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Modification of Ultraviolet Radiation Effects on the Membrane of Myelinated Nerve Fibers by Sulfhydryl Compounds

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Summary. The modification of the ultraviolet blocking of sodium channels and of the ultraviolet-induced potential shift of the gating parameters by means of the sulfhydryl compounds *l*-cysteine and 2-mercaptoethanol was investigated in the node of Ranvier under voltage-clamp conditions. The UV wavelength was 280 nm. The radiation-induced potential shift of the voltage-dependent gating parameters was prevented or even reversed by the action of the sulfhydryl compounds (internal application), while the blocking effect was not affected. It is concluded that the two radiation effects are caused by two separate photoreactions. Internally applied N-ethylmaleimide, binding specifically to protein-SH groups, exhibits an effect similar to the ultraviolet-induced potential shift, without affecting the maximum sodium permeability. Therefore, the ultraviolet-induced potential shift might be caused by a photocatalyzed oxidation of $-SH$ groups of membrane proteins changing the surface charge density at the inner side of the nodal membrane.

Key Words node of Ranvier · sodium channel · ultraviolet radiation · sulfhydryl compounds · membrane proteins · surface charges

Introduction

Ultraviolet radiation of 280 nm wavelength affects the membrane of isolated nodes of Ranvier in two separate ways: 1) by a selective irreversible blocking of sodium channels (Fox, 1974; Oxford & Pooler, 1975; Hof & Fox, 1983) and 2) by a displacement along the voltage axis of the potential-dependent kinetic parameters of sodium activation, inactivation and ultra-slow inactivation (Schwarz & Fox, 1977). In order to obtain more detailed information on the nature of the photoreactions involved, the modification of the radiation effects by the sulfhydryl-containing compounds *l*-cysteine and 2-mercaptoethanol was investigated which are known to act as radiobiological protectives (Dertinger & Jung, 1971). As a counterpart, N-ethylmaleimide (NEM) was also applied. This agent contains an activated double bond which forms additional compounds with reactive $-SH$ groups of proteins, and near pH 7 the reaction is rapid and highly specific (Riordan & Vallee, 1972).

The objective of this paper is, therefore, to investigate whether the ultraviolet radiation effects at the nodal membrane are connected to SH-containing functional groups and if so, which of the two radiation effects can be modified by SH-reacting chemicals. A second aim of this paper is to elucidate whether or not the spontaneous exponential decline with time (run-down) is connected with the potential shifts of m_∞, h_∞ and u_∞.

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Materials and Methods

Measurement of Currents and Irradiation

Single motor fibers of the sciatic nerve of *Rana esculenta* were mounted in an acrylic chamber which was connected to a voltage-clamp arrangement described by Nonner (1969). The experiments were run on-line under the control of a computer (Honeywell DDP-516, DA-converter (10 Bit), AD-converter (12 Bit, 11 μsec sampling rate).

The peak sodium inward current I^p_{Na} (corrected for leakage current) was registered with and without applying a prepulse ($V_1 = -40$ mV, $t_1 = 50$ msec). Thus the inactivation parameter

$$h_\infty(V = 0) = I^p_{Na}(V_1 = 0)/I^p_{Na}(V_1 = -40 \text{ mV}) \quad (1)$$

was easily determined. (TEA was used for elimination of the potassium current.)

Complete sodium inactivation curves $h_\infty(V)$ were measured by changing the prepulse amplitude from $V_1 = -50$ to $+50$ mV. These curves were analyzed off-line. They were approximated by the function:

$$h_\infty = 1\Big/\left(1 + \exp\left(\frac{V - V_{1/2}}{k}\right)\right). \quad (2)$$

The accuracy of determination of $V_{1/2}$ and k was extensively discussed by Hof and Fox (1983). The standard deviation of the least-squares fit of single runs of h_∞ curves never exceeded 0.2 mV. The variation (± SD) of successive determinations of $V_{1/2}$ never exceeded 0.3 mV.

The nodal membrane was irradiated with continuous or discontinuous monochromatic ultraviolet radiation of 280 ± 3 nm (± bandwidth). [For details *see* Schwarz and Fox (1977) and Hof and Fox (1983).] The ultraviolet dose rate varied between 0.6 and 2 mW/cm^2.

Determination of Ultraviolet Sensitivities of the Nodal Membrane

Both sodium currents (with and without a negative prepulse) follow an exponential decrease with irradiation dose $H \cdot t$ ($H \cdot t$ = dose rate × irradiation time; Schwarz & Fox, 1977), and therefore $h_\infty(V = 0)$ is also expected to show such a dose dependence:

$$I^p_{Na}(t) = I^p_{Na}(t = 0) \cdot \exp(-\gamma^{Na} \cdot H \cdot t) \qquad (h_\infty = 1) \tag{3a}$$

$$h_\infty(V = 0,t) = [h_\infty(V = 0,t = 0) - h_\infty(V = 0,t = \infty)] \cdot \exp(-\gamma^h \cdot H \cdot t) + h_\infty(V = 0,t = \infty). \tag{3b}$$

The rate constants γ^{Na} and γ^h define the ultraviolet sensitivity of the corresponding membrane parameters. The constant $h_\infty(V = 0, t = \infty)$ was determined to be of the order of 0.2 (*see* Hof & Fox, 1983).

γ^{Na} and γ^h were determined by fitting exponential functions to the measured values multiplied by exponential functions for run-down correction as derived from the time courses of the radiation-free intervals (for details *see* Hof & Fox, 1983). Using least-squares fitting the maximum standard deviation of the fits observed in all experiments was 3.5×10^{-2} cm^2/Wsec. This corresponds to a maximum relative error of 3.5% for γ^{Na} and of 13.5% for γ^h taking into account the lowest values of γ^{Na} or γ^h ever observed in these experiments.

Corrections for Changes of Ultra-slow Sodium Inactivation

The nodal peak sodium current measured at a fixed test voltage depends on the membrane potential, even if the normal sodium inactivation is removed ($h_\infty = 1$). This effect which is not included in the Hodgkin-Huxley formalism was investigated and quantitatively described by Fox (1976*a*):

$$I^p_{Na}(V_{1/2}, V) = u_\infty(V_{1/2}) \cdot I^p_{Na}(V) \tag{4}$$

with

$$u_\infty(V_{1/2}) = 1 \Big/ \left(1 + \exp\left(\frac{10.5 - V_{1/2}}{-11.7}\right)\right). \tag{5}$$

If the sigmoidal function $u_\infty(V_{1/2})$ is displaced by ΔV to the negative direction along the voltage axis due to irradiation as is $h_\infty(V)$, I^p_{Na} will be decreased in addition to the ultraviolet-induced block of $\bar{P}_{Na}$. To separate the two radiation effects completely from each other, $I^p_{Na}(t)$ after irradiation was corrected for changes of u_∞ by:

$$\frac{I^p_{Na}(t)}{u_\infty(V_{1/2} + \Delta V)} = \frac{I^p_{Na}(t = 0)}{u_\infty(V_{1/2})} \exp(-\gamma^{Na} \cdot H \cdot t). \tag{6}$$

Table. Ionic composition (mM/liter) of solutions

	Na^+	K^+	Ca^{++}	Cl^-	Buffer[a]	pH[b]	TEA[c]
Ringer's	110	—	1.8	121.8	5	7.3	10
Internal solution	14	105	—	119	5	6.9	—

[a] Tris(hydroxymethyl)aminomethane.
[b] At 15°C.
[c] TEA = tetraethylammoniumchloride for elimination of K^+ currents

ΔV was easily determined from changes of $h_\infty(V = 0)$. The rate constant γ^{Na} describes solely the portion of the reduction of I_{Na} due to ultraviolet-induced decrease of $\bar{P}_{Na}$. (The underlying hypothesis for an explanation of the ultraviolet-induced potential shift of $h_\infty(V)$ is that the membrane surface charges are altered by irradiation leading to an according change of field strength across the nodal membrane (Schwarz & Fox, 1977). This hypothesis is strongly supported by the fact that ultraviolet radiation displaces $m_\infty(V)$ along the potential axis by almost the same amount as $h_\infty(V)$ (Fox, 1976*b*). Therefore it seems appropriate to assume that the same potential displacement also applies to $u_\infty(V)$, particularly since not one indication was ever observed that $u_\infty(V)$ might directly be affected by ultraviolet radiation.)

Series Resistance

No correction for possible effects of uncompensated series resistance R_s was necessary, since it was shown that (under the present measuring conditions) a shift of $h_\infty(V)$ due to the reduction of $\bar{P}_{Na}$ did not exceed ±1 mV (Schwarz & Fox, 1977). Apart from that, *l*-cysteine does not affect the conductivity of the solution and therefore R_s could not account for the different amounts of radiation-induced shift under standard and −SH-containing solutions.

Solutions, Application of SH Compounds, Temperature and Notations

The solutions are given in the Table. Ten mM *l*-cysteine, 20 mM 2-mercaptoethanol or 10 mM N-ethylmaleimide (NEM) were added to the internal solution (pH 6.9) for application by diffusion along the interior of the axon, or to the Ringer's solution for external superfusion. No corrections were made for the small changes in osmolarity in either case.

Temperature was between 14 and 15°C. The sodium currents were calibrated as current densities (*see* Fox, 1974). Potentials are given as inside minus outside potential relative to resting potential, thus $V = E - E_r$.

Results

The results of a typical experiment are shown in Fig. 1. The peak sodium inward current, I^p_{Na}, and

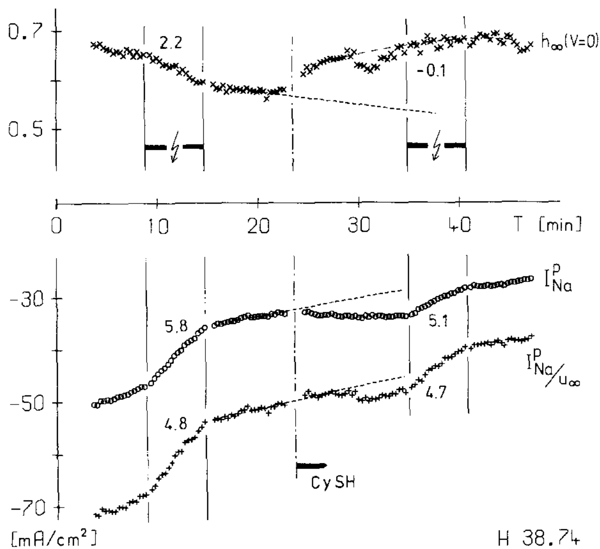

Fig. 1. Effect of *l*-cysteine (administered internally by diffusion) on the ultraviolet-induced potential shift and on the ultraviolet blocking of sodium channels. The potential shift was monitored by the change of the steady-state sodium inactivation measured at zero potential, $h_\infty(V = 0)$; the blocking of sodium channels was determined by the decrease of the peak sodium current I^p_{Na} corrected for changes of ultra-slow sodium inactivation u_∞ due to potential displacements; both plotted versus time. Periods of irradiation are indicated by horizontal bars (with arrow); period of cysteine action starting at indicated time (CySH). Broken lines = time course expected in the absence of CySH. The radiation effect is determined by the first-order rate constants of radiation-induced changes of $h_\infty(V = 0)$, of I^p_{Na} and of $\overline{P}_{Na}$ ($\sim I^p_{Na}/u_\infty$). The numbers represent the rate constants times the irradiation dose rate of 0.6 mW/cm² given in 10^{-4} sec^{-1}. Wavelength 280 nm. Motor fiber. Temperature 15°C

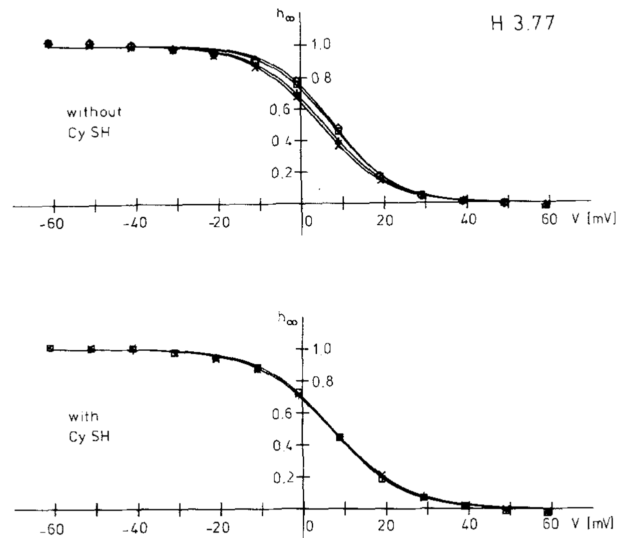

Fig. 2. Effect of ultraviolet irradiation on the steady-state sodium inactivation curve, $h_\infty(V)$, and the influence of cysteine (internal application). Design of the experiment as in Fig. 1. Upper part: ◇ = 200 sec before, □ = 30 sec before, + = 30 sec after, × = 110 sec after an irradiation of 70 sec at 280 nm. Least-squares fit: $V_{1/2}$ = 8.0, 7.5, 5.6 and 4.7, k = 7.4, 7.7, 8.1 and 8.2, respectively. Lower part: □ = 50 sec before, + = 30 sec after, × = 140 sec after irradiation of 70 sec with cysteine present. Least-squares fit: $V_{1/2}$ = 7.0, 6.8 and 7.0, k = 8.4, 8.9 and 9.0

the value of the steady-state sodium inactivation at resting potential, $h_\infty(V = 0)$, were plotted versus time. In standard solutions (without SH compounds) both I^p_{Na} and $h_\infty(V = 0)$ showed a spontaneous decline (run-down) which was taken to be exponential as described quantitatively before (Fox, 1974; 1976*a*). During irradiation an ultraviolet-induced exponential decrease with dose of I^p_{Na} and of $h_\infty(V = 0)$ was observed as known from previous work (Fox, 1974; Schwarz & Fox, 1977; Hof & Fox, 1983).

After internal application of 10 mM *l*-cysteine the peak sodium current increases slowly, but not beyond the value measured after the first irradiation period, while $h_\infty(V = 0)$ was finally increased to its initial value at the beginning of the experiment. (This restoration of the initial values of $h_\infty(V = 0)$ or I^p_{Na} was not complete in all cases.) During irradiation there was again an ultraviolet-induced exponential decline of I^p_{Na}, however, there was no effect on $h_\infty(V = 0)$.

The rate constant of radiation-induced current decrease, γ^{Na}, seems to be somewhat lower under *l*-cysteine than in standard internal solution. This is due to the fact that under cysteine no ultraviolet-induced potential displacement occurs as under normal conditions. Actually the rate constants γ^{Na} under standard and cysteine-containing solutions corrected for changes of ultra-slow inactivation according to Eq. (6) are equal. The ultraviolet-induced blocking of I^p_{Na}/u_∞, i.e. of $\overline{P}_{Na}$, was not affected by cysteine.

Figure 2 demonstrates that the radiation-induced decline of $h_\infty(V = 0)$ was due to a shift of $h_\infty(V)$ in the negative direction. After internal application of cysteine $h_\infty(V)$ returned approximately to the original position before the first irradiation. No further shift from there was measured after ultraviolet irradiation during the presence of cysteine, and neither a slow spontaneous shift occurred as would have been the case with standard internal solution. As a measure of the influence of SH compounds on the radiation effect the variable Γ is introduced:

$$\Gamma_{Na} = \frac{\gamma^{Na} - \gamma^{Na}_{SH}}{\gamma^{Na}}; \qquad \Gamma_h = \frac{\gamma^h - \gamma^h_{SH}}{\gamma^h}. \qquad (7)$$

γ^{Na} and γ^h = rate constants of radiation-induced exponential decrease of the sodium permeability (corrected for changes of u_∞) and of $h_\infty(V = 0)$,

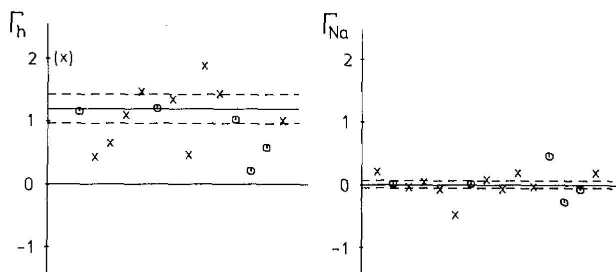

Fig. 3. Effect of the sulfhydryl compounds *l*-cysteine (CySH) and 2-mercaptoethanol (EtSH) on the ultraviolet-induced potential shift and blocking of sodium channels. Scatter plots of the variables Γ_h and Γ_{Na} as determined from 15 experiments (*see* Eq. 4). × = EtSH (20 mM), ○ = CySH (10 mM), both internally applied. The mean values ± SEM are: $\Gamma_h = 1.19 \pm 0.23$ (complete protection) and $\Gamma_{Na} = 0.01 \pm 0.06$ (no effect). (×) = value out of scale: $\Gamma_h = 4$

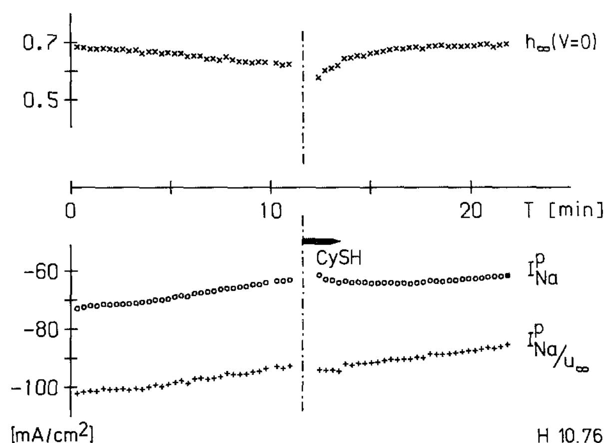

Fig. 4. Effect of internally applied *l*-cysteine (10 mM) on the slow spontaneous decline of $h_\infty(V=0)$, I^p_{Na} and I^p_{Na}/u_∞ (run-down): Prevention and restoration of initial values of $h_\infty(V=0)$ and I^p_{Na}, no effect on I^p_{Na}/u_∞. Notations as in Fig. 1

respectively, without (γ^x) or with (γ^x_{SH}) the SH compound present. Figure 3 shows scatter plots of Γ_h and Γ_{Na} determined from experiments on 16 different nodes of Ranvier (motor fibers) treated either with *l*-cysteine or 2-mercaptoethanol.

The mean (solid line) ± SEM (broken line) are also presented. The mean of Γ_h is nearly 1, the mean of Γ_{Na} is nearly zero, i.e. on the average complete protection of $h_\infty(V)$ by the sulfhydryl compounds *l*-cysteine and 2-mercaptoethanol but no effect on $\overline{P}_{Na}$. Within the limits of error no difference of the action of the two agents was observed.

It was shown in Figs. 1 and 2 that *l*-cysteine seems to restore the initial values of $h_\infty(V=0)$ and the values of I^p_{Na} measured immediately after the first irradiation, i.e. it prevents the run-down phenomena (spontaneous decline of sodium current combined with an increase of sodium inactivation), at least partly. This effect was studied in separate experiments without applying ultraviolet radiation. As shown in Fig. 4 prevention of spontaneous slow decrease (run-down) of $h_\infty(V=0)$ and partly of I^p_{Na} and—in other experiments—even restoration of initial values (measured at the beginning of the experiment) was observed in Ranvier nodes not pretreated with ultraviolet radiation.

The agent NEM (binding specifically and irreversibly to $-$SH groups), internally applied, should exert an effect similar to the ultraviolet-induced potential shift, if the radio-protective effect of cysteine and mercaptoethanol is related to restitution of $-$SH groups in the nodal membrane. This, in fact, was observed (Fig. 5): NEM reduced $h_\infty(V=0)$, but did not affect I^p_{Na}/u_∞, as expected from the results of Fig. 1. The binding of NEM to protein $-$SH is irreversible (Riordan & Vallee, 1972); accordingly, the effect of NEM could not be reversed by cysteine.

External application of SH compounds and SH reagents led to rather dramatic unspecific changes of membrane resistance as already reported from our laboratory (Keana & Stämpfli, 1974). Similar experiments were carried out though, to determine the interaction with ultraviolet radiation. No reproducible results could be obtained; however, the observations indicated that specific effects may only be obtained by using internal application of the chemicals. Significant changes of membrane resistance were never observed after internal application of cysteine, mercaptoethanol, or NEM. (Apparently no effective release of these agents occurred from the inner to the outer side of the membrane.)

Discussion

The results clearly indicate that the radiation-induced potential shift and the blocking of sodium channels are two separate photochemical processes, one being protected by *l*-cysteine and 2-mercaptoethanol, the other not (Figs. 1 and 2). Simultaneously, the spontaneous slow decline of I^p_{Na} (run-down) is prevented and at least partly reversed by the action of the SH compounds (Figs. 1, 2 and 4). This is in line with a previous observation that internal perfusion of squid giant axons with cysteine (up to 10 mM) increased the survival time of the axons (Huneeus-Cox, Fernandez & Smith, 1966). The reversed potential shift of $h_\infty(V=0)$ suggests that the type of radioprotection is restitutive rather than competitive.

The opposite effect was exerted by NEM: A reduction of $h_\infty(V=0)$, being equivalent to a shift of

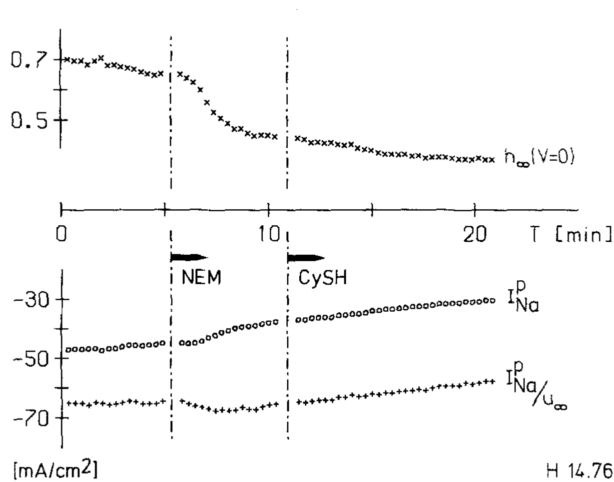

Fig. 5. Effect of internally applied *N*-ethylmaleimide (NEM, 10 mM) on $h_\infty(V = 0)$, I^p_{Na} and I^p_{Na}/u_∞: Reduction of $h_\infty(V = 0)$ without an effect on the maximum sodium permeability (I^p_{Na}/u_∞). Notations as in Fig. 1

$h_\infty(V)$ in the negative direction of the potential axis, but no change of the maximum sodium permeability. Very similar results were reported by Shrager (1975) for the crayfish axon: NEM, externally applied, reduced the sodium current slowly but progressively and with little changes in kinetics. The sodium currents could almost completely be restored by using 350-msec hyperpolarizing conditioning prepulses suggesting a negative potential shift of the sodium inactivation parameters by the action of NEM. The need of very long prepulses might now be explained by the fact that a potential shift affects the ultra-slow sodium inactivation (Fox, 1976*a*) as well (*see also* Eqs. 3-5). The reaction rate of externally applied NEM in Shrager's experiments was slow (30 min) despite the rapid binding of NEM to protein −SH (Riordan & Vallee, 1972), probably due to a rather slow penetration of externally applied NEM through the nerve membrane. In our experiments (internal application) the reaction was complete after a few minutes according to the diffusion time along the axon. Inexcitability produced by reagents which bind to or oxidize sulfhydryl groups was reported to occur in the squid giant axon (Huneeus-Cox et al., 1966), in the sciatic nerve of the frog (Marquis & Mautner, 1974*a,b*), and in the lobster axon (Smith, 1958). The reaction rate of the agents, and hence their potency to block excitability, was enhanced by frequent electrical stimulation (Marquis & Mautner, 1974*a,b*). The block of excitability by internally applied SH reagents can be explained by the higher degree of sodium inactivation (slow and ultra-slow) due to the potential shift. After external application it might also be due to a dramatic decrease of membrane resistance as was observed in giant neurones of *Aplysia* (Berry & Weisblat, 1975) and in the node of Ranvier (Keana & Stämpfli, 1974).

It was suggested (Schwarz & Fox, 1977) that the ultraviolet-induced potential shift might be due to a photocatalyzed decrease of the surface charge density at the inner side of the nodal membrane, since the voltage shift of all gating parameters occurs despite a constant membrane holding potential. The spontaneous exponential decline of $h_\infty(V = 0)$ could, correspondingly, be explained by a similar slow-rate process. If the underlying chemical reaction, in fact, is the same either spontaneous or photocatalyzed, sulfhydryl compounds should prevent any further exponential decline of $h_\infty(V = 0)$ and restore it to its initial value as it was observed. I^p_{Na}, which is also spontaneously decreasing due to the diminution of $u_\infty(V_{1/2} + \Delta V)$ (*see* Eqs. 4-6), should be increased by the restitutive reverse potential shift, but the current loss by ultraviolet blocking of P_{Na} should never be restored, as it in fact was found. Sulfhydryl reagents such as NEM (at moderate concentrations) do not significantly alter the membrane potential when blocking excitability (Berry & Weisblat, 1975; Huneeus-Cox et al., 1966; Marquis & Mautner, 1974*a*). *l*-cysteine or 2-mercaptoethanol, on the other hand, also do not cause a change of the membrane potential when perfused through a squid giant axon (Huneeus-Cox et al., 1966). These observations are in line with our results. The spontaneous and the ultraviolet-induced potential shifts occur despite a constant voltage between extra- and intracellular bulk solution. *l*-cysteine and 2-mercaptoethanol reverse them. Hence it may be concluded that a spontaneous or a photocatalyzed oxidation of −SH groups leads to a decrease of the density of the internal surface charges. This interpretation is supported by the effect of NEM (internal application) reacting highly specifically with protein −SH groups and producing a potential shift similar to the one induced by ultraviolet radiation.

Because of their high pK_a (*ca.* 8.5) it is unlikely that −SH groups themselves are these surface charges. One explanation of the described effects could arise from the assumption that the density of charges exposed to the inner side of the nodal membrane depends on the conformation of proteins. Another interpretation is suggested by the investigation of Polgar (1974) on papain: Due to the interaction with a neighboring base (the imidazole group of histidine) the thiol group of cysteine has a pK_a of about 4. Thus thiol groups of membrane proteins could exhibit a similar low pK_a value as it was found at the outer surface in our laboratory (Drouin & Neumcke, 1974).

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