Slow Sodium Inactivation in Nerve After Exposure to Sulfhydryl Blocking Reagents

PETER SHRAGER

From the Department of Physiology, University of Rochester Medical Center, Rochester, New York 14642

ABSTRACT Exposure to *N*-ethylmaleimide (NEM), a reagent that binds covalently to protein sulfhydryl groups, results in a specific reduction in sodium conductance in crayfish axons. Resting potential, the delayed rise in potassium conductance, and the selectivity of the sodium channel are unaffected. Sodium currents are only slightly increased by hyperpolarizing prepulses of up to 50 ms duration, but can be restored to about 70% of their value before treatment if this duration is increased to 300–800 ms. The time to peak sodium current and the time constant of decay of sodium tail currents are unaffected by NEM, suggesting that the sodium activation system remains unaltered. Kinetic studies suggest that NEM reacts with a "slow" sodium inactivation system that is present in normal axons and that may be seen after depolarization produced by lowering the holding potential or increasing the external potassium concentration. NEM also perturbs the fast h inactivation system, and in a potential-dependent manner. At small depolarizations τ_h is decreased, while at strong depolarizations it is increased over control values. Experiments with structural analogs of NEM suggest that sulfhydryl block is involved, but do not rule out an action similar to that of local anesthetics. *p*-Chloromercuriphenylsulfonic acid (PCMBS), another reagent with high specificity for SH groups, also blocks sodium currents, but restoration with prolonged hyperpolarizations is not possible.

INTRODUCTION

The biochemical components of excitable membranes that are associated with the control of ionic conductance are not yet well characterized. There is some evidence that membrane proteins are involved in the gating of ionic currents, both from studies with proteolytic enzymes (Armstrong et al., 1973) and from experiments utilizing group-specific reagents (Shrager, 1974, 1975). The identification of reactive groups closely associated with conductance control is important both as an extension of our ability to characterize these systems and as a possible initial step in developing a procedure for labeling components of interest. Since the solubilization of intrinsic membrane proteins generally involves conditions that result in denaturation, an identifiable ligand would be of considerable value.

A number of studies have suggested that the integrity of certain sulfhydryl groups in nerve fibers is essential for conduction (Smith, 1958; Huneeus-Cox et al., 1966; Keana and Stämpfli, 1974; Marquis and Mautner, 1974). Most of this

work has been performed on axons not under voltage clamp, making interpretation of the results difficult. There are three basic methods of altering sulfhydryl groups in proteins: reduction of disulfide bonds to individual SH residues; oxidation of neighboring SH groups to form a disulfide bond; and modification of reduced sulfhydryl groups with reagents that bind in either a reversible or a covalent manner. This paper is concerned primarily with the last of these and, in particular, with chemical modification by *N*-ethylmaleimide (NEM). This compound is highly selective for sulfhydryl groups when used at low concentration and neutral pH (Smyth et al., 1964; Morell et al., 1964; Riordan and Vallee, 1972). In initial experiments (Shrager, 1975) NEM was shown to inhibit selectively early transient currents in crayfish axons. Our further investigations, reported here, suggest that under mild conditions NEM closes sodium channels by altering an inactivation gate with very slow kinetics. A brief, preliminary report of this work has appeared (Shrager, 1976).

MATERIALS AND METHODS

Preparation

Medial giant axons from the crayfish *Procambarus clarkii* were voltage clamped as previously described (Shrager, 1974). Briefly, axons were dissected free of adhering fibers and were cannulated with a "piggy-back" electrode consisting of a low impedance potential pipette cemented to a platinized platinum wire (Chandler and Meves, 1965). Currents were collected by a guarded platinized platinum external electrode and passed into an operational amplifier acting as a current-to-voltage transducer. In later experiments this signal was amplified and fed into a 12 bit analog-to-digital converter (SHA-2A, ADC1103-003; Analog Devices, Inc., Norwood, Mass.). The signal was blanked during the first 30 μs to avoid saturation of the amplifiers by the capacitative transient. Data conversion began 10 μs after the end of the blanking signal. Each sweep was sampled at 256 points with the interval between samples variable between 5 and 800 μs. For better resolution of early transient currents the first 128 points could be sampled at a rapid rate and the remaining points at a slower rate. The digital data were stored in a temporary buffer memory and then fed into a digital computer (PDP 8/E; Digital Equipment Corp., Maynard, Mass.) and stored on magnetic tape for later analysis. Data recorded in this way agreed with those photographed directly from an oscilloscope to within 1%.

A new chamber, improved to allow better contact between the Teflon-coated aluminum cooling block and the external medium, resulted in more accurate temperature control. Except during solution changes, temperatures were held to within ±0.1°C of the set point. All experiments were run at 8.0°C.

Solutions and Reagents

The standard external saline (NVH) was that of Van Harreveld (1936) and contained (mM): NaCl, 205; KCl, 5.4; $MgCl_2$, 2.6; $CaCl_2$, 13.5; $NaHCO_3$, 2.3; pH adjusted to 7.5–7.6 with HCl. In the experiments involving *N*-ethylmaleimide, after dissection and placement of the electrodes the external bath was changed to a Tris-maleate-buffered version (TMVH) of NVH, which contained (mM): NaCl, 197.3; KCl, 5.4; $MgCl_2$, 2.6; $CaCl_2$, 13.5; maleic acid, 7.5; Tris base added to pH 6.9–7.0 at 8°C. In a few experiments, noted below, the external K^+ concentration, $[K^+]_0$ was lowered by substituting 1:1 with Na^+. In experiments at elevated $[K^+]_0$, without NEM, solutions (TMVH-II) contained (mM): NaCl, 182.3; $MgCl_2$, 2.6; $CaCl_2$, 13.5; Tris-maleate, 7.5; KCl + Tris-Cl, 20.4; pH 7.0. $[K^+]_0$

was varied by varying the relative amounts of KCl and Tris-Cl present. *N*-Ethylmaleimide was obtained from Aldrich Chemical Co., Milwaukee, Wis. (99+% reagent) or from Pierce Chemical Co., Rockford, Ill. Sources of other reagents were: β-mercaptoethanol, maleimide, and succinimide, Aldrich; *p*-chloromercuriphenyl sulfonic acid, Sigma Chemical Co., St. Louis, Mo.; dithiothreitol, Calbiochem, La Jolla, Calif.

N-Ethylsuccinimide (NES) was synthesized by catalytic hydrogenation of NEM. Dr. Leon Miller generously performed the reduction, which was done in a Parr hydrogenation apparatus. NEM (1 g) was dissolved in 50 ml of 95% ethanol. After addition of 0.3 g palladium-charcoal (5% palladium) the reduction bottle was alternately evacuated and filled with hydrogen three times, the final filling to a pressure of 30 lb/in^2. Shaking was continued at room temperature for 24 h. The catalyst was filtered off with the aid of a small amount of Filtercel and washed with a small amount of ethanol, and the clear, colorless filtrate was concentrated in vacuo with a rotary evaporator at 40°C.

The filtrate was dessicated over P_2O_5 and then crystallized by cooling to 4°C and scratching the bottle with a glass rod. IR spectra showed sharp peaks characteristic of a homogeneous product. The melting point was measured at 24.5–26.5°C, which is comparable to the published value of 26°C (Menschutkin, 1876). UV spectra of aqueous solutions showed no detectable peak at 302 nm, indicating that less than 1% of the original NEM remained (Gregory, 1955).

RESULTS

N-Ethylmaleimide

The reaction between *N*-ethylmaleimide (NEM) and sulfhydryl groups is illustrated in Fig. 1, top. The addition of the SH group across the double bond of NEM represents a covalent linkage and hence is irreversible under normal conditions. An absorption peak of NEM at 302 nm disappears after addition of a sulfhydryl group, and the reaction may therefore be followed in solution spectrophotometrically (Gregory, 1955). NEM is generally used at neutral pH since below pH 6 the reaction rate decreases markedly, and above pH 7 NEM is unstable and also loses specificity (Riordan and Vallee, 1972).

NEM was applied to crayfish axons by being added to a Tris-maleate-buffered external saline (TMVH, see Materials and Methods) at pH 6.9–7.0. On changing from the HCO_3^--buffered normal Van Harreveld's solution (NVH) at pH 7.6 to TMVH, the resting potential generally fell by 2–4 mV and potassium currents were slightly slowed (Shrager, 1974). Sodium currents measured from the original holding potential declined by about 10% during the first 5–10 min at pH 7, and this small effect was then partially reversed over the next 10–20 min, after which currents were stable. Addition of 1–3 mM NEM to the external bath resulted in a progressive decline in peak early currents with little effect on delayed currents. Fig. 2 illustrates results of an experiment that included a control for a possible nonspecific effect of this compound. Fig. 2*a* shows currents recorded 50 min after changing the bath to TMVH at pH 6.9. Fig. 2*b* illustrates effects of TMVH containing 1 mM NEM, to which 1 mM β-mercaptoethanol had been added before application to the axon. Spectrophotometric studies showed that within the few minutes required to mix the solutions, place them in a cuvette, and measure the absorption peak at 302 nm (Gregory, 1955) virtually all of the NEM had undergone reaction. Ionic currents were almost

unaffected by a 45-min exposure to this solution (Fig. 2*b*). The external bath was then switched to TMVH with 1 mM NEM alone. After 30 min peak sodium currents were reduced by 60%. Records after 70 min in NEM are shown in Fig. 2*c*, and Fig. 2*d* illustrates results 10 min after switching to TMVH containing 100 nM tetrodotoxin (TTX), a specific inhibitor of sodium channels (Narahashi, 1972). The results indicate that NEM selectively reduced inward and outward sodium currents, with relatively little effect on potassium currents. NEM generally had no effect on resting potential over the exposure times used in the experiments reported here, but the leakage current often increased, particularly with prolonged exposures. If allowed to remain in the bath, NEM eventually caused a loss of resting potential as well. Effects of NEM, which was generally applied for 30–40 min, were not reversed on washing with TMVH or NVH for up to 1 h.

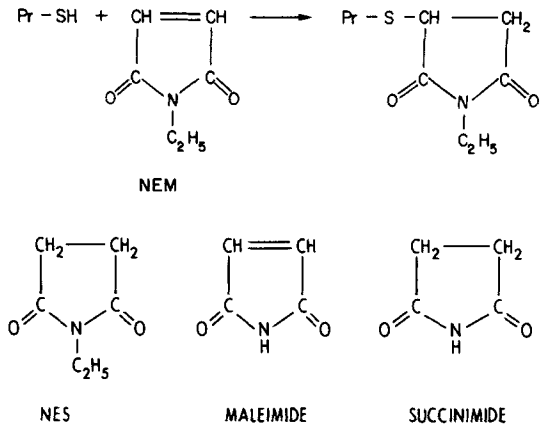

FIGURE 1. Structures of *N*-ethylmaleimide (NEM) and analogs. *Top*, The chemical reaction between NEM and protein (*Pr*) sulfhydryl groups (see, e.g., Smyth et al., 1960). *Bottom*, structures of *N*-ethylsuccinimide (NES), maleimide, and succinimide.

Sodium conductance, G_{Na}, lost after treatment with NEM, could be partially restored by a prolonged hyperpolarizing prepulse. Hyperpolarizing the axon membrane by 20–60 mV for less than 50 ms preceding a test pulse had only a small effect on G_{Na}, while sodium currents increased significantly as prepulse durations were increased beyond 50 ms. Fig. 3 illustrates families of currents obtained without and with a prepulse to −111 mV for 350 ms. After a hyperpolarization to −110 to −115 mV for 850 ms (the longest durations used in these experiments), peak sodium currents were generally restored to about 70% of the values before exposure to NEM. Potassium currents are slowed after a hyperpolarizing prepulse through the Cole-Moore (1960) effect (Shrager, 1974).

A number of experiments have been performed in which NEM was applied for a time sufficient to reduce peak sodium currents to 30–50% of their normal levels, and was then washed away by TMVH. Measurements could then be made both on remaining currents and on currents increased by long hyperpolarizations. Fig. 4 illustrates I–V curves for peak early currents before (open circles)

and after (filled circles) exposure to NEM. Fig. 4a shows results with no prepulse. The x symbols represent the NEM data multiplied by a constant factor to equalize maximum inward currents. If the effect of NEM were to render some Na channels incapable of conducting Na ions while leaving all others

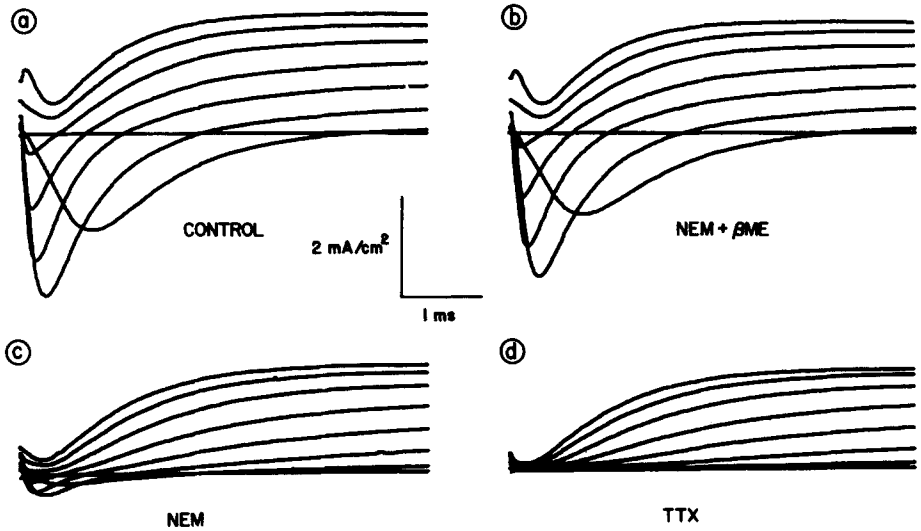

FIGURE 2. Effects of NEM on voltage clamp currents. Depolarizations from the holding potential of −74 mV to −34, −14, +6, +26, +46, +66, +86 mV. (*a*) Records in TMVH, pH 6.9. (*b*) 45 min after changing the external bath to TMVH with 1 mM β-mercaptoethanol (β ME) and 1 mM NEM mixed before applying to the axon. (*c*) 70 min after changing to TMVH plus 1 mM NEM. (*d*) 10 min after switching to TMVH plus 100 nM tetrodotoxin (TTX).

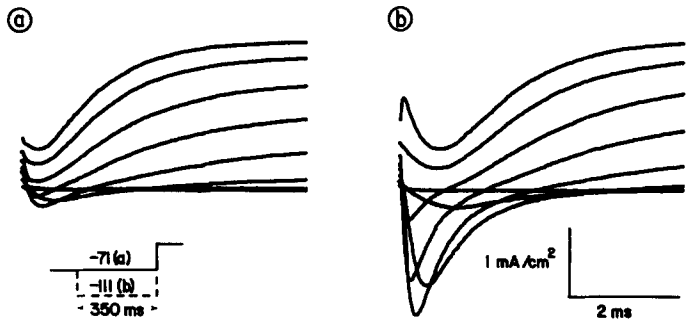

FIGURE 3. Restoration of sodium conductance by a hyperpolarizing prepulse of long duration. *Left,* Currents recorded 90 min after addition of 1 mM NEM to TMVH. Depolarizations from −71 mV to −51, −31, −11, +9, +29, +49, +69 mV. *Right,* Depolarizations preceded by a prepulse to −111 mV for 350 ms.

normal, then the x's should coincide with the control curve (neglecting the small reduction in reversal potential; see below). The shapes of the two sets of data appear similar, but the NEM points are shifted about 4 mV along the voltage axis. This shift might be at least partially due to a remaining component of

uncompensated series resistance (R_s). A residual R_s of 1.5 Ωcm^2 would be sufficient to account for the 4 mV shift in this case, and this was the approximate value estimated in most experiments (Shrager, 1974). After partial restoration of G_{Na} with a prepulse to −115 mV for 350 ms, the control sodium currents were fit more closely by a constant multiple of the currents after reaction with NEM (Fig. 4*b*). The small reduction (7 mV) in reversal potential for the early transient current is likely to be due to the gradual rise in the internal Na^+ concentration normally seen in axons held at 8°C over an extended period. This rise may be more rapid after exposure to NEM due to the increase in leakage conductance.

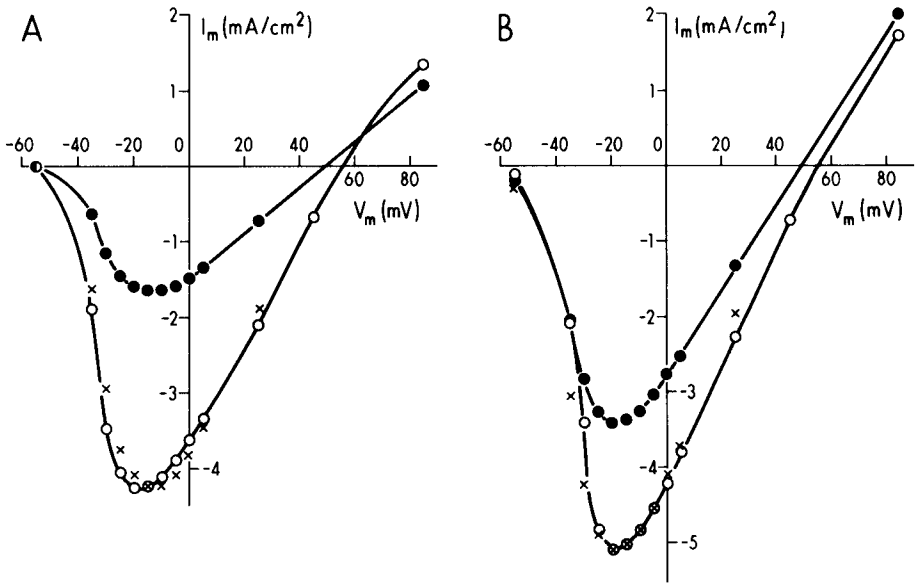

FIGURE 4. I-V curves for peak early current. (A) No prepulse. Depolarizations from −75 mV to the levels given on the abscissa. Peak currents (ordinate) are uncorrected for capacitative transients or leak currents. External medium = TMVH, $[K^+]_0$ = 2.7 mM. Open circles, before, and filled circles, after, exposure to 3 mM NEM in the above medium for 30 min. X's represent data of filled circles multiplied by 2.57. (B) Same, but all depolarizations are preceded by a conditioning hyperpolarization to −115 mV for 350 ms. X's are 1.49 × filled circles.

If the *x*'s are corrected for the reduction in reversal potential the results are only slightly affected, with the shift in the I-V curves after NEM equal to 5 mV in Fig. 4*a* and 1 mV in Fig. 4*b*. The results thus far are consistent with the idea (explored further, below) that NEM reacts irreversibly with sodium channels and renders them incapable of conducting sodium ions when depolarized from the resting potential. Sodium channels that have not reacted remain normal, at least in opening characteristics. If the reduction in reversal potential were due to an alteration of the selectivity of the channel after reaction with NEM then it might be expected that this change would be different in the case of Fig. 4*b* where presumably a higher fraction of the conducting channels have undergone reaction with NEM than is the case in Fig. 4*a*. Since reversal potentials were

unaffected by the hyperpolarizing prepulse both before and after exposure to NEM, the reduction is more readily explained by a change in concentration of internal Na^+.

Other characteristics of the sodium activation (m) system have been studied in fibers before and after exposure to NEM. The times required for the sodium current to reach its peak value after a test depolarization are given in Table I. There is no systematic alteration of this parameter by NEM, with or without a hyperpolarizing prepulse. Time constants for the decay of sodium tail currents after the termination of a depolarization are listed in Table II. Again, no significant differences attributable to NEM are seen.

The kinetics of sodium inactivation and reactivation after exposure to NEM have been investigated. Fig. 5 illustrates results of experiments to test the relationship between the duration of a hyperpolarizing prepulse and the peak sodium current elicited by an immediately following test depolarization. The points represent experimental data. In Fig. 5*a* results are shown for an axon before and after reaction with NEM. In both cases the increase in peak sodium current occurs in two phases: a small, rapid component, probably related to the *h* system; and a much slower component. The percentage increase in peak currents is greater in both phases after treatment with NEM. The curves represent a fit of the data to a kinetic model which will be discussed below. The data in this and in ensuing figures are corrected for changes in leakage conductance, assuming time invariance, but not for capacitative transients. The latter correction was small and did not significantly affect the results or the conclusions drawn from them.

The existence of a slow component of sodium inactivation in axons depolarized by lowering the holding potential or raising the external K^+ concentration has been noted by several authors (Cole, 1958; Narahashi, 1964; Baker et al., 1964; Adelman and Palti, 1969*a*,*b*; Schauf et al., 1976). Adelman and Palti (1969*a*,*b*) found that there were at least three components to the reactivation of sodium channels after depolarization by high $[K^+]_0$ with time constants of 1–10 ms (τ_h), 50–200 ms, and 30–200 s. The time course of the slower component in the open circles of Fig. 5*a* seemed to correspond to the second of these, and it may be seen that after treatment with NEM the major reactivation of sodium currents appeared to occur with similar kinetics. This point has been further investigated by examining peak early currents in crayfish axons held at depolarized potentials. Fig. 5*b* shows the results of one such experiment. The reversal of inactivation proceeds with kinetics that are similar to those of NEM-treated fibers.

In the formulation of Hodgkin and Huxley (1952*b*) sodium conductance, G_{Na}, is described by:

$$G_{Na} = \bar{G}_{Na} m^3 h, \tag{1}$$

where $\bar{G}_{Na}$ is the maximum sodium conductance with all channels open, and m and h are probability variables obeying first order kinetics. Eq. (1) may be modified to include slow inactivation. Adelman and Palti (1969*b*) called the slow process with a time constant of 50–200 ms the "p" process and that with a time constant of 30–200 s the "q" process. Since the longest conditioning pulses used

TABLE I

TIME TO PEAK EARLY CURRENT

Axon	350-ms prepulse	V_m	Time to peak	
			Control	NEM
	mV	*mV*	*μs*	*μs*
MA1075	–	−35	800	740
	–	−30	700	640
	–	−25	540	480
	–	−20	460	440
	–	−15	400	400
	–	−10	320	360
	–	−5	280	320
	–	0	240	280
	–	+5	220	260
	–	+25	180	200
	−113	−28	560	600
	"	−23	500	520
	"	−18	420	420
	"	−13	360	380
	"	−8	300	360
	"	−3	300	320
	"	+2	260	280
	"	+7	220	280
	"	+27	180	220
MA1175	–	−25	540	520
	–	−20	440	420
	–	−15	360	340
	–	−10	320	300
	–	−5	280	280
	–	0	260	260
	–	+5	220	220
	–	+25	180	180
	−115	−25	520	440
	"	−20	440	380
	"	−15	360	320
	"	−10	320	280
	"	−5	280	260
	"	0	260	220
	"	+5	240	220
	"	+25	180	160
	"	+85	80	80
MY1375	–	−23	450	460
	–	−13	320	340
	–	−3	260	290
	–	+7	220	240
	–	+27	160	220
	–	+67	–	95
	−113	−23	–	410
	"	−13	–	330
	"	−3	–	280
	"	+7	–	240
	"	+27	–	200
	"	+67	–	115
JNO975	–	−24	480	460
	–	−14	350	330
	–	−4	270	260
	–	+6	220	210
	–	+26	160	160
	–	+46	140	140
	–	+66	70	70

in the experiments reported here were under 1 s in duration, the q system is effectively constant and may be lumped with $\bar{G}_{Na}$. In analogy with Eq. (1) we consider the slow inactivation of G_{Na} occurring with a time constant of 50–200 ms to be governed by a probability variable p. In analyzing the data of Fig. 5 it was found that the reversal of this slow process proceeded with a small delay, and that a significant improvement in the fit (judged by eye) could be obtained if G_{Na} were considered as:

$$G_{Na} = \bar{G}_{Na} m^3 h p^2, \tag{2}$$

TABLE II

Na^+ TAIL CURRENT KINETICS

		Tail currents		
			τ_t	
Axon	350-ms prepulse	Vm	Control	NEM
	mV	*mV*	*μs*	*μs*
JL0975	–	−43	310	341
	–	−53	159	154
	–	−73	65	76
	−113	−43	329	320
	"	−53	202	205
	"	−73	87	92
MY1375	–	−43	269	246
	–	−53	170	173
	–	−73	75	96
	–	−93	58	82
MA1775	–	−58	153	121
	–	−78	90	84
	–	−98	43	59
	−118	−58	163	172
	"	−78	113	102
	"	−98	49	54

Time constants (τ_t) are calculated for the tail currents by a least squares fit to an exponential function.

where p obeys the equation:

$$\frac{dp}{dt} = \alpha_p(1 - p) - \beta_p p, \tag{3}$$

with the solution:

$$p = p_\infty - (p_\infty - p_0)e^{-t/\tau_p}, \tag{4}$$

where

$$\tau_p = \frac{1}{\alpha_p + \beta_p}, \tag{5}$$

and

$$p_\infty = \frac{\alpha_p}{\alpha_p + \beta_p}. \tag{6}$$

The curves in Fig. 5*a* were calculated by using Eq. (2) and were fitted by estimating initial and final h and p values from this and other experiments, and

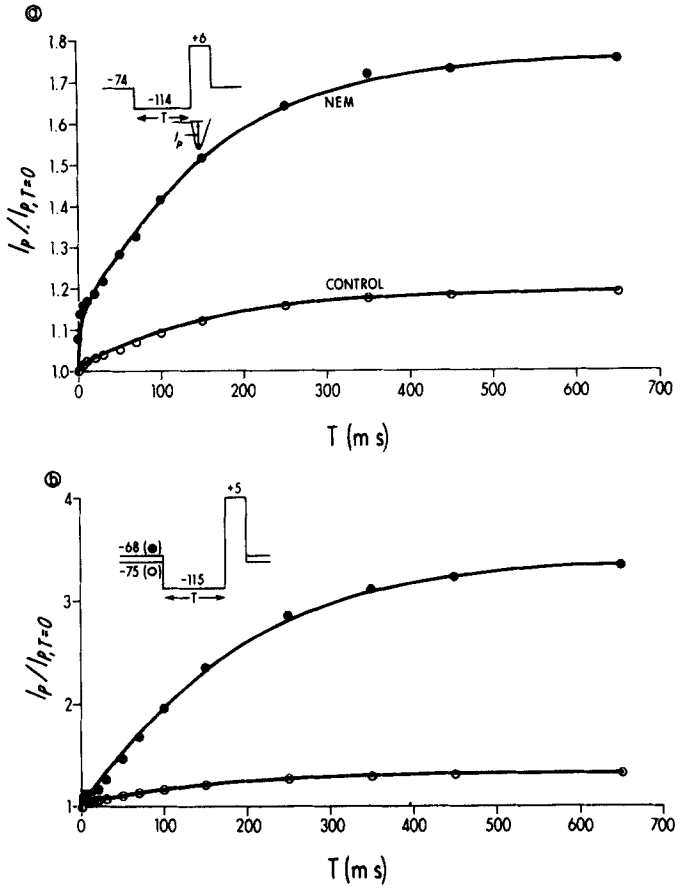

FIGURE 5. Kinetics of the restoration of sodium conductance by a hyperpolarizing prepulse. Symbols represent experimental data, measured as the ratio of peak early current with no prepulse to that with a prepulse of duration given on the abscissa. Potential sequences given in the insets. Peak early currents measured as in Hodgkin and Huxley, (1952 a, Fig. 3). (*a*) Open circles; TMVH + 1 mM β-mercaptoethanol + 1 mM NEM, mixed before applying to the axon. Results are similar in TMVH alone. Filled circles: Results after switching to TMVH + 1 mM NEM for 20 min followed by TMVH. Curves are drawn according to Eq. (2) with (for the p system at −114 mV) control; $\alpha_p = 6.3\ s^{-1}$, $\beta_p = 0.3\ s^{-1}$: NEM; $\alpha_p = 4.9\ s^{-1}$, $\beta_p = 1.9\ s^{-1}$. (*b*) Axon in TMVH-II, $[K^+]_0 = 5.4$ mM. Open circles: holding potential = −75 mV. Filled circles: holding potential = −68 mV. Both curves fitted with (at −115 mV): $\alpha_p = 6.4\ s^{-1}$; $\beta_p = 0.1\ s^{-1}$.

then judging the final fit by varying τ_p. In the case of Fig. 5*b*, all parameters were equal in the two curves except for p_0, the value of p at the beginning of the hyperpolarizing pulse. The values of τ_p were, in Fig. 5*a*, 152 ms (control) and 147 ms (NEM), and in Fig. 5*b*, 159 ms (both curves). In ensuing figures the fit

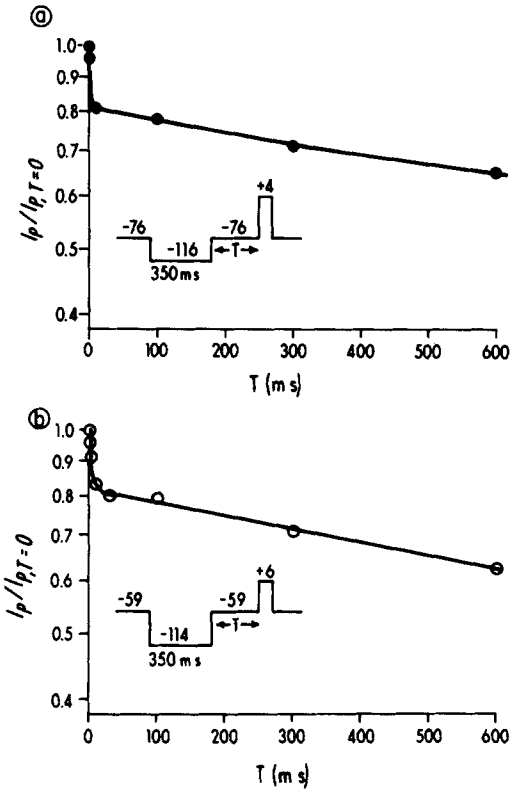

FIGURE 6. The kinetics of sodium inactivation after exposure to NEM or at elevated external K^+ concentrations. Axons were hyperpolarized by 40 mV for 350 ms, then returned to the holding potential for a variable period, T, before applying a test depolarization (see *insets*). Data given as the ratio of peak early current after T ms (abscissa) at V_h to that recorded immediately after the hyperpolarization. (*a*) Results from a fiber that had been exposed to 2.5 mM NEM for 20 min, then switched to TMVH, $[K^+]_0$ = 3.5 mM. Curve calculated from Eq. (2) with τ_p = 1.1 s at −76 mV. (*b*) Points from an axon in TMVH-II, $[K^+]_0$ = 12.9 mM. Holding potential = resting potential = −59 mV. Curve drawn according to Eq. (2) with τ_p = 3 s.

could be made more directly, calculating p and h values in succession by a least squares fit to exponential functions.

Fig. 6 illustrates results of experiments designed to measure the time course of inactivation after the conclusion of a long hyperpolarizing pulse. Fig. 6*a* shows points (filled circles) from an axon after exposure to NEM. After a pulse to −116 mV for 350 ms, the membrane potential was returned to −76 mV for a variable

period before a test pulse was applied to +4 mV (see *inset*). The data are plotted semilogarithmically and show clearly the two kinetic components of inactivation. In Fig. 6*b*, an axon was depolarized to −59 mV by raising the external K^+ concentration from 5.4 mM to 12.9 mM. The records again show two components of inactivation and again were fit by using Eq. (2). The apparent near identity of the two curves is somewhat misleading since the data are taken at different potentials, and the asymptotes at long T were different in the two cases. Nonetheless, there seems to be a close similarity between the slow sodium inactivation seen after exposure to NEM and that present after depolarization in high $[K^+]_0$.

The kinetics of inactivation have been further studied by the double pulse procedure of Chandler et al. (1965). Data are shown in Fig. 7 for two potentials: −55 and +5 mV. As illustrated in the insets V_m is returned to the holding potential for a time long compared with τ_m (see Table II) before the test depolarization. The two phases of inactivation are again visible and the curves are drawn by using Eq. (2) with $p_\infty = 0$. It may be seen from Fig. 7 that NEM affects the h system as well as slow inactivation. At −55 mV (Fig. 7*a*) τ_h was smaller after exposure to NEM (6.3 ms) than before (16.6 ms). At +5 mV (Fig. 7*b*) the situation was reversed, with τ_h slower with NEM (2.2 ms) than in the untreated fiber (1.1 ms). Records in Fig. 7 are typical of those obtained in three axons at each potential. If inactivation follows hp^x kinetics, then for $p_\infty \simeq 0$ double pulse data should be independent of p_0, and hence independent of a long hyperpolarizing prepulse. In two axons for which complete records were obtained, this was in fact found to be the case.

Khodorov et al. (1974, 1976) have shown that the tertiary amine local anesthetics procaine and trimecaine, but not benzocaine or quaternary amines, induce a slow inactivation of sodium channels when applied externally to the frog node of Ranvier. Further, an enhancement of the channel-blocking potency of other local anesthetics by repetitive depolarization (Strichartz, 1973; Courtney, 1975; Hille et al., 1975) is similar to that reported for conduction block of squid axons by NEM (Marquis and Mautner, 1974). Since NEM contains a tertiary nitrogen and carbonyl oxygen as do procaine and trimecaine, several experiments have been conducted with analogs of NEM in an attempt to determine if its action is due to properties other than reactivity to sulfhydryl residues. One such experiment has already been mentioned and the results have been illustrated in Fig. 2. In further work, the saturated analog of NEM, *N*-ethylsuccinimide (NES), was synthesized by hydrogenation of NEM, as described in Materials and Methods. NES retains the tertiary nitrogen and carbon oxygens of NEM, but has lost the ability to react with sulfhydryl groups (Fig. 1). Added to TMVH at 2.5–3 mM, NES had only slight effects on ionic currents. There was generally a 10–15% increase in peak early currents, and a small decrease in the enhancement of these currents by a 350-ms hyperpolarizing prepulse (Fig. 8), results opposite to those of NEM. On the other hand, maleimide, which retains the double bond, and the resultant ability to react with sulfhydryl groups (Friedmann et al., 1949) but which is a secondary amine (Fig. 1) acts in a manner identical to that of NEM. Maleimide (2.5 mM) was added to

TMVH after washing away the NES in the axon of Fig. 8. Peak currents fell rapidly and their enhancement by a long hyperpolarizing prepulse rose simultaneously, indicating a slow inactivation of sodium channels by this compound. The resting potential remained unchanged. Results from another axon are

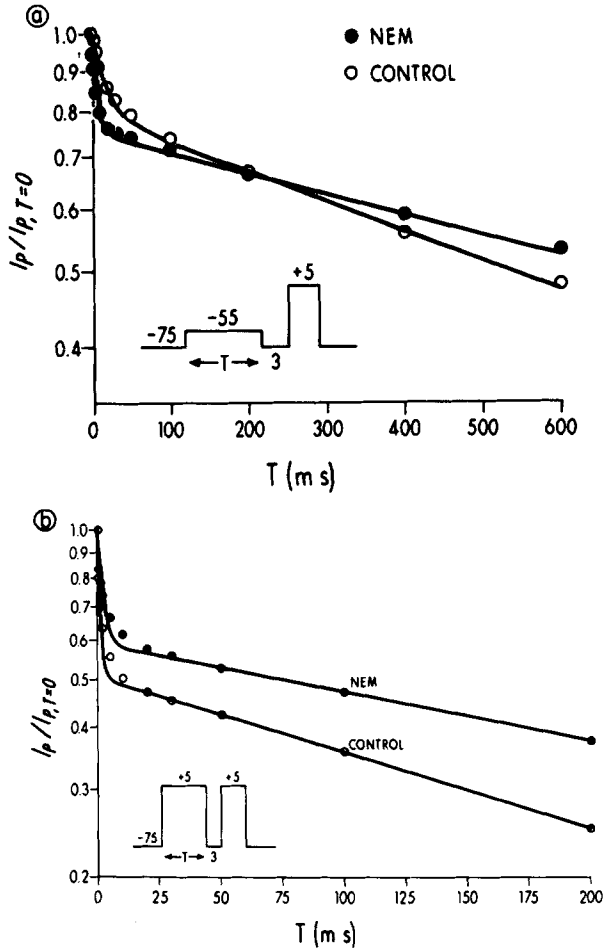

FIGURE 7. The kinetics of inactivation at depolarized potentials explored with the double pulse procedure of Chandler et al. (1965). Pulse sequences shown in insets. Open circles; Records in TMVH. Filled circles: points after exposure to 1 mM NEM in TMVH for 20 min and a return to TMVH. Curves follow Eq. (2) with: (*a*) (−55 mV) Control; τ_h = 16.6 ms; τ_p = 2.3 s NEM; τ_h = 6.3 ms; τ_p = 3.4 s. (*b*) (+5 mV) Control; τ_h = 1.1 ms; τ_p = 574 ms. NEM; τ_h = 2.2 ms; τ_p = 890 ms.

shown in Fig. 9. The currents in Fig. 9*a* and *b* were recorded 15 min after addition of 2.5 mM maleimide externally. Fig. 9*b* shows the restoration of sodium conductance by prepulses to −112 mV for 350 ms. Records in Fig. 9*c* were taken 40 min after switching to TMVH alone and show no reversal. In this

axon an additional attempt at reversal was made. 2 mM β-mercaptoethanol was added externally for 30 min, followed by an additional 20 min of washing with TMVH alone. The axon had by this time become rather leaky, but there was little change in sodium currents (Fig. 9*d*). Two experiments were performed

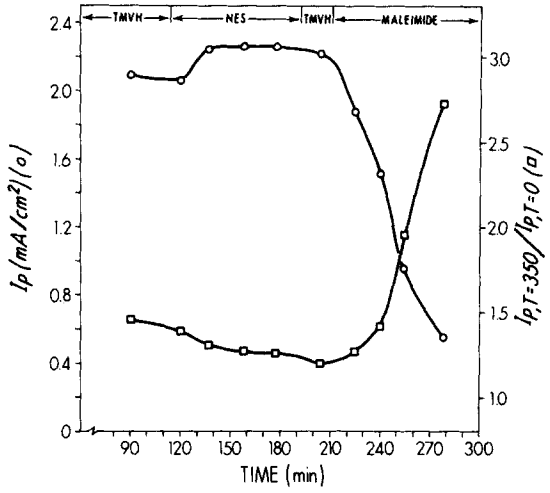

FIGURE 8. Effects of NES and maleimide on peak early currents. Circles: amplitude of peak inward current recorded with test pulses to +7 mV. Squares: ratio of peak current recorded with a prepulse to −113 mV for 350 ms immediately preceding the +7 mV test pulse to that recorded with no prepulse. NES and maleimide were added to TMVH at 2.5 mM. Lines are drawn through the points to indicate trends. The holding potential was −73 mV.

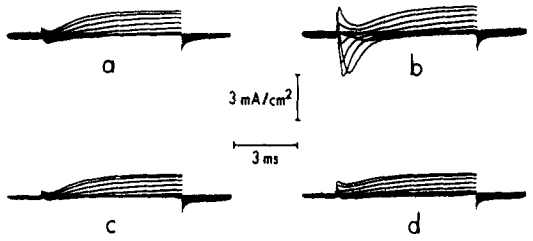

FIGURE 9. Voltage clamp currents after exposure to maleimide. Test depolarizations were from a holding potential of −72 mV to −52, −32, −12, +8, +28, +48, +68 mV. (*a*) Records 15 min after addition of 2.5 mM maleimide to TMVH. (*b*) Same, but with a prepulse to −112 mV for 350 ms immediately preceding test pulses. (*c*) Records with no prepulses taken 40 min after switching to TMVH alone. (*d*) Currents recorded with no prepulse after addition of 2 mM β-mercaptoethanol to TMVH for 30 min, followed by 20 min in TMVH alone.

with succinimide, the saturated analog of maleimide. Results were similar to those with NES although the increase in peak currents and decrease in relative restoration by a hyperpolarizing prepulse were more marked. Here, as in the case with NES, effects were opposite to those of NEM and maleimide. Small depolarizations in resting potential were noted, leading to a concern with

possible electrode artifacts. Activity of NEM and maleimide, however, occurred with no shift in resting potential.

Mercurials

p-Chloromercuriphenylsulfonic acid (PCMBS) also reacts selectively with sulfhydryl groups, but unlike NEM, PCMBS bears a net charge (−1). Fig. 10 illustrates results with this compound. PCMBS (0.2 mM) was added to normal Van Harreveld's solution for 25 min, and then was washed away. Peak early transient currents were reduced by about 50% from control values, with no change in

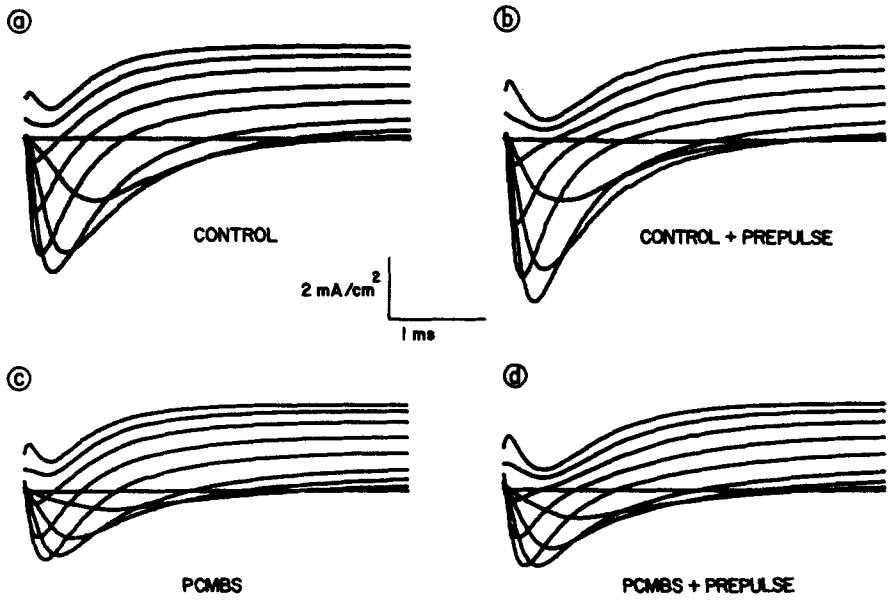

FIGURE 10. Alteration of ionic currents by PCMBS. Holding potential = −75 mV. Depolarizations to −35, −25, −15, +5, +25, +45, +65, +85 mV. In (*b*) and (*d*) a prepulse to −115 mV for 350 ms preceded each depolarization. (*a*) and (*b*), Currents in NVH. (*c*) and (*d*), Axon exposed to 0.2 mM PCMBS in NVH for 25 min, then returned to NVH.

delayed currents. However, in contrast to the results with NEM, prolonged hyperpolarizations were not effective in restoring peak early currents beyond the fractional increase seen in control fibers (in fact the fractional increase was usually slightly smaller after exposure to PCMBS). Further, the time-to-peak early current was about 20% longer after reaction with PCMBS. Attempts to suppress sodium currents completely with prolonged use of PCMBS generally resulted in a rapid increase in leakage conductance, rendering this compound less useful than NEM. The action of PCMBS was only partially reversed by 5 mM β-mercaptoethanol. Hg^{++} has been studied in only three axons. Just 4 μM $HgCl_2$ added to Van Harreveld's saline was sufficient to reduce peak early currents by 50% in 25 min, but leakage conductance increased even more rapidly than with PCMBS, making further measurements difficult.

Reducing and Oxidizing Agents

Dithiothreitol and β-mercaptoethanol (1–5 mM) added to NVH had little apparent effect on ionic currents. There is, however, a technical difficulty in working with these reducing agents, since the possibility of oxidation-reduction potentials at the metal electrode surfaces (particularly at the electrically floating wire in the potential pipette) may lead to ambiguities in the measurement of membrane potential. The resting potential generally hyperpolarized by 5–10 mV after addition of one of these compounds. The above statement that currents were only minimally altered is based on the assumption that most or all of this hyperpolarization is due to an electrode artifact. If the original holding potential is maintained (assuming the hyperpolarization to be genuine) then currents, particularly early transient currents, are significantly smaller.

Oxidizing agents are often highly nonspecific and have not been studied in this series of experiments. Earlier results with DTNB, an exception to the rule (Shrager, 1975), are mentioned in Discussion. Several years ago we found that *N*-bromosuccinimide, a highly reactive compound capable of oxidizing a number of amino acid side chains, causes a rapid and irreversible loss of resting potential in unclamped axons (unpublished observations). Keana and Stämpfli (1974) have recently reported very similar results on nodes of Ranvier.

DISCUSSION

Used under moderate reaction conditions (pH 6.9–7.0, 1–3 mM, 30–40 min), NEM affected only a limited number of events in excitation. The resting potential of the cell remained the same. Kinetic and steady-state properties of potassium conductance were not altered. Rates of opening and closing of sodium activation gates and the selectivity of the sodium channel were similarly unchanged. The normal increase in sodium conductance during a depolarization from rest was, however, almost completely absent. This "inactivation" of sodium channels could be at least partially reversed by a maintained hyperpolarization. The kinetics of this reactivation, as well as of the inactivation that followed a return to the resting level, suggested that while the h system seemed to be altered, the primary events occurred on a time scale about 50 times slower than that of h inactivation. This slow inactivation seemed not to be a new event but rather one with kinetic properties similar to those of a system that is present in normal fibers but with different voltage dependence. While slow inactivation is normally induced by depolarization and by high external K^+ concentrations (Adelman and Palti, 1969*b*), neither of these latter conditions accompanies the use of NEM.

The findings of Khodorov et al. (1974, 1976) that certain local anesthetics induce a slow inactivation of sodium channels in frog node raises the possibility that NEM is acting by virtue of "pharmacological" properties rather than by reaction with sulfhydryl residues. Testing several anesthetics, Khodorov et al. (1976) found that only the tertiary amines produced slow inactivation. In the experiments reported here, maleimide, a secondary amine, gave results identical to those of NEM. Other local anesthetics, including quaternary and primary amines, block sodium channels in the node by a reduction in $\bar{G}_{Na}$, with the blockage in some cases being dependent on the frequency of opening and

closing of these channels ("use dependence") (Strichartz, 1973; Courtney, 1975). Hille et al. (1975) have shown that tertiary amines can also block in a use-dependent manner. The distinction between use dependence and slow inactivation is not fully established by the work reported thus far. Khodorov et al. (1975) have shown that for the externally applied tertiary compounds procaine and trimecaine, slow inactivation can account for all their observations. The experiment of Courtney (1975, Fig. 6*b*) with fixed hyperpolarizing prepulse and varying postpulse levels suggests that the tertiary drug GEA 968 produces use-dependent block. Quaternary compounds appear to be primarily use dependent (Strichartz, 1973; Khodorov et al., 1976). Since the range of molecular structures active as local anesthetics is so large, and the assignment of mode of action to structure type not exclusive, the results with maleimide cannot be taken as proof that sulfhydryl block is necessary for the action of NEM. The results with the saturated analogs NES and succinimide are consistent with the requirement for sulfhydryl reaction but again are not conclusive. All local anesthetics noted above contain aromatic rings and the double bond might therefore be a structural requirement for pharmacological activity. In summary, while results of our control experiments are all consistent with the view that reaction with sulfhydryl groups is involved in the effects of NEM, it is difficult to consider the results with local anesthetics as coincidental. One rather speculative possibility is that binding of NEM via a sulfhydryl group renders it more potent in anesthetic-type block. Since NEM is permeant, it is not possible to judge its locus of action from these experiments. If a sulfhydryl residue with sufficiently low pK were located at the inner membrane surface in close association with the slow inactivation system, then NEM might exert its effect by a reduction in surface charge resulting in a depolarization of the internal membrane field.

The data have been fitted by a model in which the inactivation of sodium conductance depends on the product hp^2, where h and p are independent variables obeying first order kinetics. The fit was reasonable, but could clearly be improved, possibly by increasing the exponent of p. It might also be possible to fit the points to quite different models. One other scheme that has been considered is a system in which a channel must first undergo h or "fast" inactivation before the p gate can close. The inactivation gate would then have three states related by:

$$\text{open} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \text{closed } (h) \underset{\gamma}{\overset{\delta}{\rightleftharpoons}} \text{closed } (p).$$

This model has been fitted in only a rough manner, testing whether the solution of the resulting three simultaneous second order equations can work for any arbitrary rate constants (but with $\alpha, \beta \gg \gamma, \delta$). The match was judged slightly poorer than in the case of the hp^2 model, but since neither system fit with a high degree of accuracy, the three-state model cannot be ruled out. Extensive kinetic data are difficult to obtain because of the rather long duration pulses required. As mentioned earlier, the rate of reaction of NEM with the axon membrane may be potential dependent, and although most of the unreacted compound is washed away, some may remain, perhaps "solubilized" in membranes. After exposure to NEM, measurements involving large excursions of potential for

prolonged periods could rarely be made for more than about 60 min before the leakage conductance began to increase appreciably.

The maximum reduction of peak early currents that we have observed after adding 1–3 mM NEM externally, and allowing it to react for an unlimited time, is about 90%. After that point is reached, or if higher concentrations of reagent are used, the leakage conductance generally increases rapidly and measurements are no longer possible. After hyperpolarizing prepulses to −115 to −135 mV for up to 1 s, about 70% of the sodium conductance present before reaction is available. It is possible that NEM has modified a component of inactivation with even slower kinetics than the p system and that even longer prepulses are required to reverse it, as in the case of inactivation in high $[K^+]_0$ in squid axons (Adelman and Palti, 1969*b*). Alternatively, NEM may reduce $\bar{G}_{Na}$ by lowering the conductance of open channels, though without effect on selectivity. NEM may also act at multiple sites on a single channel. If $\bar{G}_{Na}$ is not reduced, then at least 90% of the sodium channels that have reacted with NEM are inactivated at the resting potential. However, the double pulse experiments indicate that with no hyperpolarizing prepulse τ_h may be altered by a factor of about 2 by NEM. Thus, a significant fraction of the channels with open p gates must have been affected and this might involve an SH group associated with the h system. It also follows from the above discussion that in making kinetic measurements after treatment with NEM we are observing a mixture of channels, some with modified p and/or h gates and some normal. Further information of the type noted above will be required before we can measure properties of individual populations.

There have been some previous reports of modification of nerve fibers with NEM. Smith (1958) found that 1 mM NEM blocked conduction in lobster axons in 10 ± 5 min at room temperature and depolarized the fiber by 20 mV after 90 ± 16 min. Action potentials were blocked in frog sciatic nerve by 2 mM NEM in 21 ± 9 min. Effects of this compound on both preparations were irreversible. Huneeus-Cox et al. (1966) reported that 1–2 mM NEM blocked action potentials irreversibly in squid axons when applied either externally or internally in perfused fibers. Keana and Stämpfli (1974) investigated the action of NEM on voltage-clamped frog node of Ranvier, measuring the run-down of peak early current and steady-state current. In contrast with the present work, these authors found a very rapid (20 s) 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. However, experimental conditions were also quite dissimilar to those reported here. NEM was used at very high concentrations (41 mM) for very short times. The stimulation-dependent block of squid axons found by Marquis and Mautner (1974) has been mentioned earlier.

The reduction of sodium currents by PCMBS was not reversed by hyperpolarizing prepulses effective in the case of NEM. Narahashi (1964) has similarly found that the block of action potentials by this mercurial in lobster axons is not reversed by long hyperpolarizations. PCMBS may be acting in a very different manner from NEM, or it is possible that the negative charge introduced by the mercurial alters the response to these variations of membrane potential. It is of interest that a doubly negatively charged sulfhydryl reagent, 5,5′-dithio*bis*(nitrobenzoic acid) (DTNB) reacts extensively with giant axons, as judged

by color changes, but has no effect on excitation when applied externally. NEM remains effective after exposure to DTNB (Shrager, 1975).

I am grateful to Dr. Leon Miller for help with the synthesis of *N*-ethylsuccinimide. Mr. Wing Kai Cheng and Mr. John Young provided expert assistance with the digital electronics.
This work has been supported by grants 5-RO1-NS10500 and 5-PO1-NS10981 from the National Institutes of Health.

Received for publication 2 February 1976.

REFERENCES

ADELMAN, W. J., JR., and Y. PALTI. 1969*a*. The influence of external potassium on the inactivation of sodium currents in the giant axon of the squid *Loligo pealei*. *J. Gen. Physiol.* **53**:685–703.

ADELMAN, W. J., JR., and Y. PALTI. 1969*b*. The effects of external potassium and long duration voltage conditioning on the amplitude of sodium currents in the giant axon of the squid *Loligo pealei*. *J. Gen. Physiol.* **54**:589–606.

ARMSTRONG, C. M., F. BEZANILLA, and E. ROJAS. 1973. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* **62**:375–391.

BAKER, P. F., A. L. HODGKIN, and H. MEVES. 1964. The effect of diluting the internal solution on the electrical properties of a perfused giant axon. *J. Physiol. (Lond.)*. **170**:541–560.

CHANDLER, W. K., A. L. HODGKIN, and H. MEVES. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol. (Lond.)*. **180**:821–836.

CHANDLER, W. K., and H. MEVES. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol. (Lond.)*. **180**:788–820.

COLE, K. S. 1958. Effects of polarization on membrane ion currents. *Biophys. Soc. Annu. Meet. Abstr.* **2**:22.

COLE, K. S., and J. W. MOORE. 1960. Potassium ion current in the squid giant axon: dynamic characteristic. *Biophys. J.* **1**:1–14.

COURTNEY, K. R. 1975. Mechanism of frequency dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. *J. Pharmacol. Exp. Ther.* **195**:225–236.

FRIEDMANN, E., D. H. MARRIAN, and I. SIMON-REUSS. 1949. Antimitotic action of maleimide and related substances. *Br. J. Pharmacol.* **4**:105–108.

GREGORY, J. D. 1955. The stability of *N*-ethylmaleimide and its reaction with sulfhydryl groups. *J. Am. Chem. Soc.* **77**:3922–3923.

HILLE, B., K. COURTNEY, and R. DUM. 1975. Rate and site of action of local anesthetics in myelinated nerve fibers. *Prog. Anesthesiol.* **1**:13–20.

HODGKIN, A. L., and A. F. HUXLEY. 1952*a*. The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol. (Lond.)*. **116**:497–506.

HODGKIN, A. L., and A. F. HUXLEY. 1952*b*. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)*. **117**:500–544.

HUNEEUS-COX, F., H. L. FERNANDEZ, and B. H. SMITH. 1966. Effects of redox and sulfhydryl reagents on the bioelectric properties of the giant axon of the squid. *Biophys. J.* **6**:675–689.

KEANA, J. F. W., and R. STÄMPFLI. 1974. Effect of several "specific" chemical reagents on the Na^+, K^+ and leakage currents in voltage clamped single nodes of Ranvier. *Biochim. Biophys. Acta.* **373**:18–33.

KHODOROV, B. I., L. D. SHISHKOVA, and E. M. PEGANOV. 1974. Effect of procaine and calcium ions on slow sodium inactivation in the frog Ranvier node membrane. *Bull. Exp. Biol. Med.* **77**:226–229.

KHODOROV, B., L. SHISHKOVA, E. PEGANOV, and S. REVENKO. 1976. Inhibition of sodium currents in frog Ranvier node treated with local anesthetics. Role of slow sodium inactivation. *Biochim. Biophys. Acta.* **433**:409–435.

MARQUIS, J. K., and H. G. MAUTNER. 1974. The effect of electrical stimulation on the action of sulfhydryl reagents in the giant axon of squid; suggested mechanisms for the role of thiol and disulfide groups in electrically-induced conformational changes. *J. Membr. Biol.* **15**:249–260.

MENSCHUTKIN, N. 1876. Ueber aethyl- und methylsuccinimid. *Liebigs Annalen der Chemie.* **182**:90–93.

MORELL, S. A., V. E. AYERS, T. J. GREENWALT, and P. HOFFMAN. 1964. Thiols of the erythrocyte. I. Reaction of *N*-ethylmaleimide with intact erythrocytes. *J. Biol. Chem.* **239**:2696–2705.

NARAHASHI, T. 1964. Restoration of action potential by anodal polarization in lobster giant axons. *J. Cell. Comp. Physiol.* **64**:73–96.

NARAHASHI, T. 1972. Mechanism of action of tetrodotoxin and saxitoxin on excitable membranes. *Fed. Proc.* **31**:1124–1132.

RIORDAN, J. F., and B. L. VALLEE. 1972. Reactions with *N*-ethylmaleimide and *p*-mercuribenzoate. *In* Methods in Enzymology. XXVB. C. H. W. Hirs and S. N. Timasheff, editors. Academic Press, Inc., New York. 449–456.

SCHAUF, C. L., T. L. PENCEK, and F. A. DAVIS. 1976. Slow sodium inactivation in *Myxicola* axons. Evidence for a second inactive state. *Biophys. J.* **16**:771–778.

SHRAGER, P. 1974. Ionic conductance changes in voltage clamped crayfish axons at low pH. *J. Gen. Physiol.* **64**:666–690.

SHRAGER, P. 1975. Specific chemical groups involved in the control of ionic conductance in nerve. *Ann. N. Y. Acad. Sci.* **264**:293–303.

SHRAGER, P. 1976. "Slow" inactivation of sodium conductance in nerve following reaction with *N*-ethylmaleimide. *Biophys. J.* **16**(2, Pt. 2): 75 a. (Abstr.).

SMITH, H. M. 1958. Effects of sulfhydryl blockade on axonal function. *J. Cell. Comp. Physiol.* **51**:161–171.

SMYTH, D. G., O. O. BLUMENFELD, and W. KONIGSBERG. 1964. Reactions of *N*-ethylmaleimide with peptides and amino acids. *Biochem. J.* **91**:589–595.

SMYTH, D. G., A. NAGAMATSU, and J. S. FRUTON. 1960. Some reactions of *N*-ethylmaleimide. *J. Am. Chem. Soc.* **82**:4600–4604.

STRICHARTZ, G. R. 1973. The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *J. Gen. Physiol.* **62**:37–57.

VAN HARREVELD, A. 1936. A physiological solution for fresh water crustaceans. *Proc. Soc. Exp. Biol. Med.* **34**:428–432.