0.9 msec which, by paired *t*-test evaluation, is significant to the 1% level.

In view of the postulated importance of calcium concentration in the transmission of the electrical impulse [8, 9, 29], the effects of Ca^{2+} concentration on the "stimulation effect" were investigated. Preincubation of the nerve fibers in artificial seawater containing 50 mM $CaCl_2$ for 30 min

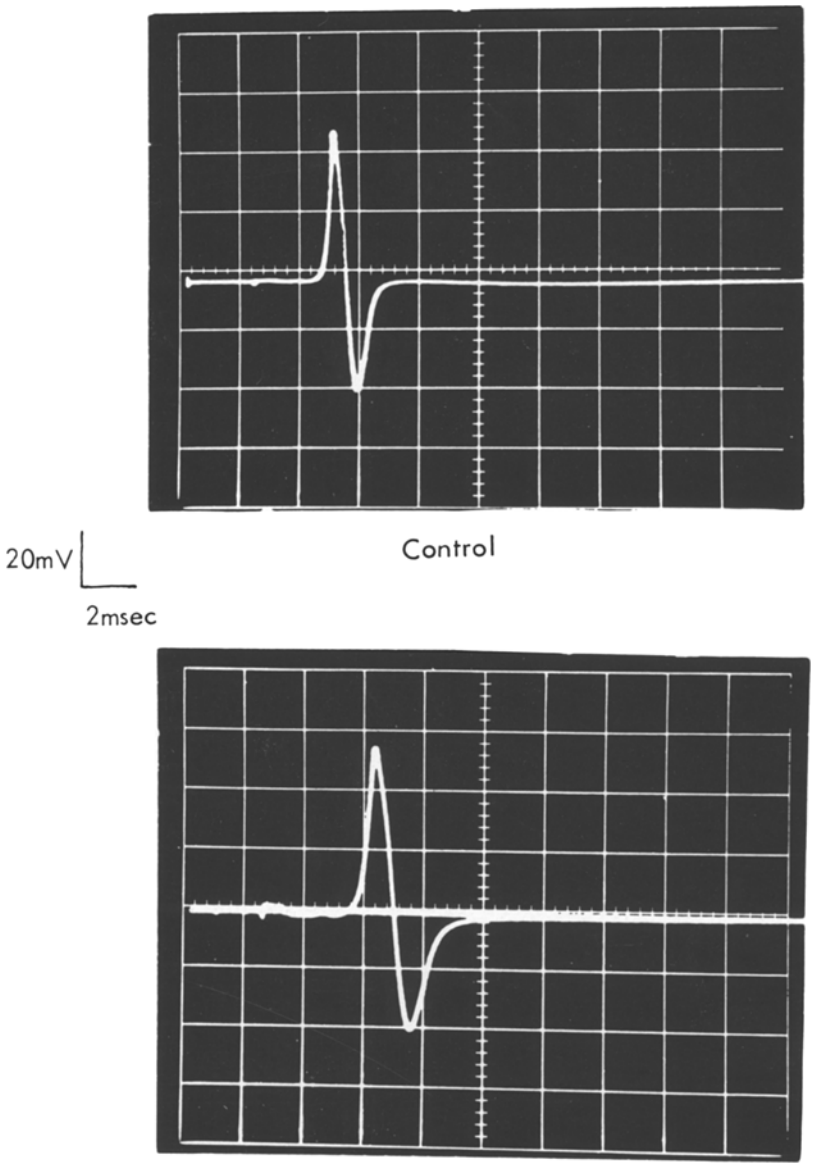

Fig. 3. The effect of 5×10^{-5} M DTNB on the duration of the action potential recorded from squid giant axon. A mean increase of 0.9 msec (n = 5) by DTNB is statistically significant ($p < 0.01$)

prior to application of the inhibitor did not alter the effects of electrical stimulation on the actions of 2×10^{-3} M NEM or 10^{-4} M DTNB.

In three different experiments, we observed that partial procaine block of a nerve fiber must be followed by an additional 10 to 20% block after

application of NEM before the stimulation effect is observed. This suggests that local anesthetics and thiol reagents may be acting at separate sites.

Fig. 4*A* and *B* are dose-response curves for NEM and mercurochrome, respectively. Plotting the number of stimuli required to produce 100% block or plotting the number of action potentials evoked, as a function of concentration of thiol reagent, yields a nonlinear curve. Controls demonstrated that the time to reach 100% block is linearly related to the frequency of stimulation (3 to 12 pulses per sec) for a given concentration of inhibitor. It was also seen that the time required to produce 20% block of conduction is linearly related to the concentration of inhibitor.

Discussion

We have demonstrated that, at levels of thiol reagent high enough to produce about 20% reduction in height of the action potential of squid giant axons, repeated stimulation of the nerve rapidly induces complete block of conduction. At lower concentrations of thiol reagent, repeated stimulation is ineffective in blocking conduction.

Dulhunty and Gage [6] observed a "stimulation effect" when maculotoxin, an as yet poorly characterized poison extracted from the salivary glands of the blue-ringed octopus *Hapalochlaena maculosa* was used to block action potentials recorded from toad sartorius muscle. More recently, a "stimulation effect" was observed when perhydrohistrionicotoxin, isolated from the Colombian arrow frog *Dendrobates histrionicus* was used to block action potentials elicited by direct or indirect stimulation of mouse diaphragm [1]. It has already been noted [19] that, in the electroplax preparation, the inhibitory effect of some thiol reagents depends on the state of depolarization of the conducting membrane.

Several interpretations of the stimulation effect can be considered. It is possible that, upon depolarization, the permeability of the axonal membrane to sulfhydryl reagents might be increased. Thus, it has been reported by Fischer and Litvak [10] that electrical stimulation increased amino acid incorporation into squid giant axons, an effect which was absent when studied in rabbit dorsal root axons [17].

It is also possible that electrical stimulation induces conformational changes resulting in the unmasking of "buried" thiol groups, inaccessible in the resting membrane. This proposal is quite compatible with studies correlating conduction of the nerve impulse with conformational changes [4, 30, 31]. The importance of the availability of thiol groups to the functions of excitable membranes has been emphasized by other workers [2, 35].

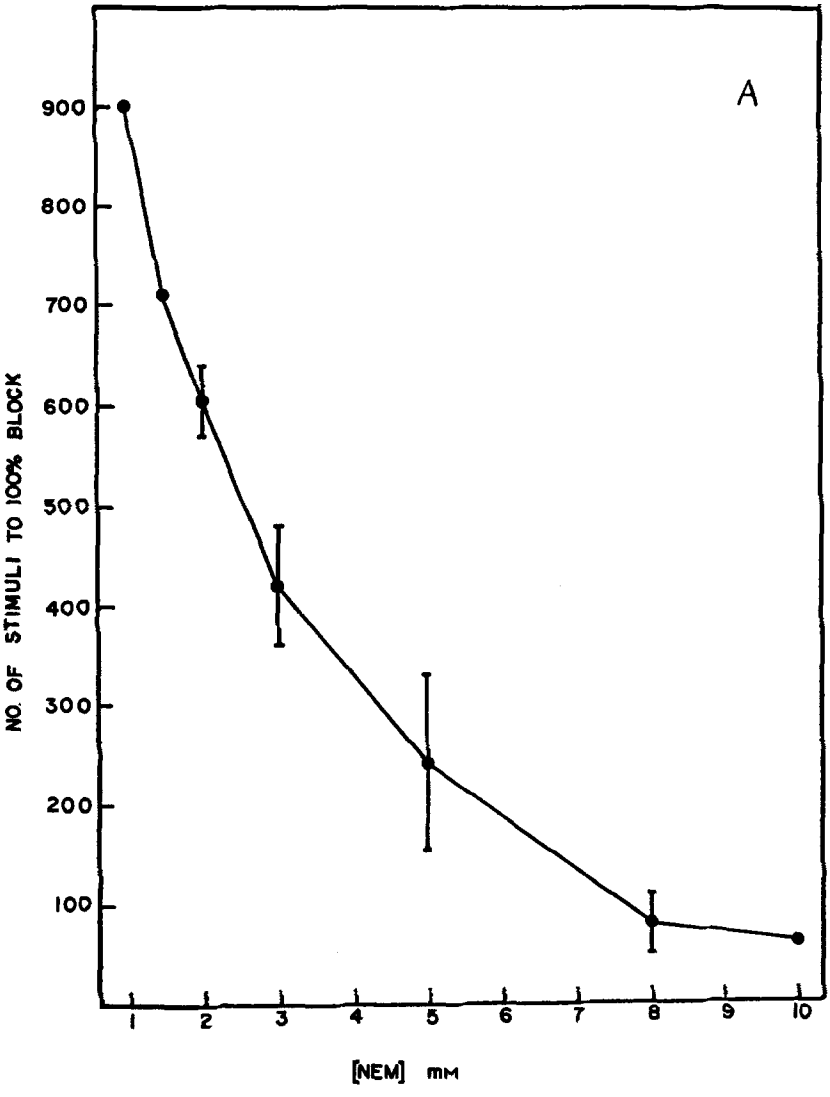

Fig. 4*A*

Fig. 4. Relationship between inhibitor concentration and number of stimuli required to induce complete block of conduction. (*A*) NEM. (*B*) Mercurochrome. Each point represents the mean of between 3 and 5 determinations obtained by extracellular recording. Vertical bars are used to indicate the range of the mean $\pm$ the standard error except where the values lie within the area of the symbol used to denote a point. The standard error is $\sqrt{\Sigma(x_i-\bar{x})^2/n(n-1)}$

Furthermore, it is possible that electrolytic reduction of disulfides might be taking place in depolarized membranes. That disulfides can be reduced to thiols electrolytically in peptides [5] and in proteins [3, 20] has been

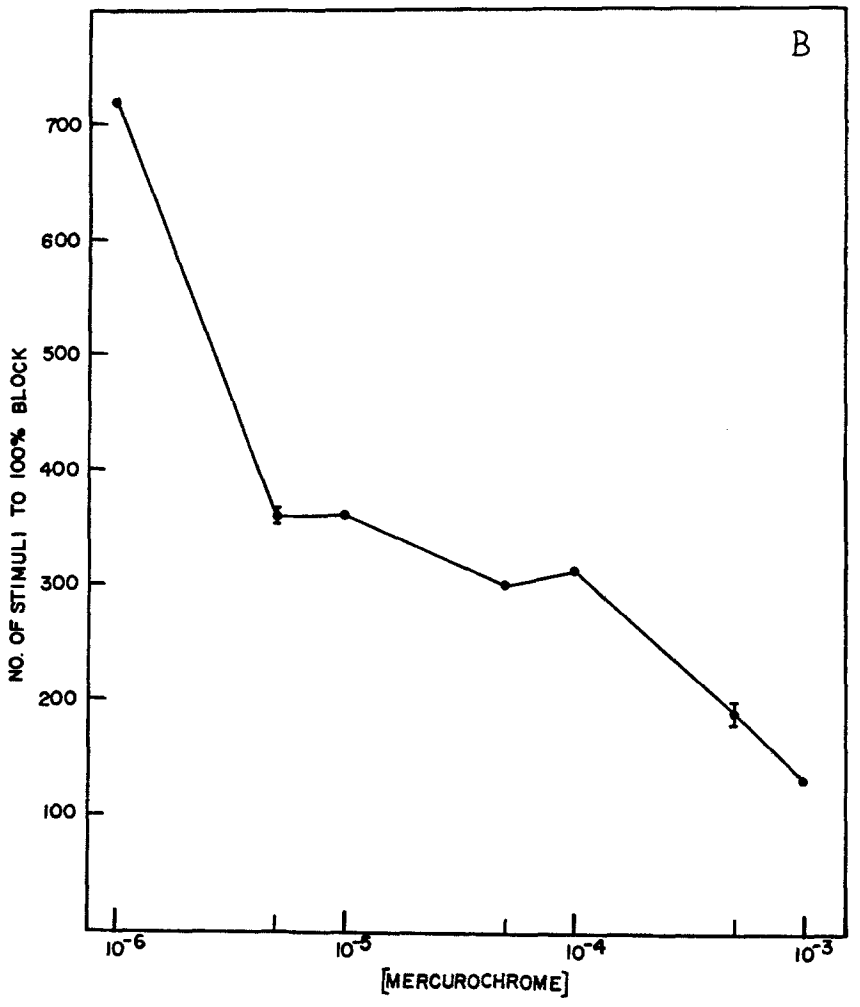

Fig. 4 *B*

known for some time. Such reactions resulting in the transient production of thiol groups might provide a trigger for the rearrangement of disulfides.

Although there is as yet no specific experimental evidence to favor any one of the suggested mechanisms above the others, the importance of disulfide rearrangement reactions has been emphasized by a number of investigators and should be considered briefly. Vincent and Lazdunski [32] have claimed that, in the interaction of trypsin and pancreatic trypsin inhibitor, intra-chain disulfides are rearranged to form interchain disulfides. Similarly, it has been postulated that disulfide exchange may be an important regulatory mechanism in fructose disphosphatase [25], hexokinase

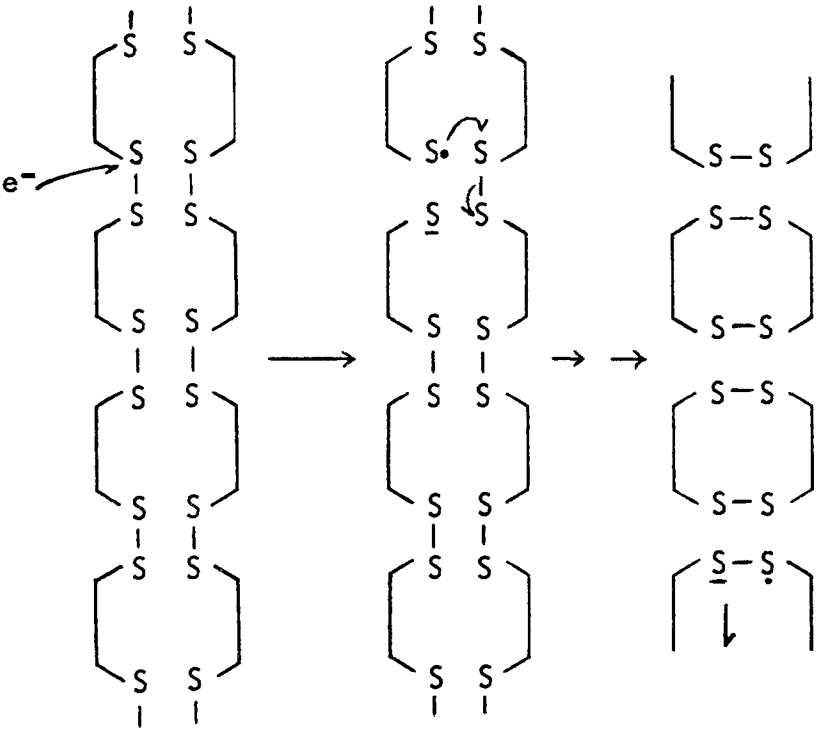

Fig. 5. A model for an electron-induced, self propagating disulfide rearrangement reaction. Details in Discussion

[23] and glycogen synthetase [7]. The possibility that electrolytic reduction of intra-chain disulfides (or conversion of inter- to intra-chain disulfides) with the newly formed disulfides differing in redox potential from the original ones, would provide a means of utilizing electron flow to induce conformational changes (*see* Fig. 5). That thiolcatalyzed disulfide exchange can take place is well established [3, 28], although the mechanism of this reaction remains obscure [18]. Such a mechanism might be involved in alterations of membrane structure responsible for changes in cation permeability. Electron-induced disulfide rearrangements might also provide an important link in mitochondrial energy transduction, a system in which the need for coupling electron flow to conformational alterations has been emphasized by Green and his co-workers [11, 12].

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