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ACTION OF SULFHYDRYL INHIBITORS ON FROG SKELETAL MUSCLE

by

EDWARD B. KIRSTEN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1969

To my wife Miriam

and my daughter Suzanne

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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INTRODUCTION

I. Purpose

Sulfhdryl (-SH) side-chain groups are important in the maintenance of normal protein conformation (Olcott and Fraenkel-Conrat, 1947; Cecil and Thomas, 1965; Yasui *et al.*, 1968) and, therefore, compounds that react with these groups modify the structural and functional properties of proteins. The -SH group inhibitors have long been used to ascertain the role of -SH groups in protein structure and enzymatic activity. These inhibitors can also be viewed, for purposes of the present study, as specific molecular tools that affect cellular function through selective interference with protein -SH groups.

The purpose of this investigation was to determine the effects and mechanisms of action of -SH inhibitors on whole skeletal muscle. There have been numerous studies of the effects of -SH inhibitors on the structural and enzymatic proteins and on subcellular components derived from skeletal muscle (Blum, 1962; Dawson and Widdas, 1963; Carsten and Mommaerts, 1964; Levy and Ryan, 1965; Hasselbach, 1966). In view of the background of essentially biochemical information available from these studies, it seemed appropriate to determine the effects of -SH inhibitors on the functional properties of whole muscle. By correlating the data obtained from the whole muscle experiments with that obtained from the previous experiments on muscle proteins and subcellular fractions, it was expected that the role of -SH groups in various aspects of muscle function would be elucidated, e.g. the role of -SH groups in excitation-contraction (E-C) coupling or in the mechanism of relaxation.

The -SH inhibitors selected for this study were the alkylating agent, N-ethylmaleimide (NEM), and the organomercurials, parachloromercuribenzonate (PCMB) and parachloromercuribenzene sulfonate (PCMBS) (Fig. 1). These particular agents were chosen because there is some

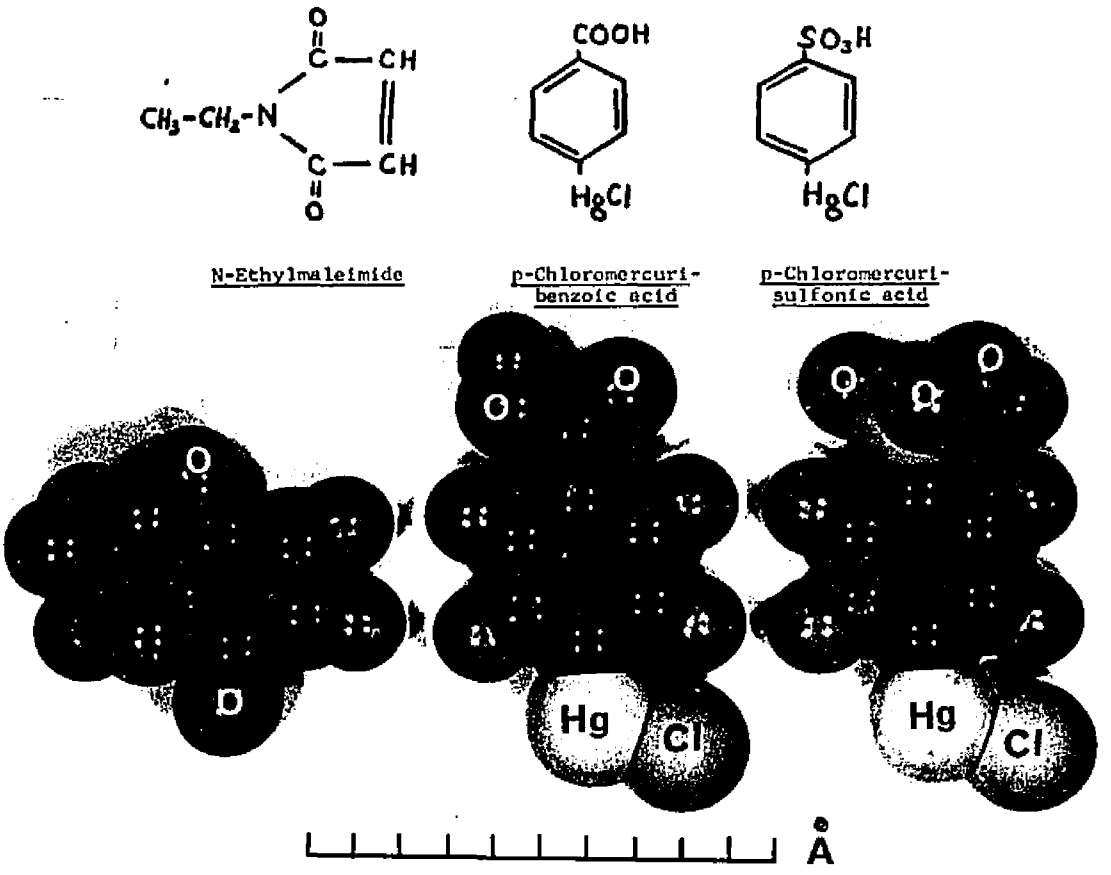


Figure 1 The molecular models and formulas of NEM, PCMB and PCMBS are shown. The black atoms are carbon; the mercury (Hg), oxygen (O) and chloride atoms are indicated. The scale at the bottom indicates 10 Angstrom(Å) units.

preliminary information about their actions on skeletal muscle (Okamoto and Kuperman, 1966) and because they have been used in many biochemical studies involving muscle (cf. previously cited references). The types of experiments carried out in the present investigation involved measurements of muscle tension during direct electrical stimulation or at rest, determinations of transmembrance resting and action potentials, and measurements of the rate of efflux of radiocalcium (Ca^{45}) from the whole muscle.

II. Mechanisms of Excitation-Contraction Coupling

Preliminary experiments have indicated that -SH inhibitors affect the mechanical, electrical and ionic mechanisms involved in the contraction of skeletal muscle. These findings suggested a role for -SH groups in the process of E-C coupling. In order to interpret and analyze the results from these studies, it seems appropriate to review the currently accepted theories of E-C coupling.

Excitation-contraction coupling in skeletal muscle depends upon events occuring at four sites: 1) surface membrane, 2) transverse tubular membrane, 3) sarcoplasmic reticulum and 4) contractile proteins. It is generally agreed that depolarization of the sarcolemma is the trigger for muscle contraction (Hodgkin and Horowitz, 1960; Sandow, 1965; Zarchar and Zacharova, 1966). The response passes from the surface membrane down the transverse tubular membrane at the level of the Z line (Huxley and Taylor, 1958; Gage and Eisenberg, 1967). From the transverse tubules (t-tubules), the response is coupled to the sarcoplasmic reticulum which results in the release of sequestered calcium (Costantin *et al.*, 1965; Winegard, 1968). The diffusion of calcium from the sarcoplasmic reticulum to the region of the contractile proteins is a prerequisite for contraction (Sandow, 1965; Ashley and Ridgway, 1968). When calcium levels at the myofilaments reach above

10^{-7} M, the myofilaments contract (Weber *et al.*, 1963; Hasselbach, 1964). Reaccumulation of calcium into the reticulum reduces the level of ionized calcium in the sarcoplasm and the muscle relaxes (Hasselbach, 1964; Costantin *et al.*, 1965; Sandow, 1965).

Several drugs (including some -SH inhibitors) have previously been studied with respect to their effects on E-C coupling, and a specific site of action has been proposed for each of them. Some of these results and hypotheses will now be reviewed along with a general consideration of drug action on membranes.

A. Surface Membrane

The bimolecular leaflet model for the molecular organization of cell membranes, initially proposed by Danielli and Davson (1935) and extended to Robertson's (1959) concept of the unit membrane, is being superseded by different models (Benson, 1966; Korn, 1966). It now appears more likely that proteins extend through the membrane in some places, forming a protein-lipid mosaic (Korn, 1966; Gainer, 1967). Such proteins or lipoprotein complexes are conceivably involved in the binding of fixed charges to the membrane (Frankenhauser and Hodgkin, 1957; Shanes, 1958a), substrate binding and transport (Fox and Kennedy, 1965; Pardee, 1968) and excitation processes (Schmitt and Davison, 1965; Rojas, 1965).

Evidence is available that calcium ions are directly associated with membrane excitation phemenona (Frankenhaeuser and Hodgkin, 1957; Koketsu, 1965). In voltage clamped squid axons, increased sodium and potassium permeabilities result from lowering the calcium concentration in the external medium (Frankenhaeuser and Hodgkin, 1957; Blaustein and Goldman, 1966). Removal of calcium from the external solution results in depolarization and inexcitability in frog skeletal muscle (Caputo and Gimenez, 1967) and inexcitability in squid axon

(Frankenhaeuser, 1957). Treatment of squid axons with low calcium also results in a decreased time-to-peak of the initial (sodium) current (Frankenhaeuser and Hodgkin, 1957). Tobias (1964), Tasaki (1965) and Koketsu (1965) have all proposed resting and active state membrane mechanisms involving the calcium ion. In squid axon and in skeletal muscle it appears that calcium is necessary for normal membrane function.

The chelator ethylenediamine tetraacetic acid (EDTA) can be used to selectively remove superficial calcium from whole skeletal muscle (Isaacson and Sandow, 1967) without penetrating into the intracellular space (Bianchi, 1965; Feinstein 1966). EDTA also produces spontaneous twitching, inhibition of the electrically evoked twitch (Feinstein, 1966) and depolarization (Koketsu and Noda, 1962) of the surface membrane in skeletal muscle.

It has been known for many years that the local anesthetic procaine depresses excitability in nerve and muscle membranes (Toman, 1952; Shanes, 1958b). In skeletal muscle, intracellular recording studies have indicated that procaine decreases action potential amplitude and rise time without altering the resting potential (Inoue and Frank, 1962). *In vitro* studies with phospholipids extracted from nerve and muscle suggests a competitive interaction between procaine and calcium (Feinstein, 1964).

While the sulfhydryl inhibitors have not been studied to the same extent as EDTA or procaine, their application to a few cellular membranes have been reported. Sulfhydryl reagents act as noncompetitive inhibitors of glucose transport in erythrocytes (LeFevre, 1948; Dawson and Widdas, 1963). Recent investigations have been concerned with delineating the role of sulfhydryl residues essential for glucose transport and cation permeability. In particular, the differential solubilities of PCMBS and PCMB have been used to localize membrane sulfhydryl

groups. PCMBS, having a more hydrophilic character, penetrates the erythrocyte membrane to a lesser extent than does PCMB (Velick, 1953; Vansteveninck *et al.*, 1965). Results indicate that approximately 5% of the reactive membrane sulfhydryl groups are superficial; about 95% are located within and/or on the inner surface (Vansteveninck, *et al.*, 1965). Inhibition of surface membrane sulfhydryl groups is sufficient to block glucose transport (Vansteveninck, *et al.*, 1965). Further studies have suggested that the sulfhydryl groups within the membrane are responsible for cation permeability (Sutherland *et al.*, 1967; Rega *et al.*, 1967).

The importance of proteins in excitation phemenona was indicated by the experiments of Rojas (1965) in which proteases, injected internally into squid axons, abolished the action potential. Gainer (1967) reported a similar depression of crayfish muscle resting potential after treatment with proteases. Schmitt and Davison (1965) have postulated a membrane limited protein undergoing a conformational change, i.e. electrogenic protein, as responsible for excitability. This electrogenic protein "valve" is thought to regulate ionic permeabilities (Davison, 1967). Evidence for this hypothesis comes from studies indicating that internal and external application of sulfhydryl reagents block conduction in squid axons without disrupting membrane integrity as evidenced by minor variations in the resting potential (Huneeus-Cox, *et al.*, 1966). More recent studies indicate that internal perfusion by antibodies directed against axoplasmic proteins also block conduction with little alteration in the resting potential (Huneeus and Fernandez, 1967). The presence of proteins and free sulfhydryl groups somewhere within the membrane appear necessary for normal excitation (Davison, 1967).

B. Transverse Tubular System

It is generally recognized that the transverse tubular membrane conducts the electrical response across the volume of the muscle fiber

to activate the contractile apparatus (Huxley and Taylor, 1958; Peachey, 1966). Sandow (1965) has postulated an electrotonic i.e. passive, mechanism for the inward spread of activation as opposed to the propagated response along the surface membrane.

Recent morphological studies indicate that treatment of frog muscle with 400 mM glycerol disrupts the t-tubules without affecting the sarcolemma (Howell and Jenden, 1967; Eisenberg and Eisenberg, 1968). After treatment, the muscle conducts action potentials but the contractile apparatus does not respond (Gage and Eisenberg, 1967). Van der Kloot (1968) has used this technique to eliminate the t-tubules as a possible compartment for calcium sequestration.

C. Sarcoplasmic Reticulum

The release of intracellular calcium is necessary for the coupling of excitation to contraction in frog skeletal muscle. Relaxation is brought about by calcium removal (Winegard, 1968; Ashley and Ridgway, 1968). The terminal cisternae of the sarcoplasmic reticulum appear to be the site of calcium storage and release, while the longitudinal reticulum and fenestration collar are considered to be the sites of calcium uptake during relaxation (Costantin *et al.*, 1965; Winegrad, 1968; Peachey, 1968).

The properties of the reticular transporting system can be studied *in vitro*. The microsomal fraction, obtained by differential centrifugation of homogenized rabbit muscle, includes the t-tubular system along with the fragmented sarcoplasmic reticulum (Ebashi and Lipmann, 1962). The fragmented membranes seal off inclosed vesicles with diameters between 300 and 2000 Å and are capable of maintaining a 500–5000 fold calcium activity gradient (Hasselbach, 1964).

A number of agents including salyrgan (Martonosi and Feretos, 1964), ADP (Ebashi and Lipmann, 1962), PCMB (Carsten and Memmaerts, 1964) and NEM (Hasselbach, 1966; Hasselbach and Seraydarian, 1966)

inhibit calcium accumulation by isolated vesicles. Hasselbach (1966) has located sulfhydryl groups essential for calcium transport on the outer surface of the sarcoplasmic membrane.

Calcium movement has also been studied on whole sartorius muscle. Calcium efflux, from muscles preloaded with Ca^{45}, consists of two components; 1) a "fast" component derived from extracellular calcium and calcium loosely bound to the membrane, and 2) a "slow" component derived from the sarcoplasmic reticulum (Shanes and Bianchi, 1959; Bianchi, 1967). Caffeine (Bianchi, 1961; Isaacson and Sandow, 1967), quinine (Isaacson and Sandow, 1967), and EDTA (Bianchi, 1965) all increase the rate of calcium efflux.

D. Contractile Proteins

Various muscle proteins, thought to have a role in the contractile process, have been isolated. The thick filaments or A bands are composed of myosin; the thin filaments or I bands consist primarily of actin. Native tropomyosin, as reported by Ebashi and Kodama (1965, 1966), is a complex of two proteins, troponin and tropomyosin. From studies using fluorescent protein and antibody techniques, it appears that both proteins of native tropomyosin may be associated with or part of the actin filaments (Endo *et al.*, 1966; Ohtsuki *et al.*, 1967).

Activation of myofibrillar contractility appears to involve two events; 1) calcium binding and 2) calcium sensitization, occuring at two separate reaction sites (Yasui *et al.*, 1968). Recent investigations indicate that the free sulfhydryl groups of troponin are essential for complex formation with tropomyosin (i.e. calcium sensitizing activity) but are not involved at the calcium binding sites of troponin (Yasui *et al.*, 1968).

METHODS

All measurements in this study were performed on isolated sartorius muscles of *Rana pipiens* (Lemberger Co.) during all seasons of the year. The frogs were maintained in tap water at 5°C for 1 to 30 days prior to an experiment. After dissection, the muscles were equilibrated at 22–24°C in Ringer solution containing (mM): 110.8 NaCl, 2.0 KCl, 1.8 $CaCl_2$, 0.1 NaH_2PO_4 and 2.02 $NaHCO_3$. The pH was adjusted to 7.2 with HCl, [tris(hydroxymethyl)aminoethane], or NaOH. Calcium-free Ringer was prepared without the addition of calcium. In some experiments Na_2EDTA was added to the calcium-free Ringer (EDTA-Ringer) solution. Choline chloride Ringer (choline-Ringer) was prepared exactly as normal Ringer except that choline chloride was substituted for NaCl. All test agents were added to the normal, calcium-free, EDTA-, or choline-Ringer before use, and if necessary the pH readjusted to 7.2. Deionized water (continental Water Corp.) was used to prepare all solutions.

The three -SH inhibitors differed in their solubilities in normal Ringer solution. The poor solubility of PCMB limited its use to concentrations of about 2.3 mM. The solubilities of PCMBS and NEM are greater than 5.0 and 20.0 mM respectively.

I. Mechanical Response Studies

In these experiments, the sartorius was excised with the pelvic bone and tibial tendon attached. This preparation was mounted in a 25 ml capacity paraffin muscle chamber. The pelvic bone was rigidly constrained within the chamber with the muscle resting horizontally on a massive platinum-iridium electrode under approximately 2 gms tension. A fine metal chain was used to connect the tibial tendon to the force displacement transducer. During the 30 min equilibration in Ringer's solution the muscle was subjected to twitch and tetanic stimulation. After

the equilibration period, the muscle bath was changed for one containing the test agent. Tubocurarine chloride (5×10^{-5} gms/ml) was routinely added to all solutions to block muscle excitation due to transmitter release from the electrically-stimulated nerve terminals.

Supramaximal electrical stimulation (Grass Inst. Co. Model S4GR) of the muscle was employed. Electrically-evoked twitches were produced by rectangular pulses of 2 millisecond duration at a frequency of 1 pulse/15 sec to 1 pulse/30 sec. A 200 millisecond rectangular pulse chopped at a frequency of 500 shocks/sec was used to produce a fused tetanus. Intervals between tetani were 1 min or longer. The isometric mechanical response to electrical stimulation was measured by an Ft-03 force displacement transducer (Grass Inst. Co.), amplified and displayed on a Model 7 polygraph recording system (Grass Inst. Co.). In some experiments the mechanical response to the test agent was recorded without electrical stimulation.

The data will be given either as originally recorded or as a graph of the particular parameters studied. In both cases, the effects will be discussed in terms of the control maximal electrically-evoked twitch or tetanus measured before adding the -SH inhibitor to the muscle bath.

Further mechanical response studies involved visual observation of asynchronous twitching under a stereomicroscope. In these experiments paired sartorius muscles were used. The control muscle was treated with the -SH inhibitor alone, while the experimental muscle was pretreated with a test agent before addition of the -SH inhibitor.

II. Spectrophotometric Studies

Spectrophotometric procedures were used to study the molecular interaction between the -SH inhibitors and the test agents used in this investigation. It had previously been reported that NEM (Gregory, 1955), PCMB and PCMBS (Boyer, 1954) show absorption peaks in the ultra-

violet and spectral shifts accompanying mercaptide formation. Absorption spectra of the -SH inhibitors were made after mercaptide formation with cysteine and also after reaction with the test agents procaine and EDTA. A difference spectrum was run with the -SH inhibitor and test agent in the sample cuvette and the test agent in the reference cuvette. Complex formation between -SH inhibitor and test agent was indicated if the difference spectrum did not match the absorption spectrum of the -SH inhibitor alone. Quartz curvettes were used in a Cary (Model 14) dual beam spectrophotometer. The spectra were recorded on a 10 inch chart recorder (Brown).

Because of the poor solubility of PCMB in normal Ringer's solution the absolute concentration of PCMB was determined by absorption measurements at 232 $m\mu$ before each experiment. The optical density (OD) was recorded and a Beer's law calculation of the concentration was made. Beer's law can be written:

$$OD = \epsilon_M \; c \; 1$$

where ϵ_M is the molar extinction coefficient, c is the concentration of PCMB and 1 is the length of the cuvette in cm. The extinction coefficient for PCMB $\epsilon_M = 1.69 \times 10^4$) was reported by Boyer (1954) and confirmed in this laboratory. These measurements were made on a Beckman DB spectrophotometer.

III. Radiocalcium Efflux Studies

Ca^{45} (New England Nuclear) was added to normal Ringer's solution to make Ca^{45} Ringer with a specific activity of 4 $\mu c/ml$. Paired sartorius muscles (50-80 mgms), with their tendons removed, were loaded with Ca^{45} by soaking for 3 hours at room temperature (22–24°C) in Ca^{45} Ringer. After loading, the muscles were rinsed with 20 ml of calcium-free Ringer's solution and gently blotted (Whatman #42 filter paper) to remove any solution that might adhere to the outer surface. The muscles

were then placed in a chamber containing 3 ml of normal or calcium-free Ringer depending upon the experiment. This collection fluid was changed for 3 ml of fresh solution at 10 min intervals through the entire washout period. At each 10 min interval 1 ml of the total 3 ml washout fluid was emptied into a polyethylene scintillation vial and 12 ml of scintillation fluor added. At certain times during the washout period (to be indicated for each experiment), the test agent was added to the collection fluid. The muscle chambers were shaken during the entire washout and at no time during the washout period were the muscles removed from the chamber.

At the completion of 180 min of washout the muscles were removed from the washout chamber, lightly blotted, and placed in silica crucibles. The muscles were dried overnight at 100°C, placed in a muffle furnace and ashed for 16 hours at 500°C. Three ml of 0.1 N HCl was added to the muscle ash and the crucibles shaken for 3 hours. One ml samples of the ashed muscle solution was then prepared for counting.

The samples obtained at each 10 min collection period and the acid-dissolved muscle ash were counted in a liquid scintillation spectrometer (Packard Tri-Carb Model 3375). After correction for decay and quenching the data was expressed as a rate coefficient curve. The rate coefficient is the percentage of the average tissue radioactivity in the tissue during the time of collection that has emerged per min during the collection period.

The scintillation fluor consisted of: 180 gms naphthalene, 36 gms 2,5-diphenyloxazole, 1.8 gms 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and 600 ml 2-ethoxyethanol all dissolved in 3 liters of p-dioxane (spectroquality).

IV. Studies on Membrane Electrical Properties

After isolation, the sartorius muscle was mounted dorsal side upward in the Plexiglas chamber (50 ml capacity) shown in Fig. 2. Both

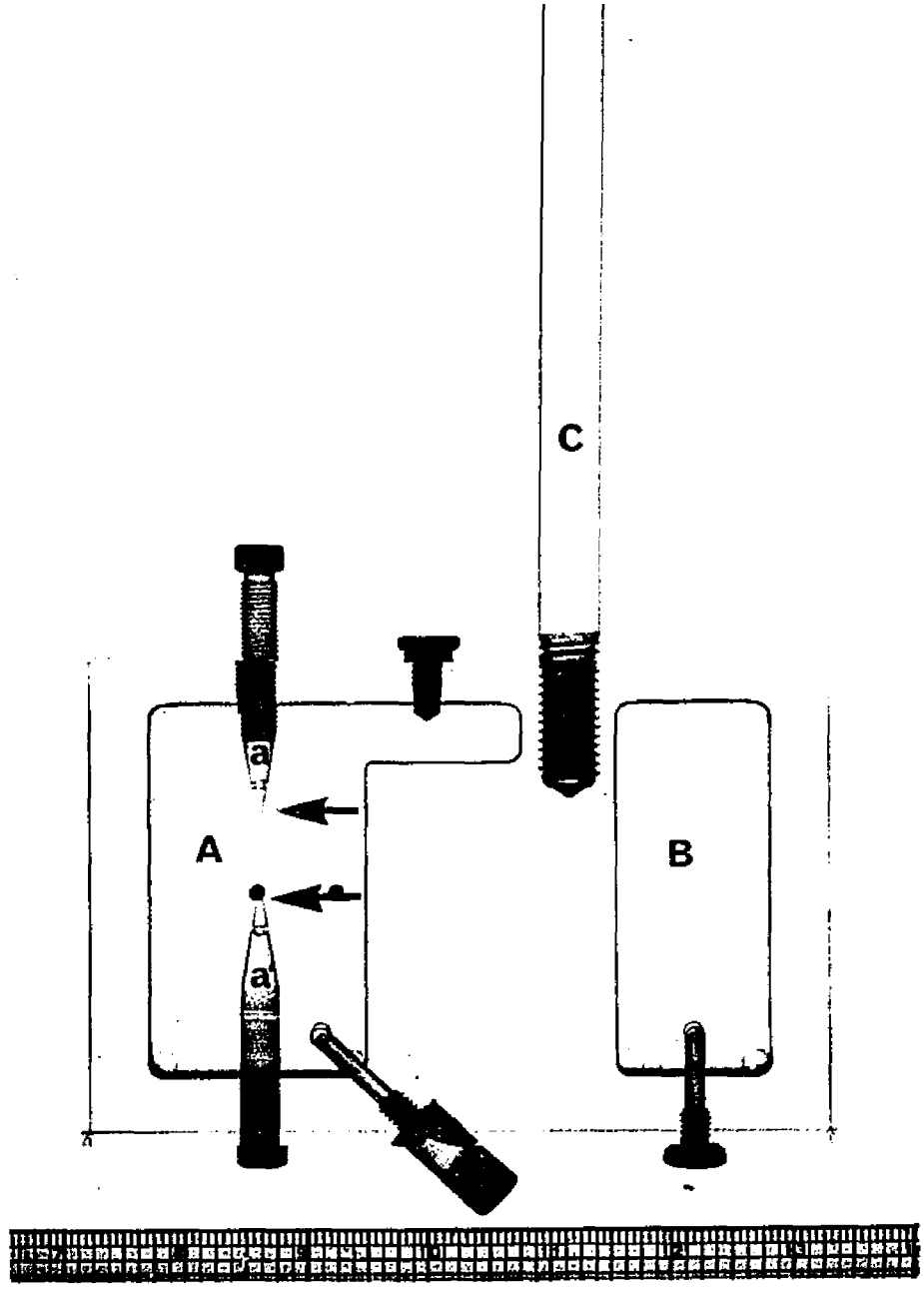


Figure 2 Plexiglas chamber used for intracellular recording studies. The sartorius muscle was secured to the support posts (a and a′) between the arrows within chamber A. The support posts were then adjusted to hold the muscle at its *in situ* length in Ringer's solution. A light source was placed below chamber A to permit observation of muscle and microelectrode. Chamber A has a 50 ml capacity. Chamber B was used to make preliminary adjustments and measurements before an experiment. A support rod (C) was used to secure the entire chamber to a vibrationless mount.

tendons were secured via nylon thread to the support posts. Muscles were mounted at rest length and allowed to equilibrate for 30 min to 1 hour in Ringer solution (22–24°C). Control resting or action potentials were recorded (depending upon the experiment) before addition of the -SH inhibitor. After addition, resting or action potentials were recorded as a function of time-zero time being the time of addition.

Transmembrane potentials were recorded by means of glass microelectrodes filled with 3.0 M KCl. Only microelectrodes with resistances between 8–15 megohms and tip potentials of 5 mv or less were used. If the resistance or tip potential changed during an experiment the microelectrode was discarded. The microelectrode was inserted into a 3% KCl-agar bridge. Both microelectrode and KCl-agar bridge were supported and positioned by a micromanipulator (Pgeigger Model PBl-2). A chlorided silver wire (.012 inch diameter) connected the KCl-agar bridge to the electrometer amplifier (Bioelectric Model NF-1). The output of the amplifier was displayed on an oscilloscope (Tektronix Model 503) and electrometer amplifier (Keithley Model 610B) and recorded on a chart recorder (Keithley Model 370).

In order to minimize liquid junction potentials the indifferent (reference) electrode was devised to cancel the potentials arising at the microelectrode side. The Ringer solution surrounding the muscle was connected in series to a Ringer-agar and KCl-agar bridge. A chlorided silver wire connected the KCl-agar bridge to the ground of the amplifier. The junction potential of the entire system was measured (without the microelectrode in place) before each experiment and was normally below 2 mv. This potential along with the microelectrode tip potential was zeroed out before each experiment.

Before any action potentials were recorded but with the microelectrode in position, the entire electrical system was calibrated and the frequency response checked. If necessary the input capacity neutral-

ization of the electrometer amplifier (Bioelectric) was adjusted for optimum frequency response.

Action potentials were elicited by short (.1 msec. duration) rectangular pulses passed between two silver chlorided wires at opposite end of the muscle. Action potentials were amplified as previously described and displayed on a storage oscilloscope (Tektronix Model 564).

V. Electron Microscopy Studies

Paired sartorius muscles were dissected from *Rana pipiens* at room temperature (22–24°C). Before dissection, the *in situ* length of each muscle was measured, with the knee joint bent to form a right angle. The experimental muscle was treated for 30 min with NEM while the control muscle remained in normal Ringer's solution. Following incubation with NEM the experimental muscle was rinsed briefly in normal Ringer and held at its new rigor length, while the control muscle was held at approximately 120% of its *in situ* length.

The muscles were fixed overnight at room temperature in a buffered 3% glutaraldehyde solution. After fixation the muscles were rinsed 3 times over a 1½ hour period in a buffered rinse solution (22–24°C). Post fixation was performed for 2 hours at 0°C in a 1% osmium tetroxide fixative. After post fixation, small bundles (0.5 mm × 3 mm long) were cut from the center region of the two muscles. The muscles were dehydrated in a graded series of ethanol-water mixtures (30%, 50%, 70%, 95%, 100%) for 10 min each. Following dehydration, the muscles were soaked for 15 min in propylene oxide and then for 1 hour in a 1:1 combination of propylene oxide and Epon. The bundles were subsequently embedded in Epon filled gelatin capsules. The capsules were set for 12 hours at 37°C and then for 36 hours at 60°C.

Longitudinal and transverse sections were cut with a Sorvall Porter-Blum MT-1 ultra microtome using glass knives. They were mounted on 200 mesh uncoated copper grids and stained with uranyl acetate (1 hour)

or lead citrate (Reynolds, 1963). Sections were examined within 2 week of staining in an RCA EMU 3-H electron microscope operated at 50 kV with a 45 μ diameter objective aperture. The electron microscope was calibrated several times during this study with a replica grating. Micrographs were made of longitudinal and transverse sections for both the NEM treated and control muscle. The micrographs were enlarged and printed by standard photographic techniques.

The fixation solution consisted of: 3% glutaraldhyde, 100 mM sodium cacodylate and 5 mM $CaCl_2$ at pH = 7.3. The buffer rinse contained: 10 gms/100 ml sucrose, 100 mM sodium cacodylate and 5 mM $CaCl_2$ at pH = 7.3. The post fixative contained: 1% osmium tetroxide, 100 mM sodium cacodylate and 5 mM $CaCl_2$ at pH = 7.3. The embedding medium (% composition, v/v): Epon 812, 46.2; methyl nadic anhydride, 25.4; dodecanyl succinic anhydride, 27.4; 2,4,6-tri(dimethylaminomethyl) phenol, 1.0.

MATERIALS

NEM	• N-ethylmaleimide (Sigma)
PCMB	• Parachloromercuribenzoic acid (Sigma)
PCMBS	• Parachloromercuribenzene sulfonic acid (Sigma)
POHMB	• Parahydroxymercuribenzoic acid (Sigma)
Tris	• Tris(hydroxymethyl)aminomethane (Sigma)
Na_2EDTA	• Disodium ethylenediamine tetracetate (Fisher)
	• Procaine-HCl (Baker)
	• d-Tubocurarine chloride (Mann Res.)
	• Choline chloride (Eastmann)
	• Cysteine (Calbiochem.)
	• Caffeine (Sigma)
	• Glycerol (Baker)
	• Sodium cacodylate (Amend Drug)
	• Osmium tetroxide (Int. Mineral and Chem.)
	• Glutaraldhyde (K & K Labs.)

RESULTS

I. Effects of -SH Inhibitors on the Mechanical Response of Frog Sartorius Muscle

A. NEM

The action of NEM on skeletal muscle is a slowly developing irreversible rigor (Okamoto and Kuperman, 1966). This effect is shown for 1.0 and 10.0 mM NEM during electrical stimulation (Fig. 3). It is interesting to note that 1.0 mM NEM produces a greater maximum rigor than does the higher concentration. The action of 10.0 mM NEM is characterized by a smaller maximum rigor followed by a partial relaxation. Relaxation from the maximum rigor tension is not observed for 1.0 mM NEM.

As shown in this experiment (Fig. 3) and others, NEM does not modify the isometric twitch response to maximal electrical stimulation. The evoked twitch tension is gradually depressed as the rigor tension increases. Treatment with equimolar concentrations of cysteine and NEM block both the rigor and twitch depression.

The dosage-response relationship for maximum rigor development in the concentration range of 0.1–20.0 mM NEM is given in Fig. 4. Rigor tension increases with concentration up to 1.0 mM; further increase (to 5 mM or greater) produces a significant reduction ($.01 < P < .02$) in rigor tension. The latent period to onset of rigor development is also concentration dependent as shown in Fig. 5. In this case the log latency is a linear function of the log concentration, as indicated by the method of least squares ($P < .001$).

It is well-known that caffeine, in concentrations of 10.0 mM or greater, produces rigor in isolated frog sartorius muscle (Sandow, 1965). Rigor is not produced by 10–20 mM caffeine in a muscle pretreated with NEM, 1–20 mM.

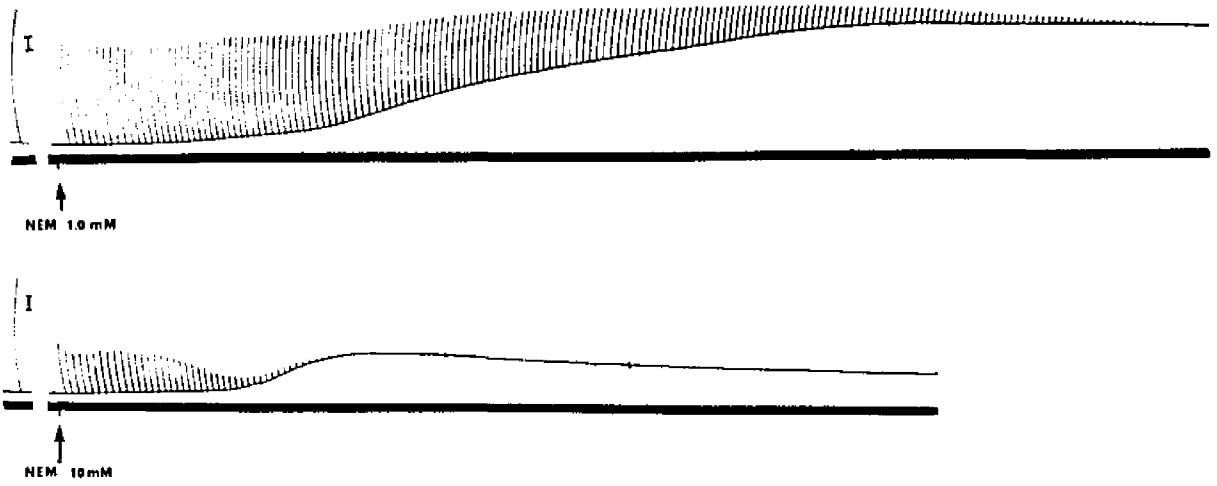


Figure 3 Effect of 1.0 and 10.0 mM NEM on the electrically-evoked twitch of isolated curarized sartorius muscle. The evoked twitch tension diminishes as the rigor tension increases until at about maximum rigor tension the muscles become inexcitable. A 200 millisecond electrically-evoked control tetanus is shown to the left of the arrows. The time markers indicate 5 sec. Paired muscles: summer frog. Calibration bar: 2.5 gms. Stimulation frequency: 1 pulse/15 sec.

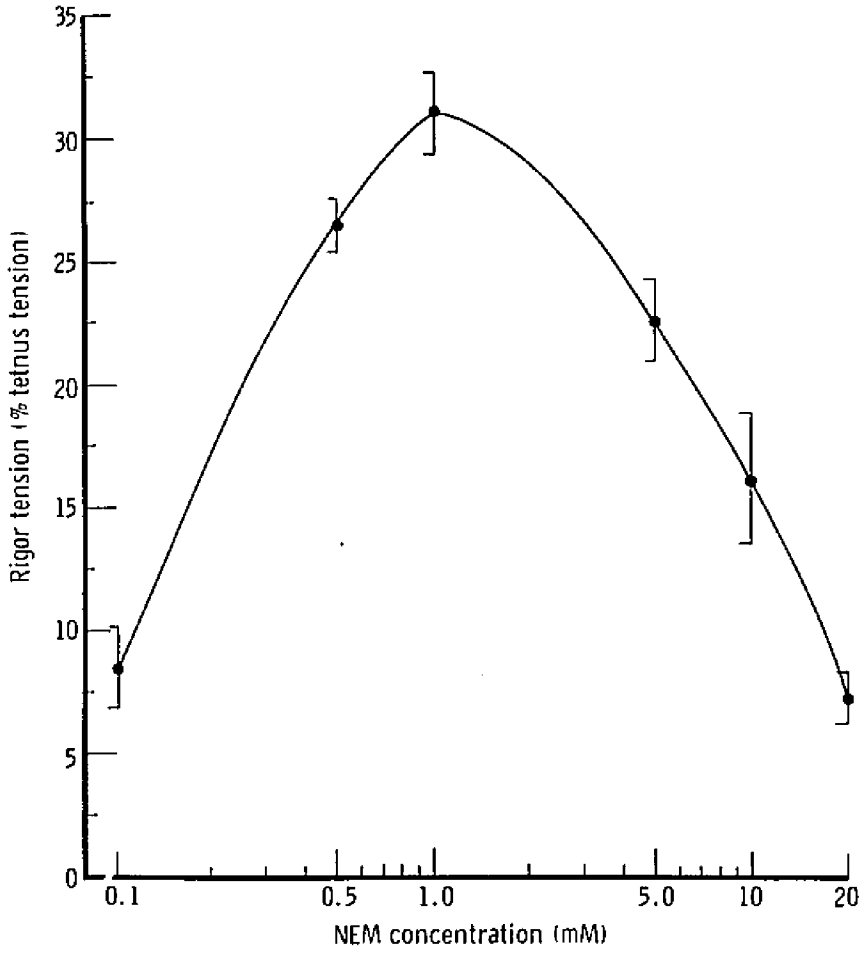


Figure 4 Dosage-response relationship for NEM on frog sartorius muscle. The rigor tension is expressed as a percentage of control tetanus tension before the addition of NEM. The muscle was not stimulated after the addition of NEM. Values given are means for 6 muscles, and the standard error is indicated (n = 6; ±SE). Winter frogs.

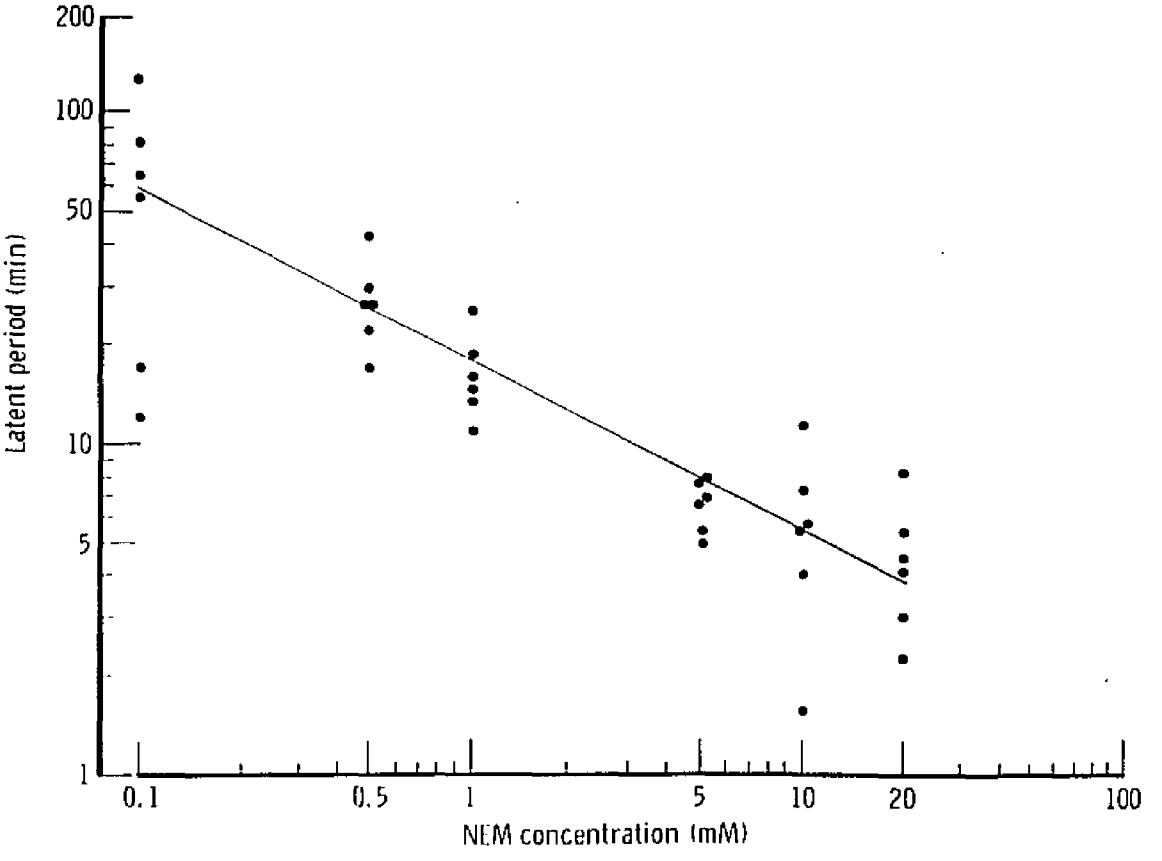


Figure 5 Latent period to onset of NEM-induced rigor tension. Each point represents a single experiment. The muscle was not stimulated after the addition of NEM. The indicated regression line is highly significant ($P < .001$). Winter frogs.

NEM produces rigor in muscles presoaked in 5.0 mM EDTA for a suffiencint time (30 min) to reduce the extracellular and membrane calcium to a very low level (Bianchi, 1965). Similarly, NEM rigor occurs in muscle pretreated with isotonic KCl. Since both EDTA and KCl cause depolarization, NEM rigor is probably not mediated by way of surface membrane deplorization. With both agents, the NEM response was somewhat less than with untreated muscle.

B. PCMB

Addition of the organomercurial PCMB to unstimulated skeletal muscle causes a period of spontaneous twitching after a latent period of 2-3 min (Okamoto and Kuperman, 1966). As observed with light microscopy, the twitching appears as asynchronous longitudinal movements of individual fibers which subside after 10-15 min. In studying this effect on paired sartorius muscles the following results were noted:

a) The asynchronous contractions are blocked by depolarizing the experimental muscle in 116 mM KCl.

b) Soaking a muscle for 30 min in choline-Ringer's before addition of PCMB also blocks this effect.

c) Neither the addition of d-tubocurarine (5×10^{-5} gms/ml) nor removal of calcium from the Ringer's solution modify the asynchronous twitching.

d) Procaine (0.25 mM) and cysteine, added in equimolar concentrations with PCMB, completely antagonize this effect.

e) After 1 hour treatment in a Ringer's solution containing glycerol (400 mM) the muscle was returned to normal Ringer's. This treatment markedly reduced the level of asynchronous twitching during subsequent application of PCMB.

From the above experiments, it appears that both sodium and a normal resting potential are required for asynchronous twitching. Disruption

of the t-tubules with glycerol (Eisenberg and Eisenberg, 1966), or treatment with a membrane "stabilizer" such as procaine (Feinstein, 1963) also blocks this effect. It is unlikely that PCMB acts by causing the release of acetylcholine from the nerve endings because d-tubocurarine does not suppress the twitching. These results indicate a membrane effect rather than an internal site of action.

Another effect of PCMB is observed only in *stimulated* skeletal muscle, and this is shown in Fig. 6. The twitch is fractionated so that two contractions are evoked by a single electrical stimulus. The second or "secondary" contraction is of longer duration than the first, and it may occur prior to complete relaxation from the first contraction. The threshold for this effect was not precisely determined but is about 0.25 mM. The time course of this effect, as produced by the maximal concentration of PCMB (2.3 mM) that is soluble in Ringer's solution, is shown in Fig. 7. With repetitive stimulation of the muscle at a rate of 1 pulse/30 sec, there is a gradual decline of both the initial and secondary contraction, the secondary disappearing first, and finally complete absence of response occurs. Parahydroxymercuribenzoic acid also produces this secondary contraction. As with the asynchronous twitching, the secondary contraction and twitch depression are antagonized by cysteine but not d-tubocurarine. Procaine (0.25 mM) blocks the secondary contraction without affecting the twitch depression.

After the PCMB treated muscle becomes electrically inexcitable, the addition of 10 mM caffeine still causes rigor.

C. PCMBS

The sulfonated analogue of PCMB, produces asynchronous twitching and depression of the electrically-evoked twitch. The asynchronous activity is similar in appearance to that produced by PCMB and is also antagonized by pretreatment with 116 mM KCl, choline-Ringer's, cys-

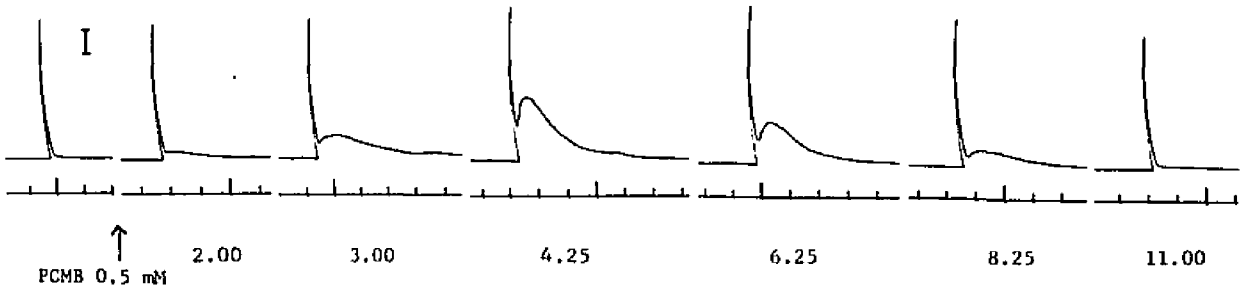


Figure 6 Effect of 0.5 mM PCMB on the electrically-evoked twitch of isolated curarized sartorius muscle. Asynchronous twitching can be observed on the secondary contraction and baseline. A maximum electrically-evoked control twitch is shown to the left of the arrow. The time markers are in seconds, and the numbers below each twitch indicate the time in min. after PCMB application. Calibration bar; 2.5 gms. Stimulation frequency: 1 pulse/15 sec.

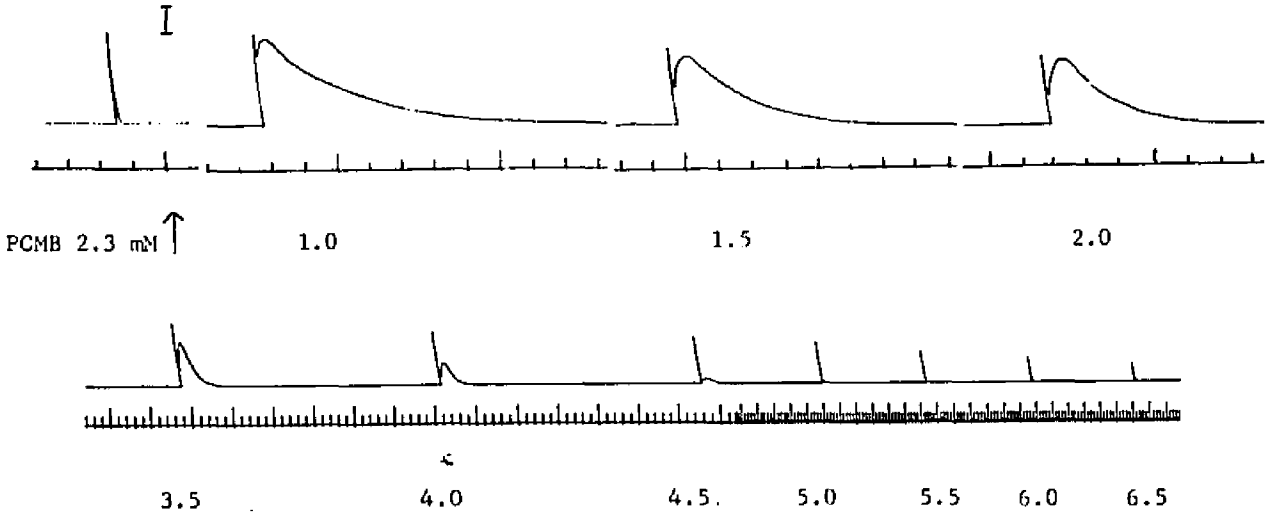


Figure 7 Effect of 2.3 mM PCMB on the electrically-evoked twitch of isolated curarized sartorius muscle. Asynchronous twitching can be observed on the secondary contraction and baseline. A maximum electrically-evoked control twitch is shown to the left of the arrow. The time markers are in seconds, and the numbers below each twitch indicate the time in min. after PCMB application. Calibration bar: 2.5 gms. Stimulation frequency: 1 pulse/30 sec.

teine, procaine and glycerol. PCMBS-induced twitching can also be blocked by increasing the calcium concentration in the Ringer's to 10.0 mM. A membrane effect appears consistent with these observations.

Cysteine will block the PCMBS-induced depression of the evoked twitch. Cysteine will not however reverse this depression.

After long exposures, high concentrations of PCMBS (5 mM) produce low rigor tensions (1-2% of control tetanus). PCMBS-treated muscles, although unresponsive to electrical stimulation, still go into rigor after exposure to caffeine. PCMBS does not produce secondary contractions in the electrically-stimulated muscle, and in this respect it is strikingly different in its pharmacological properties from PCMB.

II. Molecular Interactions Between -SH Inhibitors and Test Agents

Spectrophotometric studies were made to determine whether there is complex formation between the three -SH inhibitors and the other chemical agents used in these experiments, i.e. cysteine, EDTA and procaine. The absorption spectra and measured extinction coefficients of NEM, PCMB and PCMBS are indicated in Fig. 8. Addition of equimolar concentrations of cysteine (to the sample cuvette) along with the -SH inhibitor decreases absorption to zero. These -SH dependent absorption peaks can be used to evaluate complex formation with other test agents.

It was of interest to determine if EDTA interacts with either NEM or the organomercurials. A difference spectrum was run with the -SH inhibitor and EDTA in the sample cuvette and EDTA in the reference cuvette. The normal absorption spectra of the -SH inhibitors were not altered, suggesting that molecular interactions did not take place.

Procaine probably forms a complex with NEM and hence modifies its absorption characteristics. This effect was not observed with the

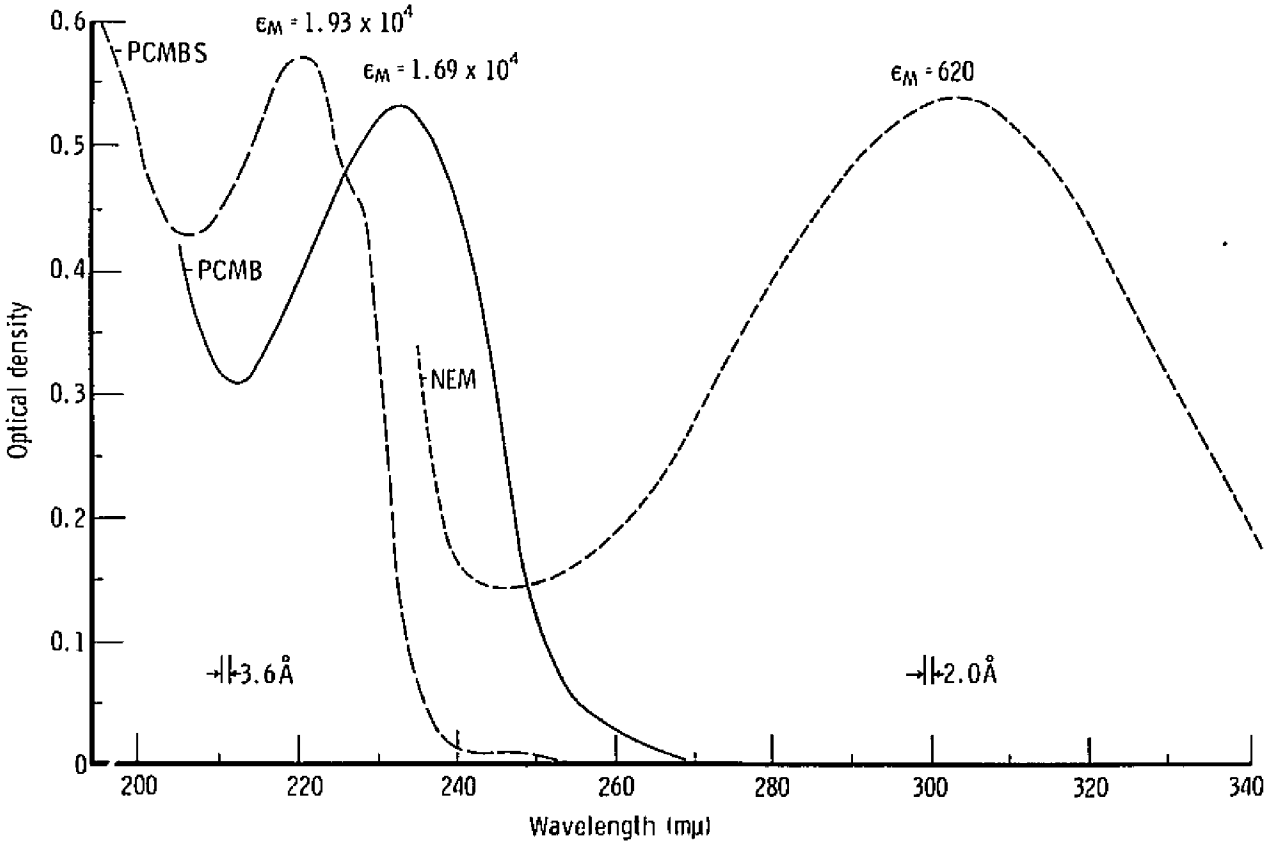


Figure 8 Absorption spectra for NEM, PCMB and PCMBS. Extinction coefficients (ϵ_M) are given at the peak of each curve. The individual absorption peaks disappear upon reaction with equimolar concentrations of cysteine or upon decomposition of the -SH inhibitor. Spectrophotometer resolution is given at bottom.

organomercurials. Accordingly, procaine was not used in any experiments involving NEM.

III. Effects of -SH Inhibitors on Ca^{45} Efflux from Frog Sartorius Muscle

A. NEM — Effects on Ca^{45} Efflux

In view of the mechanical effects described above it was of interest to determine whether NEM influenced calcium efflux from isolated sartorius muscles. The Ca^{45} release from sartorius muscle into Ringer's solution after addition of NEM is shown in Fig. 9. NEM (10 mM) produces a rapid increase in the Ca^{45} rate coefficient to about 4.1 times the base (120 min) level; the release with 1.0 mM is both slower and smaller. This Ca^{45} efflux is compared with the rate and magnitude of rigor development after addition of 1.0 or 10.0 mM NEM (Fig. 10). It is interesting that while the kinetics of both calcium release and rigor development are similar, the amount of calcium released is inconsistent with the rigor tension recorded. Maximum calcium release is produced with 10 mM; yet the maximum rigor tension developed at this concentration is less than the tension measured at 1.0 mM NEM.

To determine the origin of the Ca^{45} released by NEM, 5 mM EDTA was used to remove superficially located calcium and thus prepare the muscle for testing the action of NEM on intracellular calcium (Bianchi, 1965). As shown in Fig. 11, addition of 10.0 mM NEM releases a fraction of calcium even after treatment with EDTA. This observation implies an intracellular action of NEM or a site that is inaccessible to EDTA.

Since cysteine can block NEM rigor, it is of interest to determine whether cysteine also blocks Ca^{45} efflux. The experimental Ca^{45} loaded muscles were washed out in Ringer's solution to which 1.0 mM cysteine was added. After 120 min of washout, 1.0 mM NEM was added to the washout solution. The NEM-induced Ca^{45} efflux was completely abolished

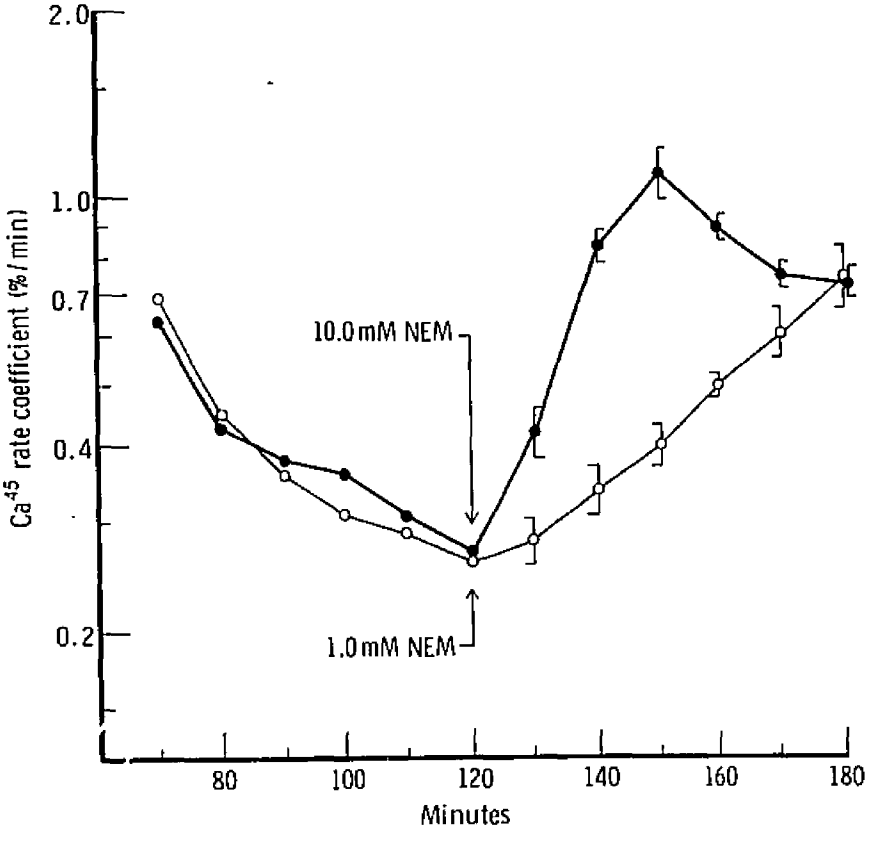


Figure 9 Time course of the changes in rate coefficients for Ca^{45} release from paired sartorius muscles in normal Ringer's solution. NEM was added to the Ringer's solution from 120 to the end of the washout. Each point is the mean of 5 separate experiments (n = 5). The standard error (±SE) is given for the later portion of the curve. Winter frogs.

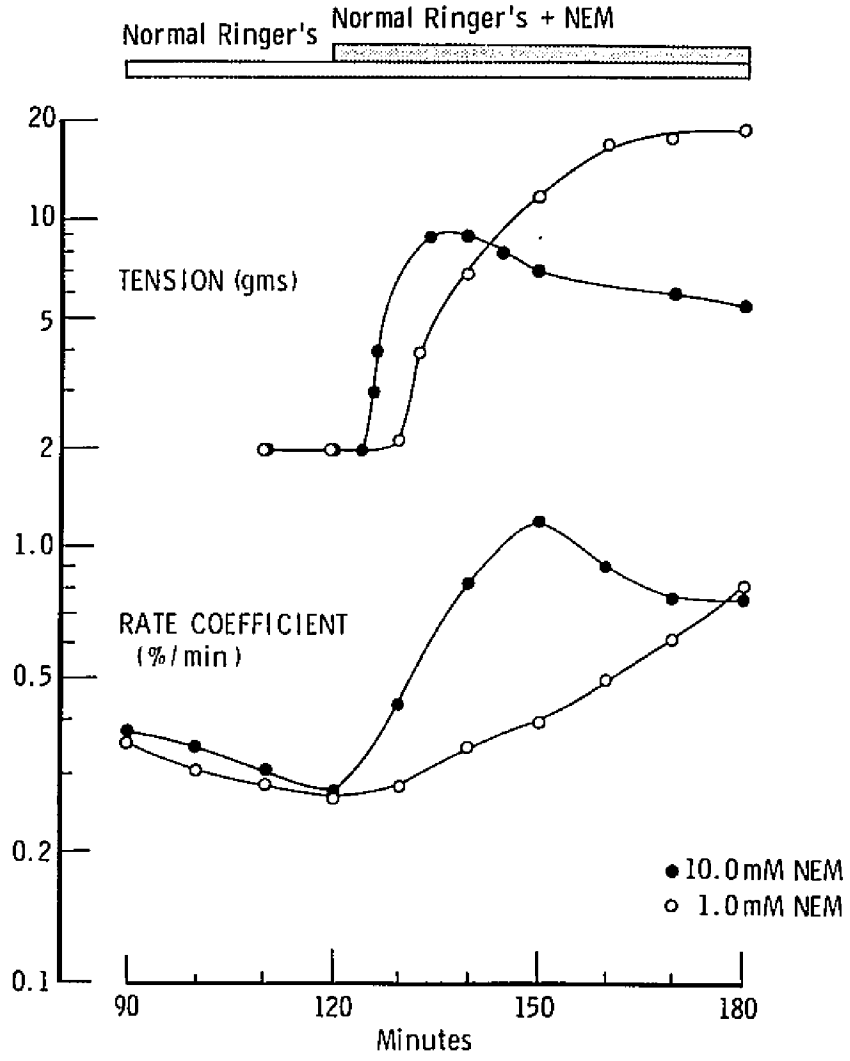


Figure 10 The development of rigor tension for 1.0 and 10.0 mM NEM (top curve) compared with the rate coefficient for Ca^{45} release (bottom curve-replot of Fig. 9). The top curve was recorded from a single pair of sartorius muscles and replotted with tension on a logarithmic scale. The muscles were held at an initial tension of 2 gms. Winter frogs.
Control tetanus tension (1.0 mM NEM): 80 gms.
Control tetanus tension (10.0 mM NEM): 90 gms.

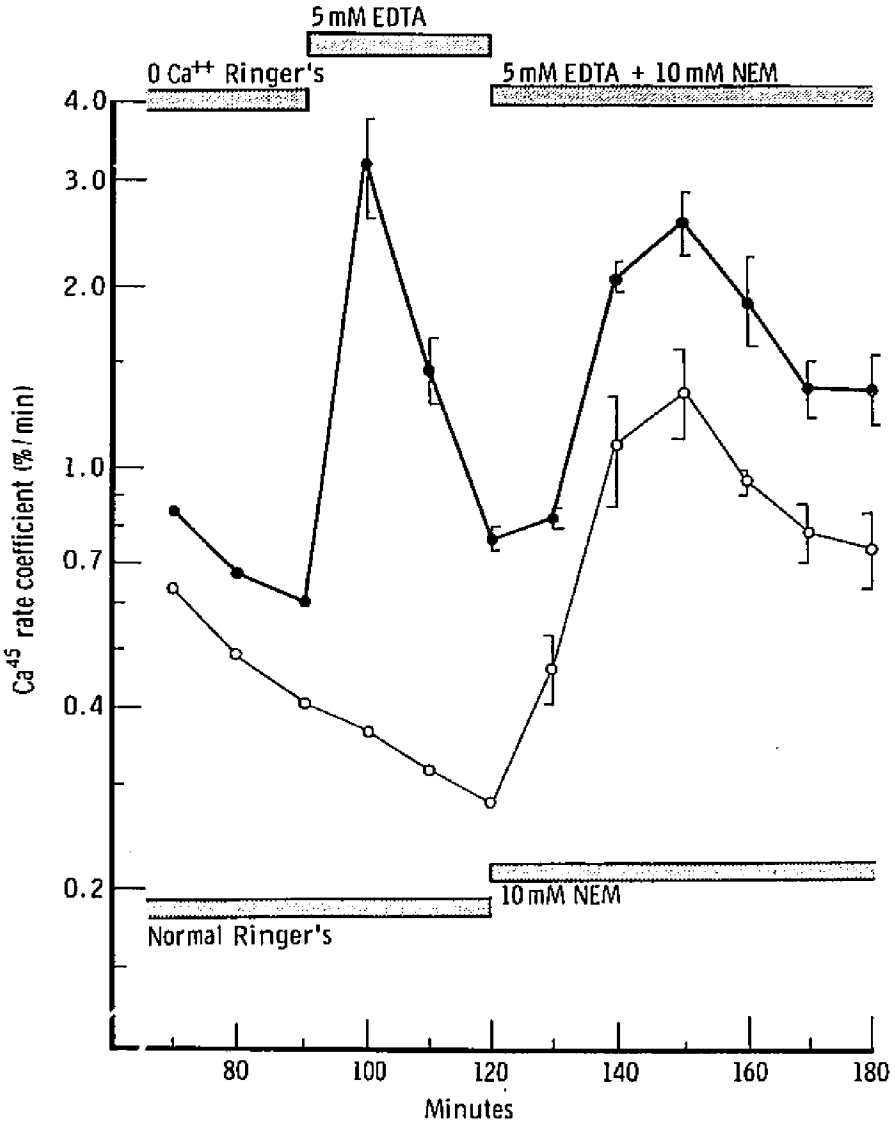


Figure 11 Effect of NEM, in the presence and absence of EDTA, on the rate coefficient for Ca^{45} release from isolated paired sartorius muscles. The experimental muscles were washed out in calcium-free Ringer to which 5.0 mM EDTA was added at 90 min and 10.0 mM NEM at 120 min. The transient EDTA-induced Ca^{45} efflux is shown. The control muscle was washed out in normal Ringer's solution for 120 min after which 10.0 mM NEM was added to the washout medium. Each point is the mean of 3 muscles (n = 3). The standard error (±SE) is given for the later portion of the curve. Notations at the top and bottom of the graph refer to the top and bottom curves respectively.

when cysteine was present in the washout medium. The paired control muscles gave a normal Ca^{45} release upon addition of NEM.

B. PCMB — Effects on Ca^{45} Efflux

The measured Ca^{45} release after treatment with 0.25 and 1.0 mM PCMB is shown in Fig. 12. For both concentrations, the calcium release is sustained for 60 min or more, whereas the PCMB-induced secondary contraction and asynchronous twitching are completed within 10 min. It is known that both caffeine and quinine, in concentrations which do not produce rigor, give a sustained Ca^{45} release similar to PCMB (Isaacson and Sandow, 1967). Perhaps PCMB, like caffeine and quinine, is releasing calcium from an intracellular compartment.

The effect of 1.0 mM PCMB on Ca^{45} efflux, in the presence and absence of 5 mM EDTA, is shown in Fig. 13. After treatment with EDTA a somewhat *reduced* increase in rate coefficient is noted (2 times the 120 min level) as compared with muscles not exposed to EDTA (3.6 times).

Cysteine reduced drastically the Ca^{45} efflux produced by 1.0 mM PCMB in normal Ringer's solution. PCMB associates with cysteine in solution to form a PCMB-cysteine complex (Boyer, 1954). This complex is formed through polar-covalent bonding, permitting some of the PCMB to exist in the undissociated state. It is reasonable to assume the undissociated PCMB binds to free -SH groups on the muscle and initiates a small Ca^{45} release. It is also possible that the complex itself causes this small Ca^{45} release.

Washout studies were also made with procaine (0.25 mM) in the washout medium. Procaine blocks the asynchronous twitching and secondary contraction but not the PCMB-induced calcium efflux.

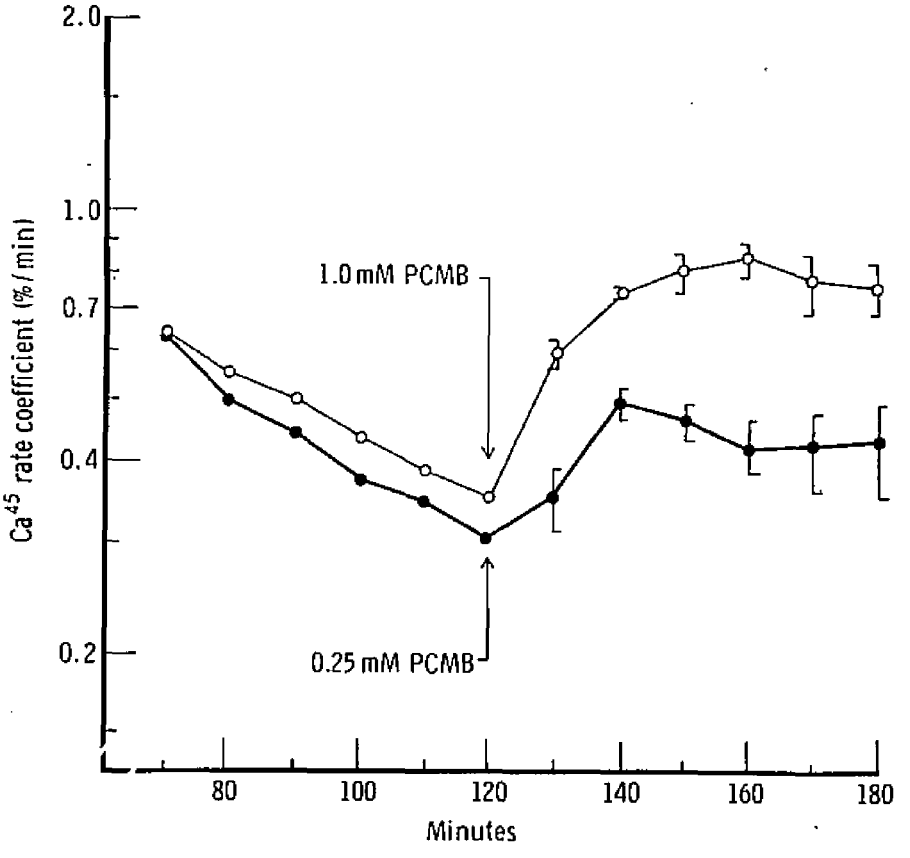


Figure 12 Time course of the change in rate coefficient for Ca^{45} release from paired sartorius muscles in normal Ringer's solution. PCMB was added to the Ringer solution as indicated. See legend-Fig. 9 for experimental details. n = 5; ±SE.

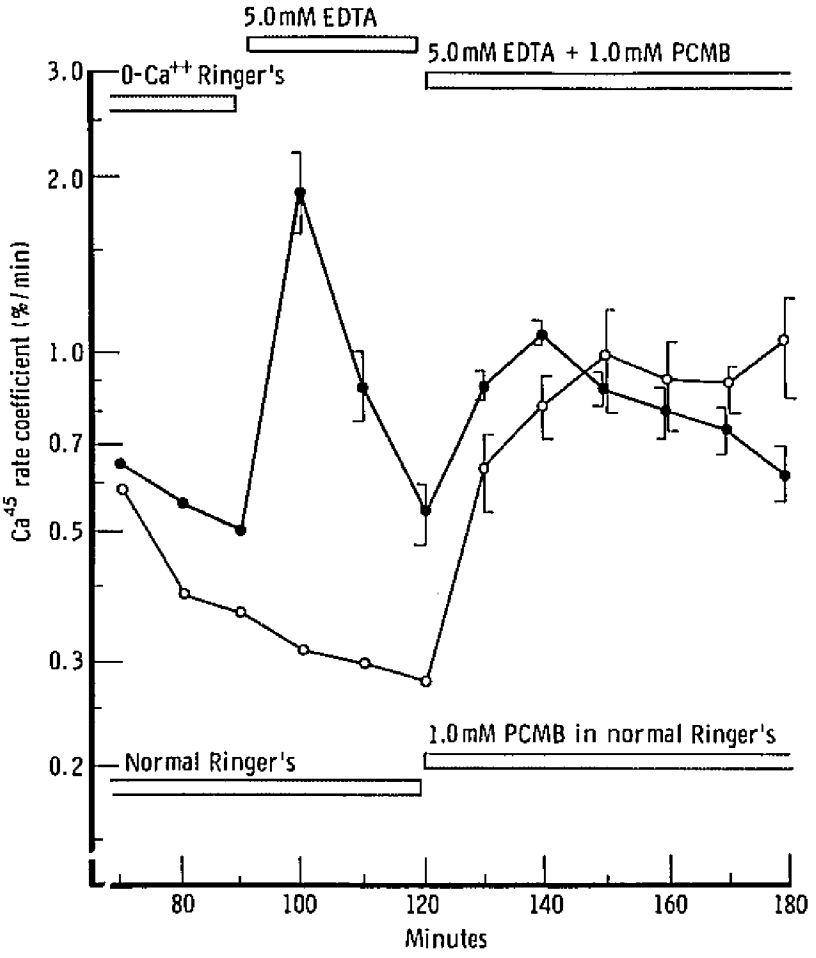


Figure 13 Effect of PCMB, in the presence and absence of EDTA, on the rate coefficient for Ca^{45} release from isolated paired sartorius muscles. See legend-Fig. 11 for experimental details. Notations at top and bottom of graph refer to top and bottom curves respectively n = 3; ±SE.

C. PCMBS — Effects on Ca^{45} Efflux

The increase in Ca^{45} rate coefficient produced by 1.0 and 5.0 mM PCMBS is shown in Fig. 14. A very rapid and transient calcium release is noted after treatment with 1.0 mM PCMBS, similar to that produced by EDTA (see Fig. 11 and 13). With the higher concentration a more prolonged efflux is noted. At the conclusion of washout, the muscles treated with 1.0 mM PCMBS were inexcitable (but not in rigor), whereas a small rigor was noted in the muscles treated with 5.0 mM PCMBS. This observation was previously noted in section I-C.

The effect of 1.0 mM PCMBS on calcium efflux after pretreatment with 5 mM EDTA is shown in Fig. 15. In this case the Ca^{45} release is completely blocked by EDTA treatment.

The addition of equimolar concentrations of cysteine and PCMBS produces an increase in rate of Ca^{45} efflux, but this increase is one-fifth of that produced by PCMBS alone. It was also observed that procaine, in concentrations (0.25 mM) sufficient to block asynchronous twitching, does not depress the PCMBS-induced Ca^{45} efflux.

IV. Effects of -SH Inhibitors on Membrane Electrical Properties

A. Resting Potential

Intracellular recording was used to study the actions of NEM, PCMB and PCMBS on the resting potential. The time-action curve of the effect of NEM on membrane potential is shown in Fig. 16. At the concentration which causes maximum rigor (1.0 mM), there is a 20 min delay to onset of depolarization, and after 60 min the maximum effect of 60 mv depolarization is noted. At 10 mM, the onset of depolarization is immediate and the rate is faster, although the maximum effect is the same.

In order to inhibit organomercurial-induced asynchronous twitching, which interfered with electrode placement, procaine (0.25 mM) was

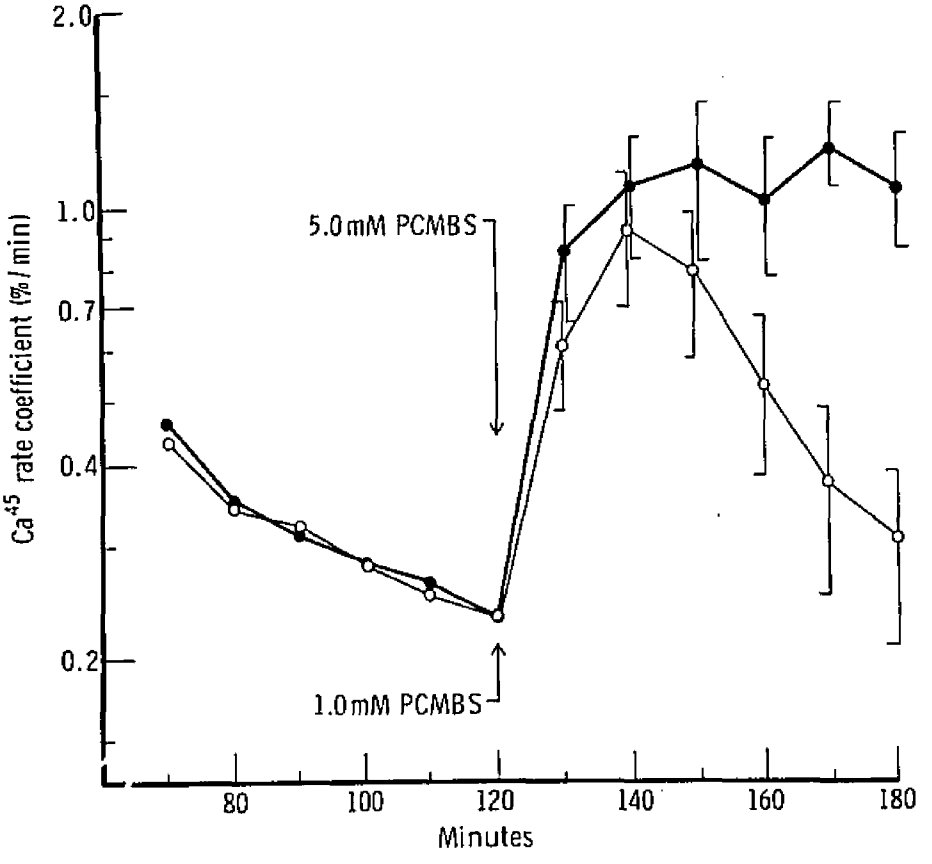


Figure 14 Time course of the changes in rate coefficient for Ca^{45} release from paired sartorius muscles in normal Ringer's solution. PCMBS was added to the Ringer solution as indicated. See legend-Fig. 9 for experimental details. n = 5; ±SE.

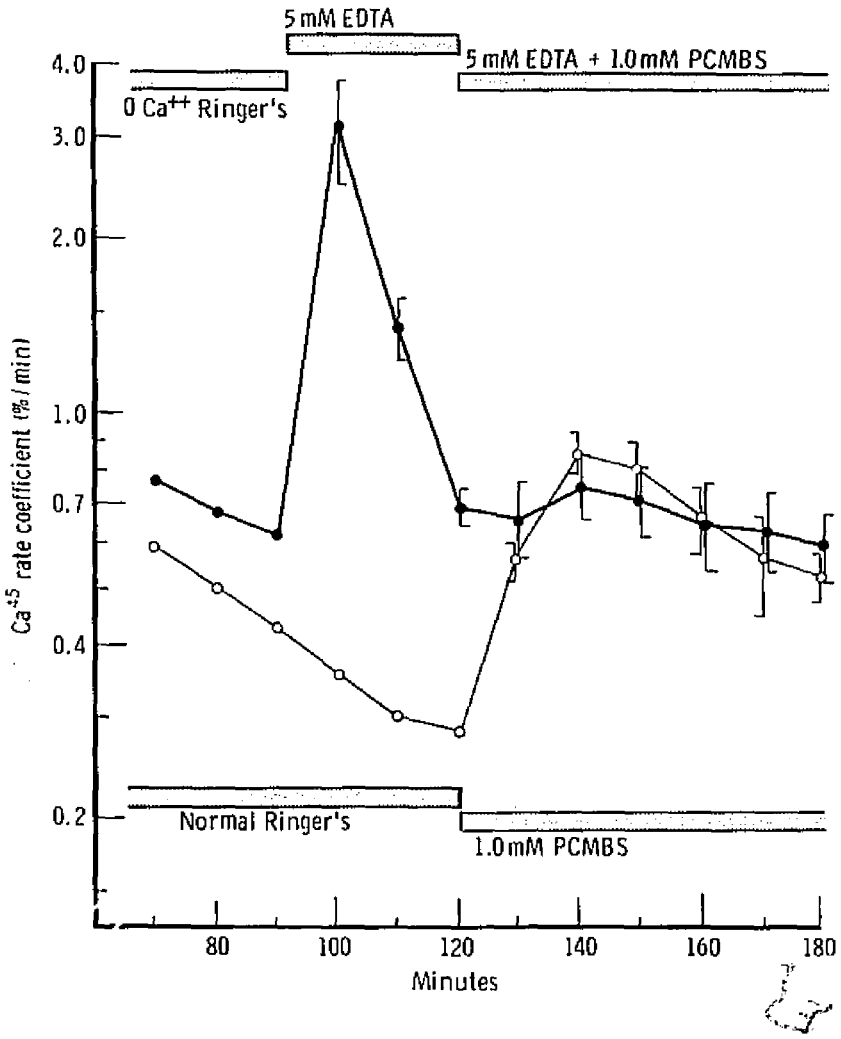


Figure 15 Effect of PCMBS, in the presence and absence of EDTA, on the rate coefficient for Ca^{45} release from isolated paired sartorius muscles. See legend-Fig. 11 for experimental details. Notations at top and bottom of graph refer to top and bottom curves respectively. n = 5; ±SE.

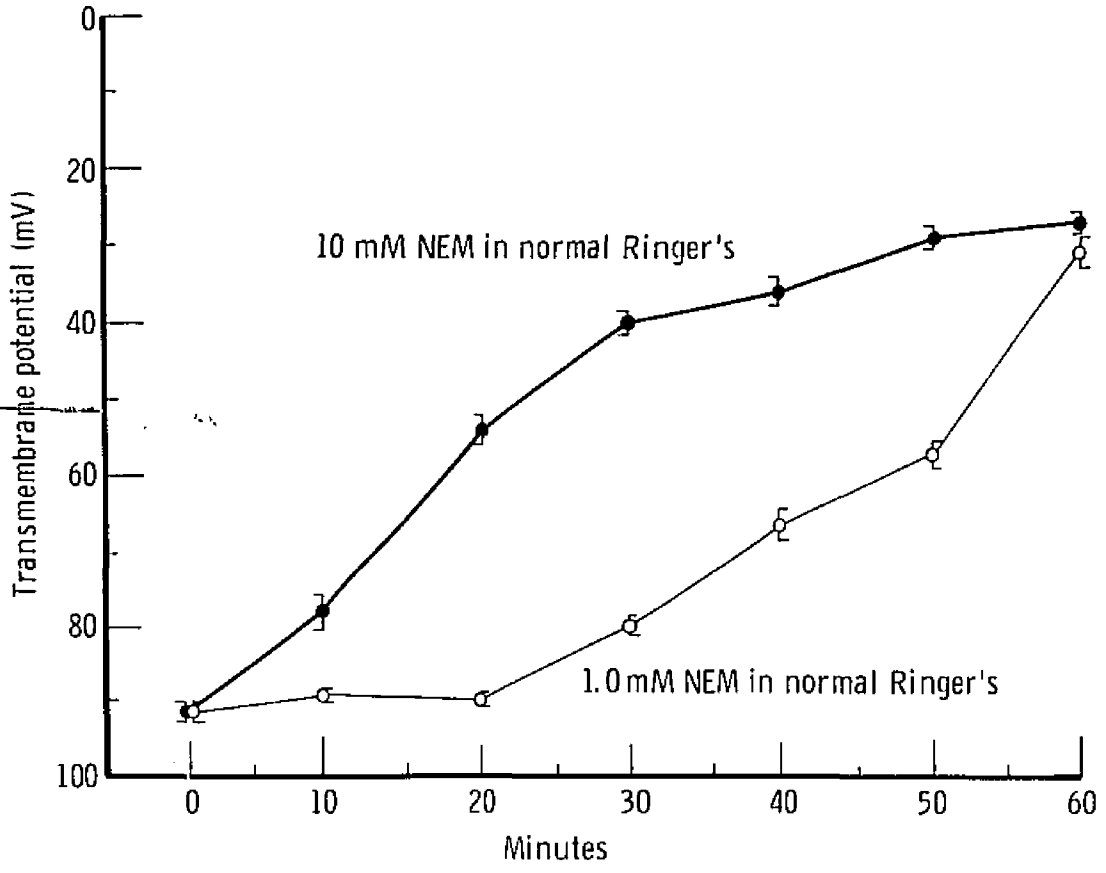


Figure 16 Effect of 1.0 and 10.0 mM NEM on the kinetics of depolarization of frog sartorius muscle. The average control resting potential is given at zero time. The values shown are pooled averages (±SE) of ±5 min from the time indicated. Each point is the average of 25 or more impalements made on 3 muscles.

added to the muscle bath. Procaine (0.25 mM) does not alter membrane resting potentials (Taylor, 1959) and only slightly decreases action potential amplitudes after treatment for 30 min or more (Inoue and Frank, 1962).

Within the first 10 min after application, 1.0 mM PCMB produced an average of 60 mv depolarization (Fig. 17). It is during this time period that both the secondary contraction and asynchronous twitching occurs. Equimolar concentrations of PCMB and cysteine do not produce depolarization (Fig. 17).

The mean resting potentials of PCMBS (1.0 mM) treated skeletal muscles are shown in Fig. 18. The kinetics of depolarization are faster with PCMB (Fig. 17) than with its sulfonated analogue although the end result is the same.

B. Action Potential

Typical action potentials obtained from muscles in Ringer's solution and in Ringer's containing the -SH inhibitors are shown in Fig. 19. The action potentials recorded after PCMBS treatment were the same as those recorded after PCMB. The first few evoked action potentials after addition of the -SH inhibitor appear normal; subsequent recording shows a diminished amplitude and an increase in duration (Fig. 19-D,G,H,I). As shown in Fig. 19, a modified action potential response was recorded when the resting potential was near normal. It appears that the mechanisms governing excitation are affected before the resting potential. Similar results on squid axon (Huneeus-Cox *et al.*, 1966) indicate deterioration of the action potential with little effect on the membrane potential.

Extracellular recording, without procaine in the bathing solution, indicates some irregular spike discharge during the period of organomercurial-induced depolarization and asynchronous twitching.

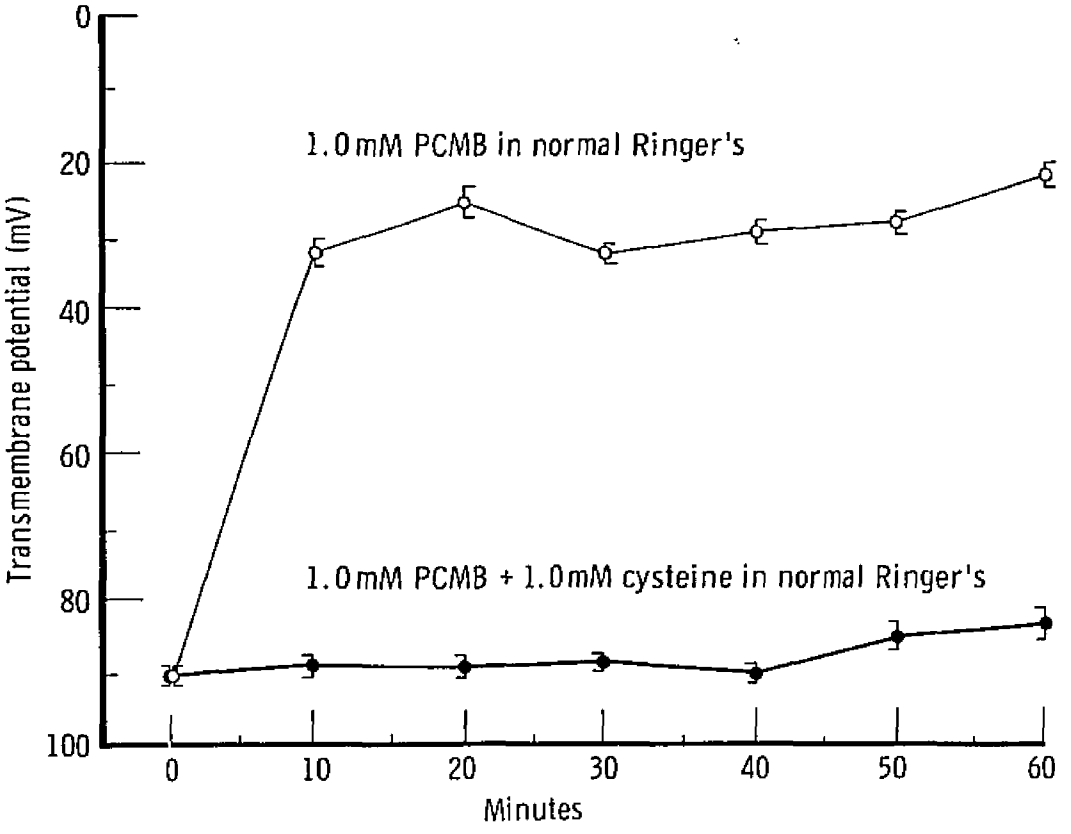


Figure 17 Effects of 1.0 mM PCMB and 1.0 mM PCMB + 1.0 mM cysteine on the kinetics of depolarization of frog sartorius muscle. The average control resting potential is given at zero time. The values shown are pooled averages (±SE) of ±5 min from the time indicated. Each point (open circles) is the average of 15 or more impalements made on 3 muscles. Dark circles represent 10 or more impalements on a single muscle. Procaine (0.25 mM) was added to the 1.0 mM PCMB experiment to block asynchronous twitching.

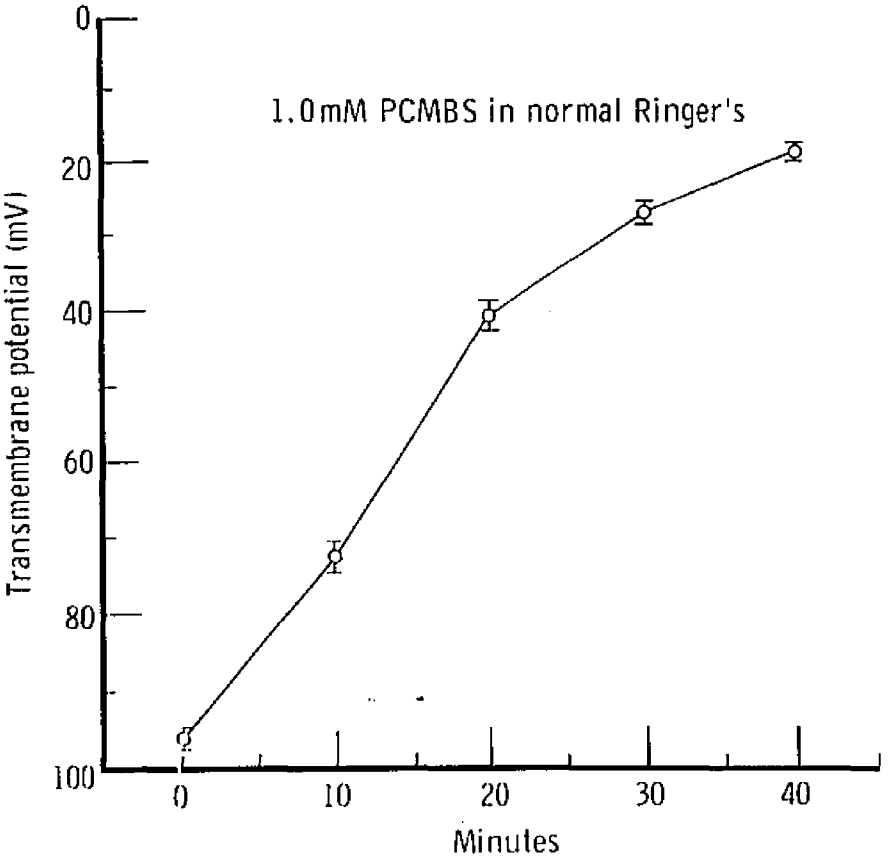


Figure 18 Effects of 1.0 mM PCMBS on the kinetics of depolarization of frog sartorius muscle. The average control resting potential is given at zero time. The values shown are pooled averages (±SE) of ±5 min from the time indicated. Each point is the average of 35 or more impalements made on 4 muscles. Procaine (0.25 mM) was added to the Ringer's with PCMBS in the above experiment to block asynchronous twitching. In a single experiment without procaine (not shown) the results were the same as those above.

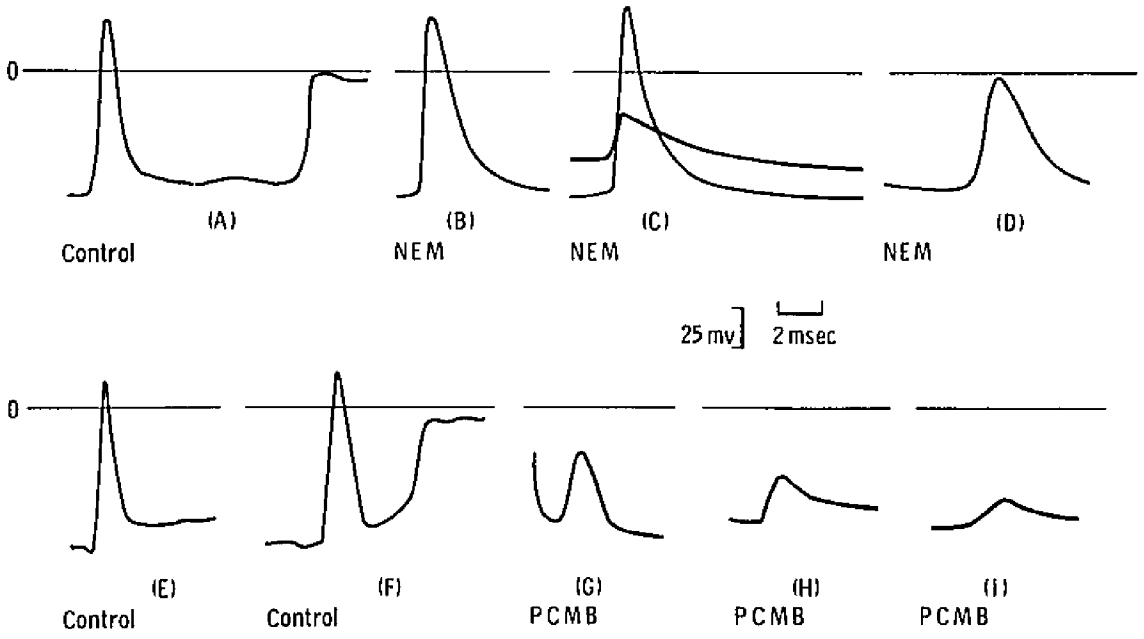


Figure 19 Effects of 1.0 mM NEM and 0.25 mM PCMB on evoked action potentials in surface muscle fibers. The zero potential line is shown. (A) Control action potential in Ringer's solution (top traces) followed by loss of membrane potential caused by movement. (B) Action potential after 10 min in 1.0 mM NEM. (C) Action potentials from the same fiber after 20 and 25 min (small spike) in 1.0 mM NEM. (D) Action potential after 25 min in 1.0 mM NEM. (E) Control action potential in Ringer's solution (bottom traces). (F) Control action potential with 0.25 mM procaine in Ringer's solution. (G), (H), (I) Action potentials recorded after about 10 min in 0.25 mM PCMB with 0.25 mM procaine. Calibration bars for both series of traces: voltage—25 mv; time—2 msec.

V. Electron Microscopy of NEM Treated Frog Sartorius Muscle

A fiber in longitudinal section is shown in Fig. 20 (top) after treatment with 1.0 mM NEM for 30 min. The lower micrograph of Fig. 20 shows a section of a control paired muscle fixed at the same time. The treated muscle shows a big increase in space between myofibrils not observed in the control muscle. A different micrograph of a treated muscle is shown in Fig. 21 under higher magnification. In neither micrograph of the treated muscle does the sarcoplasmic reticulum appear swollen. Some disruption of the sarcoplasmic reticulum is noted in these and subsequent micrographs.

Transverse sections of treated (Fig. 22-top) and untreated (Fig 22-bottom) muscles were studied. The space between myofibrils is much bigger in the treated muscle than in the control.

Two explanations for this discrepancy are possible: a) NEM treatment modifies "normal" rigor or b) pretreatment with NEM modifies normal fixation.

Figure 20 (top) Electron micrograph of a longitudinal section of fiber treated for 30 min with 1.0 Mm NEM. Sarcomere length is approximately 1.4μ. Labels indicate the Z disc (Z), longitudinal tubule (lt), transverse tubule (tt), terminal cisternae (tc), and glycogen granules (gly). Stain: uranyl acetate followed by lead citrate. Calibration bar: 1μ. 24,000 X.

Figure 20 (bottom) Control longitudinal section of a fiber from the mate of the above muscle. Sarcomere length is approximately 2.4μ. Labels indicate A band (A), H band (H), M band (M), L band (L), fenestration collar (fc) and intermediate cisternae (ic). For other labels see above. Stain: uranyl acetate. Calibration bar: 1μ. 24,000 X.


tt
gly
lt
Z
Z
tt
tc

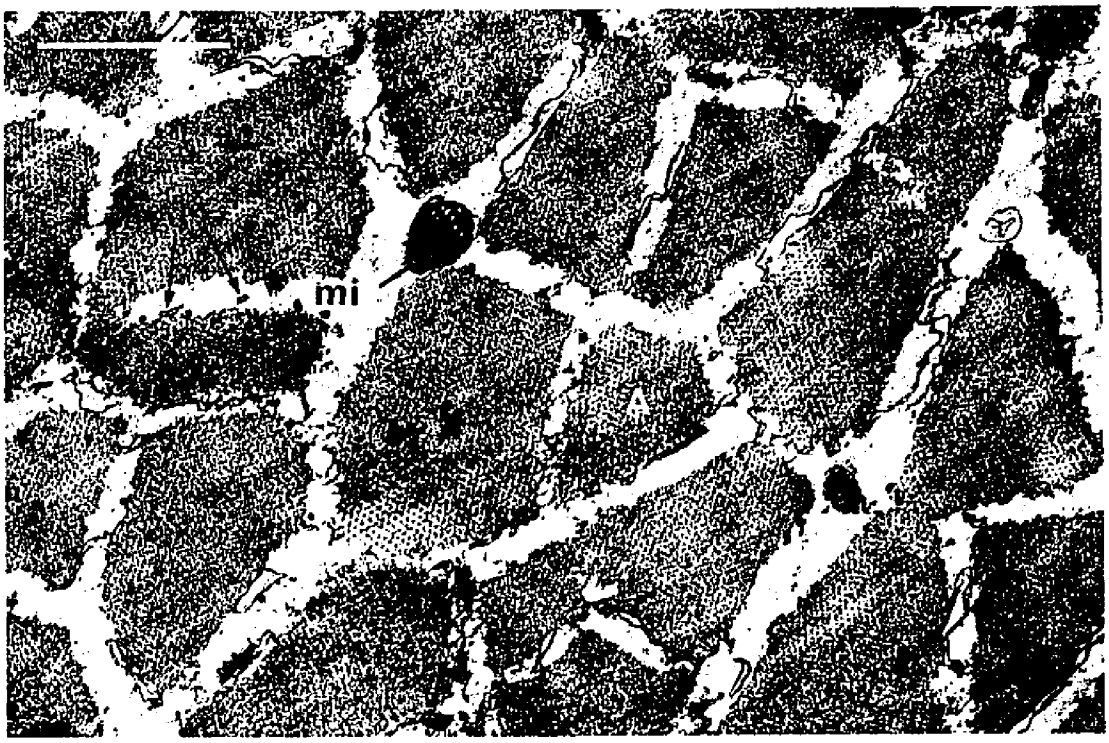

ic
tc
Z
Z

Figure 21 Longitudinal section of a fiber treated for 30 min with 1.0 mM NEM. Fiber is in rigor with a sarcomere length of approximately 1.4μ. Mitochondria (mi) are indicated, otherwise see legend-Fig. 20 for labels. Arrows indicate a rigor-induced disrupted triad. Asterisk indicates a typical triad. Stain: uranyl acetate followed by lead citrate. Calibration bar: 1μ. 42,000 X.


te
mi

Figure 22 (top) Transverse section of a fiber treated with 1.0 mM NEM. Section is taken through the A band (A) and is from the same muscle as Fig. 20 (top). Hexagonal arrays can be seen in some regions of micrograph. The increase in space between myofibrils can be observed and disruption of the sarcoplasmic reticulum is indicated. Arrows indicate some intact longitudinal reticulum. Stain: uranyl acetate followed by lead citrate. Calibration bar: 1μ. 24,000 X.

Figure 22 (bottom) Slightly oblique transverse section of a control fiber. Section shows a dark A band (A), a light I band (I) and the Z disc (Z) running diagonally on the lower right. Micrograph is from the same muscle as Fig. 20 (bottom). Mitochondria (mi) are indicated, otherwise see legend-Fig. 20. As compared with the above NEM-treated muscle, the control muscle shows intact reticulum. Stain: uranyl acetate. Calibration bar: 1μ. 24,000 X.


mi
A


It
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DISCUSSION

The effects of three sulfhydryl inhibitors have been studied on mechanical and electrical properties and calcium fluxes in the isolated skeletal muscle. Each inhibitor produces a characteristic response: NEM causes a slowly developing rigor; PCMB produces a fractionation of the evoked twitch; both PCMB and PCMBS produce asynchronous contractions of unstimulated muscle; PCMBS causes a rapid loss of excitability. Each inhibitor also produces membrane depolarization and calcium release although the magnitude and rate of development of these effects varies from one inhibitor to another. The magnitude of each of the foregoing effects is concentration dependent but the response is not always a linear function of concentration, e.g. the rigor produced by NEM. All effects are antagonized by cysteine. These results can be interpreted to suggest (1) that the -SH inhibitors are modifying some event or events in the process of E-C coupling, (2) that this modification is produced through -SH group inhibition, and (3) that this modification is also associated with a change in calcium binding and/or transport at a surface membrane or intracellular site; in other words, a fundamental relationship between -SH group inhibition and calcium is indicated.

On the basis of the data obtained, and in the context of the current views of the E-C coupling process, the site and mechanism of action of each -SH inhibitor is discussed below. An important theme that will emerge from this discussion is that each -SH inhibitor causes an effect that depends on the subcellular site or sites in the muscle fiber to which the inhibitor gains access. Although the common mechanism of action may indeed be -SH group inhibition, the pharmacologic effects will depend on the particular -SH groups that are attacked, i.e. on what molecules they are located, where in the cell these molecules play in

the sequence of events leading from membrane depolarization to contraction.

I. Assumptions and Limitations

A number of presumptions involved in the interpretation of these results and earlier studies have been made. It is perhaps worthwhile to enumerate these assumptions at this time.

Winegrad (1968) has shown from radiographic studies with muscle fiber bundles that Ca^{45} reaches the terminal cisternae after very short exposures (5-10 min) to radiocalcium. It has never been *directly* shown however that this intracellular calcium compartment is responsible for the "slow" component (half time = 500 min) of Ca^{45} efflux. In all recent efflux studies the slow component of washout is considered to originate from an intracellular site (Bianchi, 1963; Bianchi, 1965; Isaacson and Sandow, 1967).

Moreover, many studies involving caffeine (Bianchi, 1961; Feinstein, 1963; Isaacson and Sandow, 1967), quinine (Isaacson and Sandow, 1967) and IAA (Bianchi, 1963) have *assumed* than an increased Ca^{45} efflux reflects an increased calcium level within the sarcoplasm. While the mechanical effects of the above agents follow their Ca^{45} movements, there has never been any direct evidence linking Ca^{45} efflux with corresponding calcium levels within the sarcoplasm.

Although EDTA has been shown to be limited to the extracellular space (Bianchi, 1965) this does not preclude an indirect intracellular action. Furthermore, removal of membrane calcium with EDTA might upset calcium equilibrium to such an extent that intracellular calcium moves out to occupy these superficial sites.

Although NEM may effect the ATP levels in skeletal muscle, it seems plausable that the mechanism of NEM rigor could be interpreted in terms of the ionic mechanisms governing rigor and muscle contraction. The important chemical event leading to the onset of contraction

and indeed to the development of rigor seems to be the release of sufficient calcium to activate the contractile machinery. Pharmacological modification of ATP levels would only alter the effectiveness of the sarcoplasmic reticulum in sequestering calcium, thus causing further accumulation of calcium in the sarcoplasm. IAA rigor has recently been reinterpreted in terms of its effect on the sarcoplasmic reticulum calcium-accumulating system (Feinstein, 1966); the following discussion will reflect a similar viewpoint.

II. Mechanism and Site of Action of NEM

The typical response induced by NEM in the isolated sartorius muscle is rigor. This rigor is apparently produced by an action of NEM on an intracellular compartment of calcium. Two types of experiments support this conclusion. First, muscles pretreated with isotonic KCl or EDTA develop rigor tension. This indicates that the mechanism of NEM rigor does not involve membrane depolarization or the presence of calcium in the external medium. Secondly, after removal of all superficial calcium with EDTA, NEM still promotes calcium release. This suggests that NEM, like caffeine (Bianchi, 1965) and quinine (Isaacson and Sandow, 1967), releases calcium from the sarcoplasmic reticulum. Furthermore, NEM induces a more prolonged release of calcium than would be expected from a surface acting agent like EDTA.

These findings suggest the following interpretation of NEM rigor. Following addition to the muscle bath, NEM diffuses across the sarcolemma. During the latent period before rigor development, NEM reacts with -SH groups located on the surface membrane as well as intracellularly. Low calcium levels are normally maintained within the sarcoplasm by the sequestering action of the sarcoplasmic reticulum (Costantin *et al.*, 1965). Inhibition of -SH groups on the sarcoplasmic reticulum by NEM causes calcium levels to increase within the

sarcoplasm rises above 10^{-7} M (Weber *et al.*, 1963). Furthermore, the biphasic dosage-response curve suggests that in higher concentrations NEM acts at a second site, possibly at the myofilaments, to interfere with tension development.

According to the above hypothesis of NEM action, calcium release from the sarcoplasmic reticulum occurs during the latent period in amounts below the level necessary to initiate contraction. A small calcium release has been measured in NEM-treated resting muscle even before rigor development. If the muscle is electrically-stimulated during the latent period, the subthreshold amounts of calcium released by NEM should add to that released by E-C coupling, producing supernormal calcium levels within the sarcoplasm. These higher calcium levels will prolong the active state (Sandow, 1965) and result in a potentiated twitch. Twitch potentiation has been observed before onset of rigor (Okamoto and Kuperman, 1966).

In support of the above data indicating that NEM penetrates the sarcolemma, Jacob and Jandl (1962) have found a rapid penetration of NEM across the erythrocyte membrane, much faster than PCMB. Recent studies by Hasselbach (1966) have indicated the rate of calcium uptake by sarcoplasmic vesicles is diminished in direct proportion to the blockage of -SH groups by NEM. These -SH groups appear to be associated with a calcium-dependent ATPase. Also, -SH dependent calcium transport sites appear to be located on the outer surface of the sarcoplasmic vesicles, as evidenced by electron microscopy of vesicles treated with an electron dense -SH inhibitor (Hasselbach, 1966; Hasselbach and Seraydarian, 1966).

In view of Hasselbach's (1966) studies indicating a linear relationship between inhibition of -SH groups and calcium transport, it is reasonable to assume that NEM is effecting an intracellular titration of -SH groups on the reticulum. Higher concentrations of NEM should

therefore produce a faster titration of reticular -SH groups resulting in a more rapid buildup of free calcium in the sarcoplasm and a *shorter* interval to tension development. This interpretation is supported by the results showing a linear relationship between NEM concentration and the latent period prior to the onset of tension.

High concentrations of NEM produce rigor tensions which are characteristically lower than that produced by 1.0 mM. Partial relaxation at higher concentrations is also observed. One interpretation of these effects is that high concentrations of NEM not only block -SH groups on the sarcoplasmic reticulum and thereby induce calcium release, but also affect secondary site(s) needed for normal tension development. At concentrations of NEM below 1.0 mM these secondary site(s) might not be affected to the same extent, thereby producing little or no diminution of rigor tension. Myosin ATPase activity (Blum, 1962), troponin-tropomyosin interaction (Yasui *et al.*, 1968), and F-myosin-actin interactions (Bailin and Barany, 1967) are all blocked by -SH inhibitors and hence are possible secondary sites of action. The available evidence is inadequate to determine whether higher concentrations of NEM effect a secondary site(s) of action on the myofilaments or elsewhere.

Since NEM causes depolarization, it may be assumed NEM is interacting with -SH groups on the sarcolemma. As discussed above, NEM depolarization appears independent of rigor tension. Furthermore, action potentials recorded after treatment with NEM indicate that membrane excitability is depressed before the resting potential is significantly altered. Huneeus-Cox and co-workers (1968) have attributed similar data on squid axon to inhibition of protein bound -SH groups responsible for ion gating during excitation. The specific role of NEM on excitation phenomena cannot be determined from studies of this nature but must await more detailed studies of ion fluxes.

III. Mechanism and Site of Action of the Organomercurials

A number of arguments can be put forward to support the idea that both PCMB and PCMBS cause asynchronous twitching by acting on the surface membrane. The onset of activity is rapid, approximately 2-3 min for an isolated frog sartorius. In single fibers localized activity occurs at discrete regions of the fiber, implying electrotonic conduction (J. Reuben, personal communication). Propagated responses have been recorded with external electrodes during asynchronous twitching. Intracellular recordings indicate a very rapid depolarization with PCMB and a slower depolarization with PCMBS. Furthermore, this activity can be inhibited by pretreatment with the following: cysteine, KCl, choline-Ringer's, procaine, glycerol and, in the case of PCMBS, high calcium. Also, since d-tubocurarine does not suppress the twitching, it is unlikely that the organomercurials act by releasing acetylcholine from nerve endings.

From the evidence presented above, one may propose the following hypothesis. Both PCMB and its sulfonated analogue, PCMBS, cause calcium release from the outer membrane. Such an effect could be classified as a labilization of the membrane (Shanes, 1958a). This causes a non-specific increase in ionic conductances (Frankenhaeuser and Hodgkin, 1957). Localized membrane depolarizations will then occur, producing both propagated and non-propagated electrical responses. This electrical activity passes down the t-tubules and triggers the asynchronous twitching. In summary, it appears that the asynchronous activity is caused by localized depolarizations of the cell membrane, and the final cessation of activity by a depolarization block.

While the above interpretation of asynchronous twitching relates both organomercurials to a surface effect, further evidence indicates a second site of action for PCMB, internal to the surface membrane. The results of studies after treatment with EDTA indicate that PCMB releases at

least some calcium from an intracellular store. PCMBS, on the other hand, has a more superficial site of calcium releasing action.

Previous studies have indicated that intracellular calcium release results in a sustained efflux. Agents such as IAA and ryanodine (Bianchi, 1963), FDNB (Nauss and Davies, 1966), quinine (Isaacson and Sandow, 1967) and caffeine (Bianchi, 1961; Isaacson and Sandow, 1967) affect calcium binding at the sarcoplasmic reticulum. Using this criterion, the sustained calcium release augmented by PCMB is more indicative of an intracellular effect than is the efflux produced by 1.0 mM PCMBS. The kinetics of release by a high concentration of PCMBS also tends to indicate an intracellular calcium release. Indeed, a low rigor tension does develop at this concentration of PCMBS.

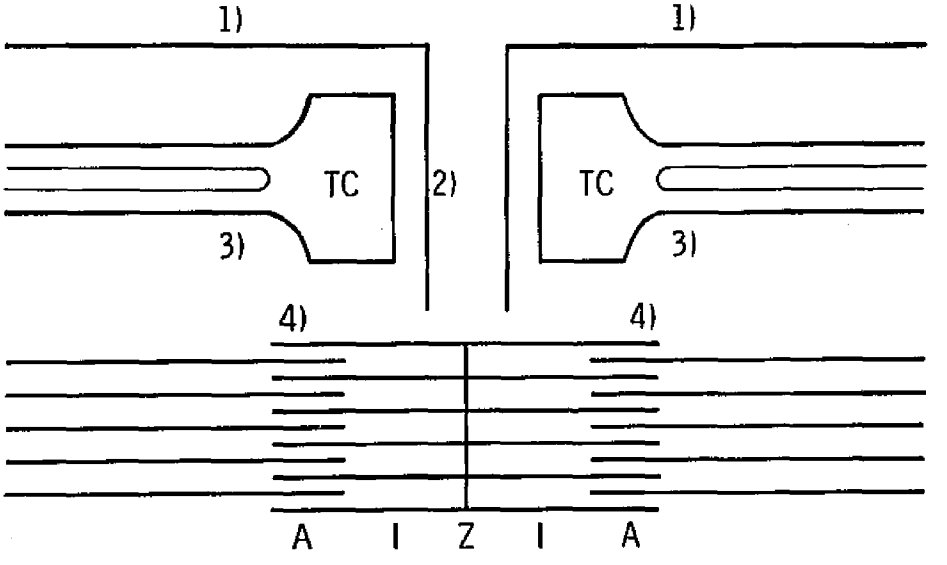
The above data indicate that PCMB penetrates the membrane to release calcium from an internal store; PCMBS (1.0 mM) acts primarily at surface sites. However, high concentrations of PCMBS reach internal calcium compartments. These findings are supported by studies on the erythrocyte membrane (Vansteveninck, *et al.*, 1965). Recent evidence indicates that PCMBS penetrates the erythrocyte membrane very slowly (Rega *et al.*, 1967), while PCMB penetrates rapidly (Jacob and Jandl, 1962; Rega *et al.*, 1967).

Electrical stimulation of a PCMB treated muscle causes a secondary contraction. It is proposed that this effect is mediated through a second site removed from the surface membrane. Sulfhydryl binding by PCMB in the region of the terminal cisternae causes a secondary and more prolonged release of calcium into the region of the contractile filaments. This binding and secondary release of calcium is dependent upon electrical stimulation. The inability of PCMBS to produce a secondary contraction results from a failure of sufficient amounts of this agent to penetrate to those intracellular sites attacked by PCMB. A second interpretation of this PCMB-related effect will be given below.

Procaine inhibits the organomercurial induced mechanical activity without affecting the calcium efflux or the kinetics of depolarization. In similar experiments, Feinstein (1963) has shown that procaine also inhibits EDTA-induced spontaneous twitching, but does not affect the EDTA-stimulated Ca^{45} efflux. Both of these effects can be accounted for on the basis of an action by procaine on trigger calcium (Bianchi, 1967). Bianchi (1967) has postulated a trigger calcium necessary in coupling the electrotonic spread of current in the t-tubules to the main calcium release at the terminal cisternae. This component of calcium would act between sites 2 and 3 of Fig. 23. It is conceivable that antagonizing the release of trigger calcium by procaine would inhibit all the mechanical effects attributed to EDTA and the organomercurials without affecting the calcium efflux. Unfortunately, the actions of procaine on muscle is so non-specific that little information can be obtained as to the origin of the PCMB-induced secondary contraction.

While the results indicate that PCMB releases calcium from an internal compartment, the calcium release from this compartment may not be associated with the secondary contraction. It is conceivable that PCMB mediates the secondary contraction by an effect on the t-tubules. Steric hindrance might prevent a similar effect by PCMBS. The internal store of calcium released by PCMB may not be related to any of the mechanical effects of PCMB.

It is well-known that high concentrations of caffeine (10-20 mM) produce rigor in skeletal muscle (Sandow, 1965). This effect is attributed to a release of calcium from the sarcoplasmic reticulum. More recently this agent has been employed to test the integrity of the sarcoplasmic reticulum (Gage and Eisenberg, 1967). Treatment of a muscle with caffeine after pretreatment with PCMB or PCMBS results in rigor. This tends to indicate a minimal effect, if any, of the organomercurials on the internal calcium sequestering system.



MUSCLE STRUCTURE

1) Surface membrane
2) Transverse tubule
3) Sarcoplasmic reticulum
4) Contractile proteins

SITES OF ACTION

1) NEM, PCMB, PCMBS
2) NEM, PCMB, PCMBS
3) NEM, PCMB (TC)
4) NEM

Figure 23 Schematic diagram indicating the possible sites of action of NEM, PCMB and PCMBSon frog sartorius muscle. TC, terminal cisternae. (Diagram reproduced by permission of C.P. Bianchi, 1967).

In general, the foregoing pharmacologic data suggest that protein bound -SH groups play a significant role in the process of E-C coupling and in excitation processes in general. It appears from this investigation and others (Robinson, 1965), that modification in -SH residues alters membrane permeability. The exact nature of this interaction and whether it involves enzymatic inactivation and/or structural alteration is not clear. Previous work has suggested that a membrane ATPase may control the passive permeability of excitable cells (Bower and Duncan, 1967). Furthermore, it has been demonstrated that membrane ATPase is inactivated by -SH inhibitors (Skou, 1964). It has also been suggested that -SH groups are important in maintaining structural integrity (Smith, 1958; Robinson, 1966). Interaction of -SH groups with either NEM, PCMB or PCMBS could therefore alter permeability either by affecting enzymes that function in membrane transport or by affecting the conformation of structural proteins.

Data from this investigation support Hasselbach's (1966) study indicating the presence of -SH groups on the sarcoplasmic reticulum. In both studies the inhibitor NEM appears to have modified the calcium binding properties of this internal membrane-limited compartment. Due to slow penetration, the organomercurials appear to affect the sarcoplasmic reticulum only to a limited extent.

SUMMARY

1. Experiments were done on frog sartorius muscles to study the effects and mechanisms of action of the -SH inhibitors NEM, PCMB and PCMBS. A schematic diagram indicating the sites of action of these inhibitors is shown in Fig. 23.

2. The characteristic action of NEM is muscle rigor. It is hypothesized that NEM produces rigor by an intracellular titration of -SH groups at the sarcoplasmic reticulum. The binding of internal -SH groups causes calcium levels to increase within the sarcoplasm, thereby inducing rigor. The biphasic dosage-response curve indicates that high concentrations (5-20 mM) may affect the myofilaments.

3. NEM produces membrane depolarization. This surface effect appears unrelated to rigor development. Depolarization most likely accounts for the loss of twitch response during electrical stimulation.

4. The organomercurials produce a depolarization of the membrane which is associated with a period of asynchronous twitching and followed by inexcitability. It is proposed that organomercurial-induced calcium release causes changes in the cell membrane resulting in both electrotonic and electrogenic responses. This electrical activity passes down the t-tubules and triggers the asynchronous twitching. Cessation of twitching might result from a depolarization block.

5. A single electrical stimulation of a PCMB treated muscle results in a double contraction, i.e. a normal twitch followed by a more prolonged secondary contraction. The more soluble sulfonated analogue of PCMB does not produce this effect. It was proposed that the secondary contraction results from an effect on an internal compartment of calcium, one to which PCMBS does not gain access. A second explanation is that PCMB mediates the secondary contraction by an action on the t-tubules. Steric hindrance might prevent PCMBS from causing a similar effect at this site.

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