THE ROLE OF SULFHYDRYL AND DISULFIDE GROUPS OF MEMBRANE PROTEINS IN ELECTRICAL CONDUCTION AND CHEMICAL TRANSMISSION

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ABSTRACT. The chemical reactions of sulfhydryl and disulfide groups in proteins are discussed and the use of reagents specific for these groups as a tool in electrophysiology is reviewed. The drastic and specific changes seen when these group-specific reagents are used demonstrate the critical role of sulfhydryl and disulfide groups in electrical excitability, synaptic transmission and, particularly, postsynaptic receptor function. Sulfhydryl groups have been shown to be involved in the slow inactivation process of the voltage-dependent sodium channel, in the activation of voltage-dependent calcium channels and in sodium channel conductance. Sulfhydryl and disulfide groups have been shown to intervene in the function of the acetylcholine receptor at the vertebrate neuromuscular junction and in invertebrate glutaminergic receptors. The release of neurotransmitter from the presynaptic terminals of these neuromuscular junctions is also sensitive to sulfhydryl and disulfide group modification. Although in most instances the site of action of the reagents has not been resolved, their use has produced a clearer picture of receptor and channel structure-function relationships. *(Key Words: Sulfhydryl Reagents, Electrical Conduction, Chemical Transmission, Voltage-dependent Channels, Postnynaptic Receptors)*

THE critical role that membrane proteins play in neuronal function is demonstrated by the dramatic and quite specific changes in electrical excitability, synaptic transmission and postsynaptic receptor function that occur when these proteins are chemically modified. These chemical modifications can be accomplished by a variety of reagents that are known to react with specific chemical groups of proteins. Because of the prevalence and critical role of disulfide and sulfhydryl groups in protein structure and the availability of reagents specific for these groups, use of these reagents has allowed insight into the role of sulfhydryl and disulfide groups in membrane protein function. This review is designed to provide a general knowledge of the nature of the chemical reactions of disulfide and sulfhydryl groups and then to review the literature on the application of this chemistry to explore the role of these groups in membrane proteins that function as voltage-gated channels and receptor-channel complexes, focusing primarily on work done with intact cells.

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BIOCHEMISTRY OF SULFHYDRYL AND DISULFIDE GROUPS

Nearly all proteins contain sulfur as the side chains of three different amino acids. One is methionine (CH_3-S-CH_2-CH_2-$CHNH_2$-COOH), which has a nonpolar or hydrophobic R group (-SCH_3) with very lit-

tle chemical activity. The other two are cysteine ($HS-CH_2-CHNH_2-COOH$) and cystine ($HOOC-CHNH_2-CH^2-S-S-CH_2-CHNH_2-COOH$) which have the singular property of being interconvertible, cystine being the oxidized form of cysteine.

Both S-S bonds and SH groups are important determinants of conformational and, therefore, functional properties of protein molecules. There are only two covalent linkages between amino acids: the peptide bond and the disulfide bond of cystine. The peptide bond determines the primary sequence of amino acids into chains. The S-S bond serves as the only covalent cross-link between two separate polypeptide chains (interchain disulfide bond) or between loops of a single chain (intrachain disulfide bond). When these disulfide bridges are cleaved, proteins unfold and their biological activity is altered.

Sulfhydryl groups in proteins can be divided broadly into two kinds, reactive and unreactive. The reactive SH groups are those which behave in the same way towards SH reagents as to simple thiols (simple organic compounds containing the SH group). The unreactive SH groups are those which show a low reactivity in the native protein but normal reactivity after the protein has been denatured. Unreactive SH groups are involved in hydrophobic bonding. They are known to contribute to the quaternary structure of some proteins by binding together their various subunits (1). Unreactive SH groups have also been shown to be involved in intrachain bonding (2). Reactive SH groups are involved in labile intramolecular linkages but to a lesser extent than the unreactive groups (3).

The major role of SH and S-S groups lies, then, in determining and stabilizing the three-dimensional structure of proteins thereby preserving their functional properties.

In the early 1950's, chemicals which modify sulfhydryl and disulfide groups of proteins began to be used as tools for the study of the relationship between membrane macromolecules and excitability. Not all sulfhydryl reagents are useful for these electrophysiological experiments, since factors such as pH, temperature and ionic composition should be compatible with both reactions of the chemicals and normal functioning of the tissue. Worthwhile information on the location and functional role of membrane protein sulfhydryl groups can be obtained by the use of several types of sulfhydryl reagents. In addition, if there is a reagent that can be used *in vivo* to reverse the action of the sulfhydryl reagent in question and this reagent also reverses the change in the physiological effect, this action provides further evidence that the reaction is specific for a sulfhydryl group critical to the physiological function. Extensive discussions of sulfhydryl reagents and their reactions have been published by Webb, Jocelyn, and Friedman (4, 5, 6).

The reactions of most of the important sulfhydryl reagents can be classified into four types: a) oxidation of SH groups, b) mercaptide formation, c) alkylation of SH groups and, d) addition of SH groups to double bonds. Disulfide bonds are less reactive than SH groups; however, several nucleophilic (electron-donating) reagents can cleave them. A separat description now follows of the reaction of sulfhydryl and disulfide groups.

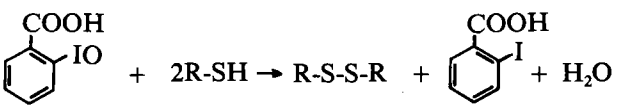
A. Oxidation of SH Groups

Under mild conditions, thiols are oxidized to form disulfides:

$$2RSH + \text{oxidant} \rightleftharpoons \underset{\text{reductant}}{RSSR} + 2H^+ + 2e^- +$$

Oxidation can occur by a variety of mechanisms. The reagent 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), for example, oxidizes SH groups by means of a thiol-disulfide exchange reaction (7). DTNB contains a disulfide bond which is reduced upon reacting with the SH groups of a protein; the SH groups, in turn, are oxidized.

Oxidation of SH groups by o-iodosobenzoic acid (o-IB) involves a different mechanism (8):



In this reaction o-iodobenzoic acid is formed. It is unclear whether a free radical or sulfenium ion intermediate is involved in the oxidation of SH groups by o-IB (9).

Diamide reacts with thiols probably via nitrogen bound intermediates to form disulfides (10):

$$2RSH + (CH_3)_2\ NCON=NCON(CH_3)_2 \longrightarrow (CH_3)_2\ NCONHNHCON(CH_3)_2 + RSSR$$

Diamide is highly reactive, its rate with glutathione is such as to give 50% completion of the reaction in less than 1 second at pH 7.4. It is also very stable, with a half-life for hydrolysis in aqueous buffer at pH 7.4 of 3,000 hours (11).

The formation of disulfide groups by oxidation requires the presence of SH groups that are close enough to link together in S-S bonds. The disulfide bonds formed in the oxidation may be either intramolecular or intermolecular.

B. Mercaptide formation

Mercurials are among the most reactive reagents and seldom are SH groups resistant to mercurials and ca-

pable of reacting with other SH reagents. The term "mercapto" was used in the past to refer to the SH group. This emphasizes the fact that thiols form very strong complexes with mercuric ions. Reaction of a simple thiol with mercuric chloride gives mercaptides of the types $(RS)_2Hg$, $(RS)_2Hg_2$ and $(RS)_2Hg_3$ (12). Native proteins will more often give the half-mercaptides, Prot. SHgX, whereas denatured proteins normally form (Prot. $S)_2Hg$. These differences arise from the valence of mercury and whether or not it is sterically possible for two sulfurs to react with the mercuric ion.

Organic mercurials of the type RHgX have a specificity for SH groups comparable to that of mercuric ion (13, 14). They have the advantage of being univalent and having, therefore, a more dependable stoichiometry. $HgCl_2$ is bifunctional in the sense that it can react with two ligands (L) to form L-Hg-L complexes, whereas organic mercurials are monofunctional, i.e., they react with only one ligand to give R-Hg-L. Mercuric ions can also form cyclic mercaptides with two adjacent SH groups but organic mercurials cannot. Organic mercurials, therefore, react with SH groups according to the following equation:

$$RHgX + R'SH \rightleftharpoons RHgSR' + HX$$

R can be aromatic ($-C_6H_5$, $-C_6H_4COOH$, and $-C_6H_4SO_3H$) or aliphatic ($-CH_3$). The aklyl and the unsubstituted phenylmercurials are more soluble in lipids than the substituted phenylmercurials (14). The COO^- and SO_3^{2-}groups reduce the ability of the mercurial to penetrate into cells.

Other heavy metal ions react with thiols but are less specific and give complexes of much lower stability. These are silver, arsenic (arsenite and organic arsenicals), copper, iron, cobalt, lead, and cadmium (5).

C. Alkylation

Iodoacetate and iodoacetamide are the most commonly used reagents for alkyl substitution reactions. Thiols are alkylated to give sulfides according to the following equation (15):

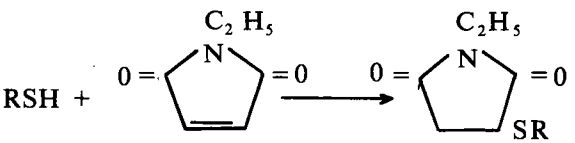
$$R\text{-}SH + R'\text{-}X \longrightarrow R\text{-}S\text{-}R' + HX$$

The reactive species in such alkylation reaction is RS^- and the reaction proceeds by the classical bimolecular nucleophilic substitution, S_N2, displacement mechanism. Therefore, the rate of reaction of a given simple thiol decreases with decreasing pH of the medium. The rate of reaction of a simple thiol with the alkyl halide also decreases when iodine is replaced by bromine, chlorine and fluorine atoms (1). However, although much is known regarding the effects of structure, steric hindrance and environment on the rates of nucleophilic displacement for simple organic compounds, the use of such information in protein chemistry is limited by the complexity of the molecules and the scarcity of data on surface topology. Interaction between the reagent and the protein environment can result in decreased (or enhanced) reactivity of a functional group in the protein. For example, the SH groups of oxyhemoglobin react with iodoacetamide but not with iodoacetate (16). The immediate inference is that negatively charged groups in the vicinity of the sulfhydryls hinder the approach of the negatively charged reagent.

D. Addition to double bonds

The addition of thiols to double bonds provides another way for alkylating SH groups. Because nucleophilic addition to double bonds has stereochemical and mechanistic features which differ from those of S_N2 displacement, this reaction provides an additional tool for exploring the subtleties of protein structure and reactivity. In both S_N2 and nucleophilic addition reactions, the nucleophile is RS^-. The rate of addition of RS^- to isolated double bonds is very slow unless there is strong base catalysis and, therefore, could not be expected to occur spontaneously in cells. However, if the double bonds are activated by conjugation, thiols add spontaneously to them. The most important conjugated system is the α, β-unsaturated carbonyl grouping, $-C=C-C=O$, and N-ethylmaleimide (NEM) is the most extensively used reagent in this group. Other reagents include acrylonitrile, maleate and quinones.

The advantages of NEM as a SH reagent may be summarized as (a) high selectivity for SH groups at near neutral pH, (b) reaction with only certain accesible SH groups on proteins, (c) fast reaction near neutral pH involving no change in pH, and (d) reasonably good penetration into cells due to the uncharged nature of the compound (17, 18). NEM reacts with thiols according to the following equation:



The reactivity of thiols toward alkylation may be different with NEM than with an alkyl halide. Taking the example used in the section on alkylation, the SH groups of oxyhemoglobin react with NEM but not with iodoacetate (16). This is so because negatively charged groups in the molecule hinder the approach of the negatively charged iodoacetate but have no effect on

the uncharged NEM. The sulfhydryl groups of deoxyhemoglobin, on the other hand, react with NEM but not with iodoacetamide or iodoacetate. The electrostatic explanation is no longer sufficient and the relative sizes of the reagents do not support the use of steric hindrance arguments. An alternative to consider is the infrequently recognized differences in mechanism of reaction of the different reagents. Nucleophilic displacement of halogen from tetrahedral carbon and addition of a nucleophile to an activated double bond (trigonal carbon) can be affected differently by the polarity of the local environment. The stereochemical requirements of their transition states differ significantly and the two reactions will not be similarly affected by other functional groups in the vicinity of the sulfhydryl.

E. Nucleophilic cleavage of disulfides

The best known and most studied of the reactions of disulfides are those with nucleophiles such as simple thiols and sulfite (19, 20). The cleavage reactions of greatest biological interest are those between a thiol and a disulfide which result in the reduction of the disulfides to thiols. The thiol-disulfide exchange reaction is the most specific method available for reducing disulfides (3):

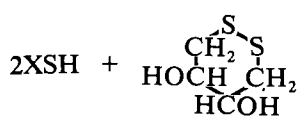
$$RSH + XSSX \rightleftharpoons RSSX + XSH$$

$$RSH + RSSX \rightleftharpoons RSSR + XSH$$

The monothiols most commonly used are glutathione (GSH), cysteine, thioglycollic acid, and 2-mercaptoethanol (2-ME). They are added in large excess to minimize mixed disulfide (RSSX) formation. Since this reaction involves the nucleophilic cleavage of the disulfide, the rate of reaction is proportional to the concentration of RS^- rather than that of RSH.

The dithiol, 1, 4-dithiothreitol (DTT) is an excellent reductant for disulfides. It forms, upon oxidation, a stable cyclic disulfide (21):

$$SXXS + HSCH_2(CHOH)_2CH_2SH \rightleftharpoons$$



The equilibrium constant for reduction with this dithiol is several orders of magnitude greater than that with monothiols, probably due to the formation of the stable six-membered disulfide (21). A lower concentration of DTT is, therefore, needed to accomplish the reduction of disulfide bonds.

Sulfite ions react with disulfides to forms S-substituted thiosulfates and a thiol (20):

$$XSSX + SO_3^{2-} \rightleftharpoons XS\text{-}SO_3^- + XS^-$$

Sulfite ions are obtained from bisulfite. The equilibrium constant is highly favorable for cleavage near pH 7, where the thiol is protonated and much of the sulfite remains in the dianionic form (22):

$$HSO_3^- \rightleftharpoons SO_3^{2-} + H^+ \qquad pK \doteq 7$$

Sulfitolysis and reduction of disulfides are the two main methods used *in vitro* for cleaving the S-S bond of proteins.

Some general remarks concerning protein modification by SH reagents are pertinent at this point. Reduction of disulfides and oxidation of sulfhydryl groups could possibly alter the protein conformation since the function of these groups is to stabilize the three-dimensional structure of proteins. Disruption of the functional properties of the protein could then be due to the alteration of its structure. The formation of mercaptides, alkylation of SH groups and addition of SH groups to double bonds all introduce new side chains into the protein. The alteration of protein function in these cases may be as much related, if not more, to the properties of these new side chains as to the disappearance of free SH groups. These new groupings have varying sizes and they also frequently have electrical fields. They may cause an alteration or prevent changes in the conformation of thé protein molecule and, if the new side chains are electrically charged, they may increase the instability of the molecule by altering the local electrostatic fields.

Effects of Sulfhydryl Reagents on Membrane Proteins

A. Axonal Membranes

The earliest studies of the effects of sulfhydryl reagents on excitable membranes were done on nerve preparations and pointed indirectly to the relationship between SH groups and function. It was first shown that arsenite ions block the action potential in frog nerves (23). Treatment with cysteine or glutathione delayed the onset of the rapid decline of the action potential. However, treatment with these reducing agents did not prevent the extinction of the action potential and it was not possible to reverse the effect of arsenite. Iodoacetate, iodoacetamide and cupric ions depolarized frog nerves after long periods of exposure (24). Excitability and impulse conduction were irreversibly blocked; attempts to restore nerve function by washing with Ringer's solution were not successful.

However, bundles of frog nerve fibers blocked by low concentrations of heavy-metal ions (i.e. Ag^+) became excitable within seconds after immersion in Ringer's containing cysteine or GSH (25). It was suggested, on those grounds, that excitability and conduction might depend upon the integrity of free SH groups of proteins at the surface of the nerve fibers (26).

The above experiments did not prove that nerve block resulted from the combination of the metal ions with SH groups. In fact, with the exception of Hg^{2+}, the metal ions used (Cd^{2+}, Cu^{2+}, Hg^{2+}, Ag^+, UO_2^{2+}) are not highly specific for SH groups since they also react with hydroxyl, carboxyl and guanido groups. For this reason, the mechanism of the blocking effect of the metal ions on nerve was re-investigated, testing, on nerves blocked by metal ions, the reversal effect of compounds containing sulfhydryl, hydroxyl, carboxyl, amino or guanido groups (27). Moreover, in addition to frog nerve, isolated lobster axons were tested for the effects of NEM and the organic mercurial p-chloromercuribenzoic acid (PCMB). The results confirmed that the blockade of SH groups was indeed responsible for the loss of excitability as well as for a decrease of the resting potential. It was observed that the action of metal ions and PCMB could be reversed with GSH or cysteine. NEM exerted a profound blocking effect on nerve, but the action of this compound, which forms a covalent bond with SH groups was irreversible.

The action currents of single isolated nerve fibers of the Japanese toad were measured by the air-gap method and it was found that Co^{2+} and Ni^{2+} induced plateu formation in the current (28). This could be due to delayed activation of potassium conductance. The plateau disappeared completely upon addition of cysteine. PCMB had no effect on the current, but it inhibited the effect of Co^{2+} and Ni^{2+}.

Using the giant axons of the squid *Loligo pealei* and *Dosidicus gigas*, sulfhydryl reagents were applied to both the outside surface membrane and to the inside by internal perfusion (29). Internal application of reducing agents decreased the duration of the action potential without affecting its amplitude, but at higher concentrations, an irreversible deterioration and eventual block of the action potential was observed. Oxidizing agents tended to increase the duration of the negative afterpotential of the action potential. If applied internally, they produced a slow and irreversible deterioration of the action potentials. The application of mercaptide-forming reagents to either side of the membrane caused a deterioration of the resting and action potentials and conduction block developed within 5 to 45 min. This effect was reversed by 2-ME. $HgCl_2$ blocked the action potential in 9 minutes and decreased the resting potential. In *Loligo*, the 2-ME reversal of the $HgCl_2$ induced block was accompanied by a brief but intense hyperpolarization. The alkylating agents iodoacetate and iodoacetamide were without effect, but NEM blocked the action potential irreversibly when applied externally or perfused internally.

The above studies showed that the integrity of certain sulfhydryl groups in nerve fibers is essential for conduction. More recent investigations have tried to elucidate the mechanism responsible for the blocking effect. In lobster giant axons it was found that PCMB decreased the resting potential and blocked excitation (30). The rising phase of the action potential was slowed faster than the falling phase and it was also observed that the block of action potentials by this mercurial was not reversed by long hyperpolarizations. On this basis, it was suggested that PCMB selectively inhibits the mechanism of sodium activation.

Working with the voltage-clamped node of Ranvier from the sciatic nerve of *Rana esculenta*, the action of NEM on peak early current and steady-state current was investigated (31). A very rapid (20 sec) and reversible partial decline in peak early current, a slower and irreversible fall in steady-state current, and an eventual sharp increase in leakage current was found. No definite evidence for the presence or absence of a functionally important SH group on sodium channels was obtained.

The action of NEM on the voltage clamped node of Ranvier has recently been reinvestigated (32, 33). Externally applied NEM shifted the voltage dependence of sodium inactivation to more negative potentials, but had little effect on the time course of sodium activation and inactivation. It produced a small decrease of the sodium current which was reversed almost completely by hyperpolarizing prepulses (32). When internally applied, NEM also shifted the voltage dependence of sodium inactivation to more negative potentials with no change in the peak sodium current (33). These results suggested that NEM enhanced the slow sodium inactivation mechanism normally present in the nodal membrane.

Methionine-specific reagents have recently been shown to irreversibly slow and inhibit sodium channel inactivation of frog nodes of Ranvier (34). Externally applied N-bromoacetamide, N-bromosuccinimide, chloramine-T and N-chlorosuccinimide altered the inactivation but not the activation of sodium channels, suggesting that methionine residues are critical for the function of the inactivation process.

When applied to voltage-clamped medial giant axons from the crayfish *Procambarus clarkii*, NEM was shown to inhibit selectively early transient currents; DTNB had no effect on excitation (35). The normal increase in sodium conductance during a depolarization from the resting potential level was almost completely abolished by NEM. This reduction in so-

dium conductance suggested that NEM induced slow inactivation, since sodium currents could be restored to about 70% of their value before treatment by hyperpolarizing pre-pulses of 300-800 ms duration (36). NEM did not affect the resting potential, the delayed rise in potassium conductance, the selectivity of the sodium channel or the sodium activation system. In contrast with NEM, the organic mercurial p-chloromercuriphenylsulfonic acid (PCMBS) reduced the peak early transient currents to about 50% of control values and prolonged hyperpolarizations were not effective in restoring these currents, suggesting an irreversible block of sodium conductance (36).

Methylmercuric chloride, applied to voltage-clamped giant axons from *Loligo pealei*, caused a steady increase in the threshold for excitation and an eventual block of action potentials without changes in the resting membrane potential (37). Also, the peak transient current decreased after exposure to the organic mercurial. These results were in agreement with those obtained when using PCMBS (36).

Axonal membranes have also been used to study the effects of electrical stimulation on the reactivity of the membrane with sulfhydryl reagents, in particular, what has been called the "stimulation effect", i.e., reagent induced acceleration of conduction block by repeated, brief electrical stimulation of the nerve fiber (38). Giant axons of the squid *Loligo pealei* were used and $HgCl_2$, PCMB, NEM, and DTNB produced this "stimulation effect". DTT did not affect conduction, while iodoacetate and iodoacetamide blocked conduction irreversibly but did not exhibit the "stimulation effect". Possible explanations of the "stimulation effect" are: altered permeability of the nerve membrane, unmasking of buried SH groups in the membrane, or electrolytic reduction of disulfides. On the basis of later data, it was suggested that electrolytic reduction of disulfides may be involved in a conformational change in membrane proteins during electrical excitation (39).

The electrophysiological and biochemical studies have some morphological support. Electron microscopic studies of crayfish (*Procambarus clarkii*) abdominal nerves showed that axonal membranes displayed ultrastructural changes if the axons were fixed, during electrical stimulation, by aldehydes followed by osmium (40, 41). These changes consisted of an increase in electron opacity and thickness associated with the axon surface membrane, the membranes of the endoplasmic reticulum and the outer membranes of the mitochondria. This phenomenon did not appear if the axons were exposed to maleimide or NEM before treatment with osmium. These findings again suggested that the SH groups of axonal membranes are unmasked as a result of electrical stimulation.

B. Muscle Membranes

Sulfhydryl reagents have been widely used in the study of skeletal muscle. Additional properties, such as synaptic transmission and contraction, are involved in the study of muscles. However, skeletal muscle is more difficult to use for voltage-clamp experiments than large nerves and no study has been made to date of the effects of SH reagents on ionic conductances in skeletal muscle.

A variety of SH reagents have been demonstrated to affect muscle membranes. Iodoacetate caused contracture of toad sartorius and the development of this contracture was not accompanied by depolarization of the muscle. Instead, the muscle became depolarized after developing contracture (42). On the frog sartorius muscle, PCMB caused repetitive low-amplitude contractions and a moderate increase in muscle tension (43). PCMBS also caused repetitive low-amplitude contractions but with no increase in tension. On the other hand, NEM had a striking action on muscle: an initial contractile effect which was immediate in onset, reached a peak within a few seconds and was short-lived, and a subsequent slow-rising increase in tension which was not reversed by repeated washings in normal Ringer's. The effects of NEM and organic mercurials were later studied in greater detail (44, 45). The primary effect of NEM was found to be muscle contracture (45). In agreement with early experiments (42), it was found that membrane depolarization is unrelated to contracture development. Peak contracture was observed before any effect of NEM on resting potential was recorded. In addition, NEM caused a substantial increase in ^{45}Ca efflux from whole muscle. It was concluded that the primary site on NEM action was the inhibition of calcium uptake by the sarcoplasmic reticulum, thereby producing contracture. It had already been observed that NEM inhibited the uptake of calcium by isolated vesicles of the sarcoplasmic reticulum and SH groups located on the outer surface of the vesicles were implicated in active calcium transport (46). NEM also severely depressed the amplitude and the rates of rise and fall of the electrically evoked action potential of frog sartorius muscle. NEM and organic mercurials had several effects in common: spontaneous contractions, membrane depolarization associated with a period of asynchronous twitching followed by inexcitability, and a depression of the electrically evoked twitch. PCMB produced a fractionation of the evoked twitch into a fast and slow component. PCMBS lacked this effect, probably due to the difference in the rate of diffusion of the two compounds across membranes (44).

Iodoacetate blocked contraction and the action potential of frog sartorius muscle (47). It also was shown to shorten the after-potential and to reduce in ampli-

tude the action potential in this preparation. There was no recovery of the action potential upon washing in fresh Ringer's for 1 hr, instead, the after-potential disappeared and the spike duration increased even more. This after-potential is the result of the activity of the Na, K-pump and these results suggest that the pump is blocked by iodoacetate (48). Iodoacetamide was later shown to block the Na, K-pump of frog sartorius muscle (49).

Several sulfhydryl reagents were compared for their effects on the frog sciatic nerve-sartorius muscle and the rat phrenic nerve-diaphragm preparations (50). Conduction blockade of the motor axons, depolarization of the muscle membrane, sustained contracture and depression and prolongation of the muscle action potentials were observed with every SH reagent used. The drugs tested were the mercaptide-forming reagents phenylmercuric acetate and PCMB, the alkylating reagents NEM and iodoacetamide, and the oxidizing reagent o-IB. NEM, phenylmercuric acetate and iodoacetamide blocked impulse propagation in the nerve fiber before direct paralysis of the muscle. PCMB and o-IB had a weaker effect, inducing conduction block after paralysis of the muscle. NEM and phenylmercuric acetate induced spontaneous twitching and fibrillatory movement of the muscle. NEM and iodoacetamide increased the frequency of the miniature endplate potentials (mepp).

Surprising results were found when the action of sulfhydryl reagents on invertebrate muscle was studied. In contrast to vertebrate muscle, some of these reagents induced excitability in the ventroabdominal flexor muscles of the shrimp *Atyas occidentalis*, which are normally electrically inexcitable. Exposure of the muscle to NEM caused the firing of repetitive action potentials, with a remarkable low degree of adaptation, upon depolarization by injected current (51). NEM changed neither the membrane resting potential nor its input resistance. Altering the ionic composition of the external solution suggested that the rising phase of the action potentials was due to the activation of calcium channels since they could be blocked by Mn^{2+} but not with tetrodotoxin (52). NEM appeared to exert this excitability-inducing effect by combining covalently with SH groups since, if before applying NEM, the muscle is exposed to Hg^{2+} or organic mercurials, the effect is prevented. This blocking action was reversed by cysteine. The effects of NEM and organic mercurials were clearly not equivalent and, therefore, the NEM-effect was not simply the result of binding free SH groups. It was proposed that the induction of excitability depended on the conversion of $-CH_2SH$ side chains to thioethers having carbonyl groups. To test this hypothesis, 4-cyclopentene-1, 3-dione, a compound similar to maleimide but with a methylene group instead of a nitrogen, was used. This compound induced action potentials and was also found to combine covalently with the sulfhydryl groups of cysteine and the cysteinyl residue of the protease pinguinain (53, 54).

Although voltage clamp methods have not been employed to study the effects of sulfhydryl reagents on skeletal muscle, voltage clamped cardiac muscle have been examined. Using bullfrog atrial muscles and a double sucrose gap method, NEM was shown to transiently increase inward calcium current and contractile tension. In addition, resting membrane potential was depressed and steady state outward and background currents were increased (55).

C. Somatic Neuronal Membranes

NEM applied to the giant neuron of the abdominal ganglion of *Aplysia californica* produced a 5-10 fold decrease in the cell's input resistance while the resting potential remained relatively unchanged (56). The results indicated that the reaction of NEM with membrane constituents brought about a large increase in conductance to chloride and potassium ions, without significantly affecting sodium conductance, and without producing a significant change in resting potential. This is paradoxical, however, in that the equilibrium potentials for chloride and potassium ions are normally more negative than the resting potential of this cell and thus an increase in permeability of these two ions alone would be expected to hyperpolarize the cell. No hyperpolarization was observed even transiently.

These results have recently been confirmed. NEM indeed reduced the membrane resistance of *Aplysia* giant neurons by about 90%, it did not change the resting membrane potential and no outward current large enough to trigger an action potential could be injected (57).

D. Synpatic transmission: presynaptic effects

A large number of sulfhydryl reagents have been found which modify the release of neurotransmitter at the presynaptic terminal. The precise site of action of these reagents is not known and could involve any of the many proteins associated with transmitter release. These proteins include the Na, K, and Ca channels of the presynaptic axon and terminal membrane, internal calcium sequestering proteins, either cytoplasmic or mitochondrial, a Na, K-ATPase in the terminal membrane or an actin-like molecule linking the membrane and the secretory vesicle (58-61). Synapsin I, a protein specific for synaptic vesicle membranes is another potential target for sulfhydryl reagents (62-64). Although sulfhydryl reagents of all classes have been shown to modify transmitter release, the action of all

reagents tested, with the possible exception of the reducing agent DTT, produce similar effects: an increase in spontaneous transmitter release accompanied by either an increase or decrease in evoked release (65). This is often accompanied or preceeded by a block of the presynaptic action potential.

The earliest experiments on the presynaptic effects of sulfhydryl reagents were done on the cat superior cervical ganglion using lead as the reagent. Lead ions produced a block of transmitter release which could be restored by an increase in the calcium concentration of the media. The lead application did not affect the postsynaptic receptor since the response to iontophoresed acetylcholine (ACh) did not change (66). NEM, applied to the same preparation, produced a triphasic effect. The immediate effect, an enhancement of the postganglionic spike, was followed in 10 to 45 minutes by a decreased or abolished response to stimulation of the presynaptic nerve. Finally, at 45-150 minutes post application, spontaneous pre-and postganglionic asynchronous discharges appeared (67).

Using the rat and frog neuromuscular junction, and application of the oxidizing agent o-IB, the mercaptide reagents phenylmercuric acetate and PCMB, and the alkylating agents iodoacetic acid and NEM, only NEM and iodoacetic acid produced an increase in spontaneous release of transmitter. Evoked release was not studied (50). These experiments were re-examined in the frog neuromuscular junction using NEM and PCMB and an increase in both spontaneous and evoked release was found, even in the absence of extracellular calcium. It was concluded that the action was on a intracellular site for calcium sequestration (68).

Diamide, a sulfhydryl oxidizing agent, was applied to the neuromuscular junction of the frog where it produced an increase in spontaneous and evoked release of transmitter. This was attributed by the authors to an action specific for intracellular glutathione which they proposed was involved in the transmitter release process (69, 70). When applied to rat cerebral brain slices, diamide produced an increased release of ACh, GABA, norepinephrine and dopamine (71). The action of diamide on transmitter release was also assessed at the glutaminergic neuromuscular junction of the lobster where it produced an increase in release. This action was independent of external calcium (72). The mechanism of action of diamide at the above synapses has not been resolved.

Sodium bisulfite, which adds a nucleophilic sulfonate group to one side of a disulfide bond (22), was applied to the frog neuromuscular junction and produced results similar to those seen with other reagents above. The most apparent effect was an increase in mepp frequency which could be reversed by application of the oxidizing agent DTNB and which was not dependent on extracellular calcium or sodium. DTNB, because of its two carboxyl groups, should be relatively impermeable. Therefore, its action in reversing bisulfite-induced mepp frequency increase, indicates an extracellular action of bisulfite. DTNB alone, at higher concentration and longer latency, produced an increase in mepp frequency. An increase in evoked transmitter release followed by a decrease was usually seen in response to bisulfite. Block of the presynaptic spike was common in later stages. These events were variable in time course and occurence (73).

Similar results were seen when a series of reagents were used in an attempt to distinguish between the actions of reagents which would readily penetrate the presynaptic terminal and those which would not. The results were equivocal since it appears that penetrability may be a matter of time. Even a reagent such as PCMBS, which would be poorly soluble in the membrane because of its sulfonate group, did, with longer periods of application, produce essentially the same results as PCMB. The reagents assayed included NEM, DTNB, sodium bisulfite, PCMB, PCMBS, and DTT (Steinacker, unpublished observations). In these experiments, it was not possible to distinguish clearly whether the action of a reagent in modifying transmitter release was on a receptor or channel in the terminal membrane or on a protein inside the terminal which was linked to transmitter release.

Since transmitter release is dependent upon the level of depolarization of the presynaptic terminal which is, in turn, dependent upon an active Na, K-pump in the membrane, a simple explanation of many of the above effects of sulfhydryl reagents on transmitter release and action potential generation is interference with the function of a Na, K-ATPase in the membrane. This pump uses ATP as its substrate and it is known that sulfhydryl reagents inhibit this enzyme (74, 75). The accumulation of Na^+ in the terminal would produce a long lasting depolarization and could produce the presynaptic neuronal effects of sulfhydryl reagents (76).

The few experiments addressing this question do not resolve the issue. Sodium bisulfite, applied to rat brain slices in concentrations that produce an increase in norepinephrine release, produced no change in ATP levels (77). The effect of NEM, PCMB and PCMBS on Na pump activity, ATP content and membrane electrical properties was studied in the crayfish stretch receptor (57). These reagents produced a depolarization of the resting membrane potential with resultant spontaneous firing, increase in membrane resistance, a decrease in action potential amplitude and lowering of the trigger level of the action potential. It was concluded that all three reagents, when applied to the stretch receptor, produced results consistent with inhibition of the sodium pump similar to that produced by ouabain, a specific inhibitor of the membrane

bound Na, K-ATPase (74). However, the ATP content of the stretch receptor neuron was decreased only by NEM and not by PCMB or PCMBS (57).

E. Synaptic transmission: postsynaptic receptors

1. Somatic neuronal receptors

The effects of disulfide bond reduction and reoxidation on the postsynaptic response of neurons in *Aplysia californica* abdominal ganglion cells were investigated and three kinds of cholinoceptive neurons, nicotinic depolarizing (D-), nicotinic hyperpolarizing (H-), and muscarinic H-types, as well as gamma-aminobutyric acid (GABA) H-, and dopamine H-type neurons, were used for the study (78). The effects of DTT and DTNB on their postsynaptic responses were studied by measuring the conductance changes produced by each transmitter. The acetylcholine-induced responses of both nicotinic types (D- and H-) were depressed by DTT and restored by oxidation with DTNB. The responses of the muscarinic H-, GABA H-, and dopamine H-cells were not affected by either DTT or DTNB. The authors concluded that the disulfide bond was a crucial element in both types of nicotinic receptors (D- and H-), and that this bond is related to the activation process of the receptors, regardless of their ionic specificities.

Two kinds of cholinoceptive neurons in the central ganglion of the marine slug *Anisodoris nobilis* have been identified: depolarizing (D-) neurons, and Cl^- neurons; in the latter ACh increases membrane permeability to chloride ions (79). Exposure to the organic mercurial PCMBS did not inhibit the cholinergically induced response in D-neurons. However, PCMBS caused a gradual increase in membrane leak conductance without changing the resting potential of Cl-neurons. This effect could be partially reversed by the reducing agent 2-ME. Furthermore, PCMBS inhibited the response to carbamylcholine (a nicotinic agonist), but it did not prevent carbamylcholine from binding to the ACh receptor. In addition, the PCMBS-induced activation of chloride permeability could be transiently blocked by the binding of carbamylcholine. This suggested that the binding of the agonist was associated with a conformational change in the receptor, such that PCMBS could no longer bind to it. Conversely, when PCMBS was bound to the receptor, it blocked the agonist-binding sites.

2. Neuromuscular junction

The postsynaptic ACh receptor at the neuromuscular junction is perhaps the best membrane protein in which to discuss the role of sulfhydryl and disulfide groups in membrane function, since the primary structure of this protein and the location of sulfur containing groups is now reasonably well known, and site specific affinity labels developed and used for some time (80-82, see reference 83 for review). Most of this work has been done using the isolated receptor from the electroplax (a modified neuromuscular junction) of *Torpedo californica* and *Electrophorus electricus*. The receptor is composed of five polypeptide chains, two α and single β, δ and γ chains, all of which span the membrane (84, 85). These chains contain intra- and interchain disulfide bonds and sulfhydryl groups. The receptors usually occur as dimers in the native membrane, cross-linked by a disulfide bond between δ chains (86-88).

There are two α chains, each of which contains an ACh binding site, and one each of the other three chains (89-92). Since there are two α chains and three other nonidentical chains, each must see a different environment and react differently to some reagents (83). For instance, in the isolated receptor from *Torpedo*, although there are two ACh binding sites, one on each α chain, it was initially reported that only one site is susceptible to reduction and affinity labeling (93, 94). In rat skeletal muscle, however, the two chains were reported to be equally reactive to sulfhydryl labeling (95). It has since been shown that the second α chain can be labelled using higher concentrations of affinity label (96). In addition to the affinity labels for the α chain disulfide groups, there are two oxidizing agents which show site specifity on receptor interchain bonding: diamide and copper phenanthroline. Diamide cross-links monomers via the β chain in the reduced receptor, while copper phenathroline cross-links reduced receptor monomers via the β and δ chains (97).

Electrophysiological evidence for a well-defined role of SH and S-S groups in the function of the ACh receptor was first shown using the eel *Electrophorus* electroplax (see 83 for review). The original work involved reduction of a disulfide bond on what is now known to be the α chain of the receptor and was followed by the development of several very elegant and useful affinity labels for this site. Reduction of this bond with DTT resulted in a decrease in the response of the receptor to ACh which could be restored by oxidation with DTNB. By first reducing the disulfide bond and then reacting the preparation with quaternary ammonium alkylating or acetylating agents of different chain lenghts, it was determined that this disulfide bond lay about 10 Å from the ligand quaternary binding site (98, 99). The results from these experiments were then applied to the ACh receptor in many other preparations: denervated rat muscle, frog neuromuscular junction, sympathetic ganglia, leech

muscle and molluscan soma (65, 100-103). In all cases, reduction of this disulfide bond resulted in a decreased response of the receptor to ACh. In the most detailed experiments, using the frog neuromuscular junction, reduction with DTT produced a three-fold decrease in the affinity of the receptor for ACh and decrease in single channel open time and conductance with no change in the ionic selectivity of the channel (104). There was no change in the dipole moment of the ionic channel gating protein since there was no change in the slope of the rate of decay of the endplate current plotted against the membrane potential (105).

In the above experiments, the sulfhydryl reagents were bath-applied for long periods of time before the agonists were added. Since some sulfhydryl reagents themselves could have agonist-like effects, and since prolonged exposure of the receptors to cholinergic agonists can result in receptor desensitization (106), the effect of sulfhydryl reagents applied iontophoretically was investigated (107, 108). It was found that DTT inhibited the depolarizing action of ACh but that much weaker reducing agents, such as 2-ME and GSH, enhanced the action of ACh.

Other modifications of a disulfide bond are possible which produce groups more complex than the sulfhydryls produced by reduction. Among these is the formation of a thiosulfate and thiol via nucleophilic attack and heterolytic cleavage of a disulfide bond with sulfite. Sodium bisulfite at pH 7.0 was used as a source of sulfite to add the nucleophilic sulfonate group to the same α chain disulfide group involved in the reduction and affinity labeling experiments by Karlin and coworkers, mentioned above (98,99). The site of attack of the sulfite group was determined by the use of an affinity label for this site (109). Sulfonation at this site, unlike reduction, increased the response of the receptor to ACh, as evidenced by an increase in mepp amplitude. Under voltage clamp at the lizard neuromuscular junction, a cholinergic synapse, the miniature endplate current (mepc) rise and decay time is increased. This increase is not due to the destruction of endplate acetylcholinesterase. Nor is it due to an increase in single channel open time or conductance, as measured using spectral analysis of endplate current fluctuations (110). Voltage jump current relaxation experiments show in increase in the relaxation time equal to the increase seen in mepc decay time (111). This data indicates an effect on either agonist binding or channel opening which will have to be resolved by the use of patch clamp methods (112, 113). This technique permits the calculation of rate constants from the observation of opening and closing of individual channels (114-116).

Application of diamide, an oxidizing agent, to the same preparation produced somewhat different effects than those of bisulfite. The same increase in mepc decay time was produced but no effect on the rise time was seen. No changes in single channel parameters as measured by spectral analysis were observed (110). However, unlike sulfonation, oxidation produced no change in voltage jump current relaxations (Steinacker and Zuazaga, unpublished observations). Clearly, the two reagents have complex and very different effects on receptor function. Diamide has been shown to produce dimers or oligomers in the isolated receptor (88, 97). If it has the same site of activity in the receptor *in situ*, it may be that receptor proximity or clustering influences the kinetics of binding and/or channel opening. A definitive answer to this must be provided using patch clamp analysis.

The only non-cholinergic neuromuscular junction studied using sulfhydryl reagents were glutaminergic: the excitatory neuromuscular junction of the grasshopper *Romalea microptera*, and of the lobster, *Homarus americanus*. In the grasshopper, NEM, PCMB and iodoacetic acid blocked the neurally evoked mechanical response of the isolated nerve-muscle preparation and also blocked the contractions evoked by external application of glutamic acid. The PCMB blockade could be reversed completely with DTT, while the NEM and iodoacetic acid blockade could not be reversed. Protection could be achieved by prior mixture of SH reagents with equimolar concentrations of cysteine or GSH. Cysteine, GSH and DTT were without appreciable effect on the neurally evoked mechanical activity. The authors concluded that free SH groups are also essential in glutaminergic neuromuscular function (117). Diamide increased the response of the lobster neuromuscular junction to glutamate, applied either by superfusion or iontophoresis (72).

Concluding Remarks

The proteins of electrically excitable membranes, which endow them with their conduction and excitability properties, are stabilized in their three dimensional conformation to a great extent by the presence of SH and S-S groups. Chemical modification of these groups alters the function of many membrane channel proteins. Moreover, different reagents often produce different alterations of function. For example, NEM induces slow inactivation of the sodium channel in crayfish axons, while PCMBS blocks sodium channel conductance; the potassium channel in this preparation is unaffected by these two reagents (36). In addition, cleavage of the same disulfide bond in the ACh receptor by DTT or sulfite produces different re-

sults. DTT decreases the response of the receptor to ACh by decreasing mean channel open time and conductance, while sulfite enhances the reponse to ACh without changing open time or conductance (104, 105, 109, 110, 118). Furthermore, a reagent may affect different types of channels differently. Thus, NEM reduces sodium conductance in crayfish and squid axons, but increases calcium conductance in heart and invertebrate muscle (29, 36, 52, 55).

The extent to which chemical modification is useful is in large part determined by knowledge of the site specificity of the reagents. The most elegant example of this was the use of reduction and subsequent affinity labelling of a disulfide bond on the α chain of the ACh receptor; by tailoring the length of the affinity label, it was shown that the distance of the S-S bond from the ACh binding site was 10Å (98,99). Furthermore, the loss of the affinity of the receptor for ACh after reduction of the bond demonstrated the role of this disulfide bond in receptor affinity.

A defined role for sulfhydryl and disulfide groups in receptor function has been implicated not only in the well-studied neuromuscular ACh receptor but also in a wide variety of receptor types as diverse as the receptors for progesterone, estrogen, and insulin, the enkephalin receptor, the β-adrenergic receptor, the ACh receptors of the central nervous system and vasopressin receptors (119-129). These receptors have sites sensitive to sulfhydryl reagents as assessed by alteration of their function after chemical modification. Sulfhydryl groups are also known to be involved in the function of gap junction channels and of the ACh receptor in heart muscle (130-134). It should be pointed out that the earliest evidence for a role of sulfhydryl groups of proteins in biophysical phenomena was obtained by the Russian physiologist Ch. S. Koschtojanz during his studies of cardiac synaptic physiology (134). The information on synaptic physiology available at that time was so limited that no definite function could be assigned to the proposed sulfhydryl-containint proteins and, for this reason, Koschtojanz's work, though resting on solid experimental observations, never reached the mainstream of neurophysiologial work. Even today, with very few possible exceptions, the functional significance of sulfhydryl and disulfide groups is not apparent and will not become apparent until careful work has been done in tailoring reagents which are clearly site specific. This information can then be combined with purely chemical data on membrane protein structure to give a more concise picture of receptor or channel structure-function relationships.

The approach to the problem of the site of action of reagents in a complex system of interrelated parts, such as the presynaptic terminal, rather than a specific postsynaptic protein typified by the ACh receptor, is more complex. The resolution of the site of action of sulfhydryl reagents at the presynaptic terminal perhaps lies in the use of isolated cellular components of the terminal and their reaction with the reagents *in vitro*.

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RESUMEN

Se discuten en este trabajo las reacciones químicas de los grupos sulfidrilo y bisulfuro y se revisa el uso de reactivos específicos para estos grupos como una herramienta en electrofisiología. Los cambios drásticos y específicos que se observan cuando se aplican estos reactivos demuestran el papel crítico que juegan los grupos sulfidrilo y bisulfuro en los procesos de excitabilidad eléctrica, transmisión sináptica y, particularmente, en la función de los receptores postsinápticos. Se ha demostrado que los grupos sulfidrilo intervienen en el proceso de inactivación lenta de los canales de sodio dependientes de voltaje, en la activación de canales de calcio dependientes de voltaje y determinan la conductancia de los canales de sodio. Tanto los grupos sulfidrilo como los bisulfuro juegan un papel en la función de los receptores colinérgicos nicotínicos de la sinapsis neuromuscular de los vertebrados y en la de los receptores glutaminérgicos de los invertebrados. La secreción de neurotransmisor por las terminaciones nerviosas presinápticas en estas uniones neuromusculares es también afectada por la modificación química de los grupos sulfidrilo y bisulfuro. Aunque en la mayoría de los casos no se ha esclarecido aun el sitio de acción de estos reactivos, los resultados de su utilización permiten una visión mas clara de las relaciones entre la estructura y la función de los canales y receptores existentes en las membranas excitables.

LIST OF ABBREVIATIONS

ACh	acetylcholine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	1,4-dithiothreitol
GABA	gamma-aminobutyric acid
GHS	reduced glutathione
o-IB	o-iodosobenzoic acid
NEM	N-ethylmaleimide
2-ME	2-mercaptoethanol
mepc	miniature endplate current
mepp	miniature endplate potential
PCMB	p-chloromercuribenzoic acid
PCMBS	p-chloromercuriphenylsulfonic acid

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