

Simulation of Na Channel Inactivation by Thiazin Dyes

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ABSTRACT Some dyes of the methylene blue family serve as artificial inactivators of the sodium channels when present inside squid axons at a concentration of ~ 0.1 mM. The dyes restore a semblance of inactivation after normal inactivation has been destroyed by pronase. In fibers that inactivate normally, the dyes hasten the decay of sodium current. Many dye-blocked channels conduct transiently on exit of the dye molecule after repolarization to the holding potential. In contrast, normally inactivated channels do not conduct during recovery from inactivation. Kinetic evidence shows that inactivation of a dye-blocked channel is unlikely or impossible, which suggests that dye molecules compete with inactivation "particles" for the same site. In the absence of tetrodotoxin, the dyes do not affect the ON gating current unless the interpulse interval is very short. If sufficient equilibration time is allowed during a pulse, the initial amplitude of the OFF gating current is reduced to near zero. This suggests that a dye molecule in a Na channel completely blocks that channel's gating current, even the fraction that is resistant to normal inactivation. Dyes block I_{Na} and I_g with the same time course. This provides the strongest evidence to date that virtually all of recorded "gating current" is associated with Na channels. Tetrodotoxin greatly slows dissociation of dye molecules from Na channels and reduces gating current during both opening and closing of the channels.

INTRODUCTION

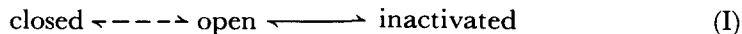
In normal function the sodium channels in axon membrane activate (open) after depolarization and then quickly inactivate, so that the sodium permeability is increased very briefly. Activation and inactivation result from the functioning of two separate gates, and the latter can be destroyed by internal perfusion with pronase (Armstrong et al., 1973; Rojas and Rudy, 1976) or *N*-bromoacetamide (Oxford et al., 1978) without affecting activation. A number of substances have been found that restore a semblance of inactivation after it has been removed by pronase. These substances are quite varied in structure and include tetraethylammonium derivatives (Rojas and Rudy, 1976), *N*-

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methyl strychnine (Shapiro, 1977; Cahalan and Almers, 1979), local anesthetics (Cahalan, 1978; Cahalan et al., 1980), pancuronium (Yeh and Narahashi, 1977), and *n*-alkylguanidines (Kirsch et al., 1980). Some of these have been shown clearly to compete with the normal inactivation mechanism, i.e., when blocked by pancuronium, for example, a channel cannot be inactivated: block and inactivation are mutually exclusive. In contrast, it has been postulated that some local anesthetics (Courtney, 1975; Hille, 1977) and other substances (Cahalan et al., 1980) enhance inactivation caused by the normal mechanism.

Recent additions to the list of inactivation simulators are the common dye methylene blue and its relatives azure A and thionin (Croop and Armstrong, 1979). These dyes initially interested us because they serve as photo-oxidizing agents capable of modifying proteins on exposure to light (Pooler, 1972; Oxford et al., 1977). Our results suggest that the photodynamic damage suffered by Na channels in the presence of methylene blue is preceded by a specific interaction of dye and channel: the dye may have to enter the channel as a prelude to photodynamic damage.

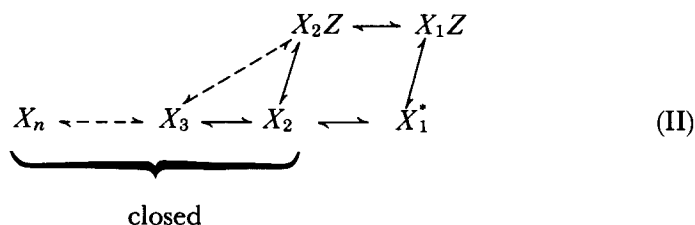
Artificial inactivators are of interest because they may help in understanding the normal mechanism of inactivation. In this regard it is useful to review current knowledge about inactivation and its relation to activation. Hodgkin and Huxley (1952) initially suggested, in part because it simplified computation, that activation and inactivation are completely independent of each other. Kinetically, however, it is equally reasonable that the two processes interact (are coupled), and that at least partial activation must occur before inactivation is possible (Bezanilla and Armstrong, 1977). Specific evidence favoring a coupled hypothesis is (a) after depolarization, inactivation begins after a lag, consistent with the idea that partial or full activation must precede inactivation. (b) If the inactivation gate is independent of activation, it must have a voltage-sensing mechanism that would necessarily generate a measurable gating current as the gate opens or closes. No such gating current has been observed. If, on the other hand, the two gates interact, inactivation can derive its apparent voltage dependence from coupling to the activation gate: activation, which is strongly voltage dependent, must proceed to some degree before a channel can inactivate. This obviates the need for inactivation gating current and is thus consistent with the experimental observations. (c) Gating charge is immobilized by inactivation of Na channels, which is consistent with the idea that inactivation prevents the activation gating structure from returning to its resting configuration. Immobilization of gating charge has the time course of inactivation, and no longer occurs after inactivation has been removed by pronase. These observations initially suggested that activation and inactivation might be related by the following scheme:



in which the closed-to-open transition is a multistep process that is highly voltage dependent, and the open-inactivated transition is not significantly voltage dependent. With such a scheme, both the kinetics of inactivation and

the degree of steady state inactivation appear to be voltage dependent, because activation and inactivation are coupled (Bezanilla and Armstrong, 1977).

This scheme had to be modified because it predicts that during recovery from inactivation, there should be a measurable Na current as channels reflux through the open state in transit from inactivated to closed. Such a current has not been seen, and firm evidence on this point is presented in this paper. Channels recovering from inactivation thus bypass the open state, suggesting a scheme of the following type:



where X_1^* is the open state, and X_1Z and X_2Z are two inactivated states. During recovery from inactivation, channels pass from X_1Z to X_2Z to X_2 and to the left, bypassing the conducting state (Armstrong and Gilly, 1979). The inactivation-resistant component of gating current is associated with the X_1Z -to- X_2Z transition.

A final modification of the scheme is useful in accounting for the voltage dependence of the kinetics of recovery from inactivation. This modification is indicated by the dashed arrow that joins X_2Z to X_3 . This transition, which is the rate-limiting step in recovery from inactivation, would involve gating charge movement and thus be voltage sensitive. A necessary consequence of this scheme is that channels can inactivate after partial activation (from states X_3 and X_2) and need not fully open. This is consistent with recent evidence presented by Bean (1981).

We report here an analysis of the "inactivation" caused by the methylene blue family of dyes. Among the more interesting findings are (a) the dyes immobilize virtually all gating charge movement when the channels are closing, without (in the absence of tetrodotoxin [TTX]) altering gating charge movement as the channels open; (b) a significant fraction of the channels are blocked by the dyes before they open fully.

METHODS

Experiments were performed on segments of squid giant axons from *Loligo pealei*, obtained at the Marine Biological Laboratory, Woods Hole, MA. The axons were perfused and voltage clamped by standard methods (Bezanilla and Armstrong, 1977), and membrane currents were recorded on magnetic disks for later analysis. The solutions used are listed in Table I together with their compositions.

Azure A was obtained from Matheson Coleman and Bell, Norwood, OH, thionin from Aldrich Chemical Co., Arlington Heights, IL. Because heavy-metal contamination of the dyes is likely, some experiments were performed with 2 mM EDTA added to the dye solution. Results were indistinguishable from those recorded without EDTA.

Dye experiments were performed in subdued light to prevent photodynamic damage. In general, reversibility of the dye effects was excellent, with currents usually returning after washing a dye away to within 5 or 10% of their value before the dye.

Theoretical computations were performed by describing the appropriate kinetic scheme with simultaneous first-order linear differential equations, which were solved by numerical integration with the aid of a computer.

RESULTS

Thiazin Dyes Simulate Na Inactivation

Fig. 1 illustrates the effects of azure A on gating and sodium currents, for depolarizations to the membrane potentials indicated. The control traces are

TABLE I
SOLUTIONS

External solutions	Trizma 7.0*		NaCl	CaCl ₂	
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
ASW	10		450		50
1/4 Na SW	360		113		50
Tris SW + TTX‡	480		—		50
10 Ca Tris SW	540		—		10
Internal solutions§	K	TMA	F	Glutamate	Sucrose
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
275 KFG	275	—	50	225	420
200 TMA	—	200	50	150	560

* Tris(hydroxymethyl)aminomethane (Sigma Chemical Co., St. Louis, MO): pre-mixed crystals of Tris and Tris hydrochloride.

‡ Tetrodotoxin, 200 nM.

§ Internal solutions were buffered with 10 mM Trizma 7.0.

|| Tetramethylammonium ion.

the average of records taken before applying the dye and after washing it away. They show an initial outward current, the gating current associated with channel opening (I_g , ON), followed by an inward sodium current (I_{Na}) that increases in amplitude (activates) and then decays (inactivates). With increased voltage during the pulse, I_g ON becomes larger, and activation of Na channels, as measured from the I_{Na} time course, is faster. At -40 mV, no inactivation is apparent by the time the trace ends. Sustained I_{Na} at this voltage is predicted by a coupled model of inactivation (Bezanilla and Armstrong, 1977).

Azure A reduces I_{Na} at -40 mV without much altering its time course. At higher voltages (0, 40, and 60 mV), peak amplitude is reduced and the decay of the current is speeded. This could be interpreted as a speeding of the normal inactivation mechanism, but the experiments below show that instead the dyes simulate inactivation and compete with the normal mechanism (cf. Shapiro, 1977; Yeh and Narahashi, 1977; Cahalan and Almers, 1979).

The effects of the dye were quite reversible provided the fiber was shielded from light. In Fig. 1, current after the dye was even slightly larger than before.

I_g ON is not detectably affected by the dyes, although, as shown below, it is altered in the presence of TTX, and I_g OFF (gating current associated with channel closing) is severely reduced with or without TTX.

All the dyes of this family that we tested (methylene blue, azure A, thionin) were similar in their effect on membrane current: we can make no distinctions without further study and greater certainty regarding purity.

Fig. 2 shows that azure A restores a semblance of inactivation after the normal mechanism has been destroyed by internal perfusion with pronase. The top frames are I_g plus I_{Na} during pulses to 0 and 60 mV, together with the large current tails generated by a step back to -80 mV after the intervals

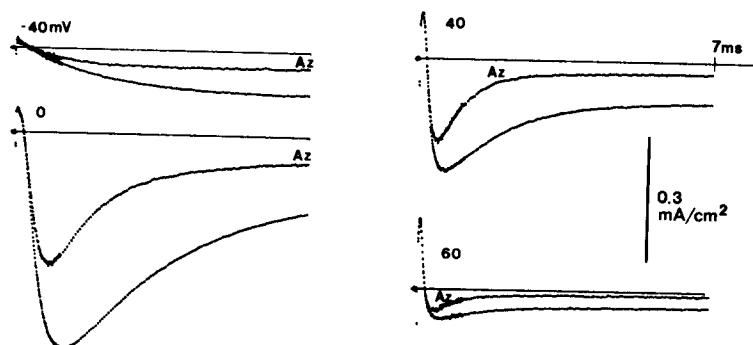


FIGURE 1. Azure A appears to speed inactivation. The control traces are the averages of currents recorded before perfusion with 0.25 mM azure A and after washing it away. The dye does not affect the transient outward current (gating current), which is most visible at 60 mV. Experiment JL179B, HP -80 mV, 8°C . $1/4$ Na ASW//200 TMA + 0.25 mM Azure A.

indicated. At 60 mV there is a rapid phase of activation, followed by a much slower one (cf. Matteson and Armstrong, 1982), which makes the tail after 15 ms of depolarization larger than the one after 5 ms. When azure A is added to the internal perfusion medium, I_{Na} is reduced and appears to “inactivate” as dye molecules enter the open channels. The initial amplitude of the tails falls in proportion to the current during the pulse. At 0 mV the decay of the current is not very complete, and there remains a substantial steady state current. The tails after this pulse have a fast component of decay, as the channels that are not blocked by the dye close normally. After longer pulses the fast decay is followed by a slow phase. As described later, the slow phase is generated by channels that lose their dye molecules on repolarization and conduct transiently before closing.

I_{Na} decays almost completely during the pulse to 60 mV. As the duration of this pulse increases, the tails acquire a rounded time course and a slow decay phase. That is, current amplitude increases for a time after the step back as

azure A comes out of blocked channels, and the tail current is prolonged for the reasons just described.

As in Fig. 1, I_g ON is not detectably affected by the dye. From the low initial amplitude of the current repolarization, one can deduce that the OFF gating current is much reduced, as will become clearer below.

Fig. 2 thus shows that azure A simulates inactivation, rather than speeding it, for its action is the same after inactivation has been destroyed by pronase. It is also clear that, at least after pronase treatment, dye-blocked channels conduct transiently as they recover from block, giving rise to slow, rounded

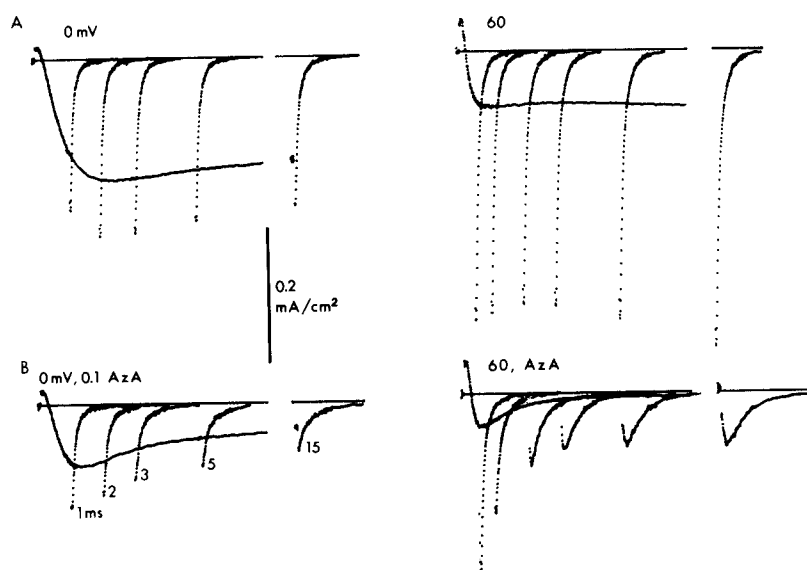


FIGURE 2. Azure A restores a semblance of inactivation to a pronase-treated axon. A. The axon was treated internally with pronase until most inactivation was removed, as evident from the large steady current and the large initial amplitude of the inward tails on repolarization. B. When azure A (0.1 mM) is added to the internal medium, the current appears to "inactivate" as channels become azure A blocked, and tail amplitude decreases with pulse duration. Experiment JL209B, HP -80 mV, 8°C . $1/4$ Na SW//200 TMA + 0.1 mM azure A.

tails. The next section shows that normally inactivated channels do not conduct during recovery.

Na Channels Do Not Conduct during Recovery from Inactivation

Before examining the relation between dye block and inactivation, it is worthwhile to document a point that has been asserted but not rigorously proved in the literature, that Na channels do not conduct during inactivation recovery. The simple foot-in-the-door model (scheme I in the Introduction) predicts that channels open transiently on repolarization. That is, if V_m is returned to the holding level when the channels are inactivated, there will be

a measurable conductance as channels reflux from inactivated through the open state and then close. The magnitude of the conductance and current can be predicted from a knowledge of the rate of recovery from inactivation and the closing rate of the activation gates.

Fig. 3 shows the result of a search for this component. Part A depicts the decay of I_{Na} at -80 mV after activating pulses to 0 mV for either 1 or 15 ms. At the end of the 1-ms pulse there was little inactivation, whereas after the 15-ms pulse it was nearly complete. If recovery from inactivation involved reflux

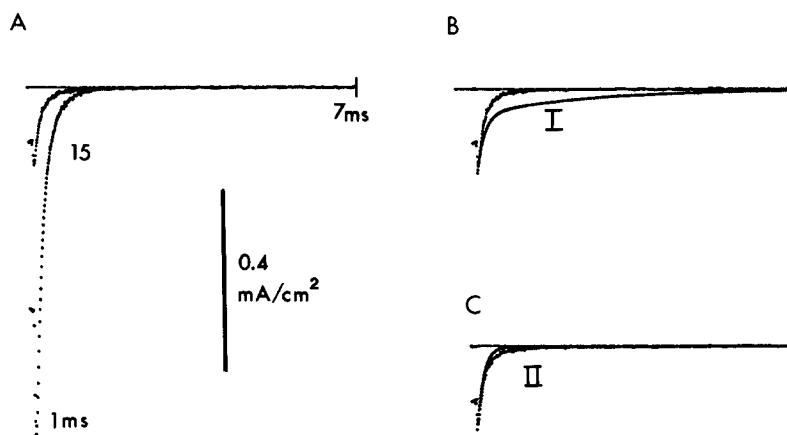
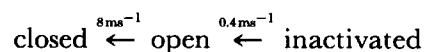


FIGURE 3. Sodium channels do not conduct during recovery from inactivation. A. I_{Na} tails recorded at -80 mV after a 1- or 15-ms pulse to 0 mV. The traces never cross. B. The 5-ms tail from part A of the figure, together with the prediction of scheme I, for which the recovery path includes the conducting state. The prediction has a prominent slow component that is missing from the experimental trace. Rate constants are given in the text. The calculation assumes that 19% of the channels are in the conducting state (X^*) and the remainder inactivated at the beginning of the tail. C. The 15-ms tail and the prediction of scheme II, for which the recovery path bypasses the conducting state. The fit is not perfect, but much better than for scheme I. Rate constants are in the text. JL179B, HP -80 , 8°C . $1/4$ Na SW//200 TMA.

through the open state, the 15-ms tails should have a visible slow component, but none can be seen in the traces: the 15- and 1-ms traces never cross.

In part B of the figure, the current after the 15-ms pulse is compared with a theoretical trace calculated from the simple model (scheme I in the Introduction) with the following rate constants:



The rate constants for the open \rightarrow closed transition were determined from the decay of I_{Na} tails after the 1-ms pulse. For the inactivated \rightarrow open step, the rate constants were approximated by following the recovery from inactivation using a two-pulse procedure (see below). The theoretical curve in Fig. 3B has

a slow component that is missing from the experimental records. From parts A and B of the figure one can conclude that Na channels do not conduct significantly during recovery from inactivation.

Part C of Fig. 3 compares the experimental traces with curves calculated using scheme II from the Introduction. In this model the recovery path bypasses the conducting state, X_1^* . The predictions are in good agreement with the experimental traces and support the idea that the recovery path bypasses the open state. Scheme II embodies this idea and is sufficient for the present data, but is certainly not the only possibility.

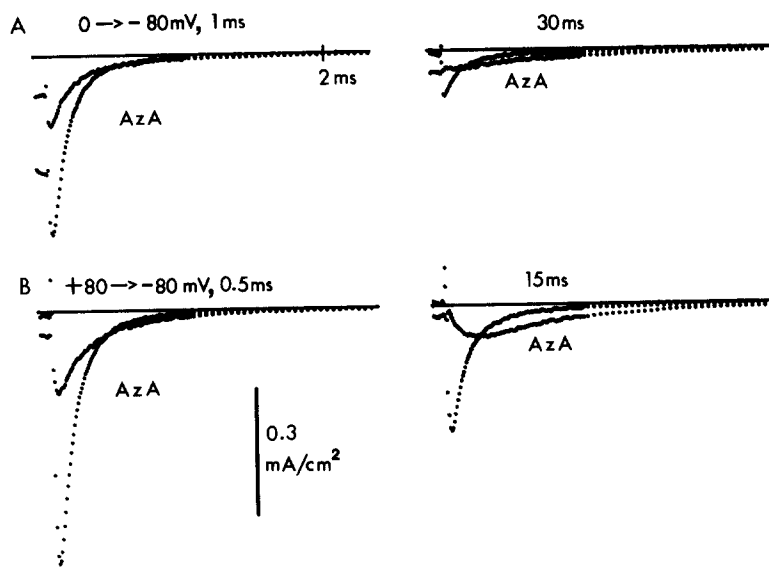


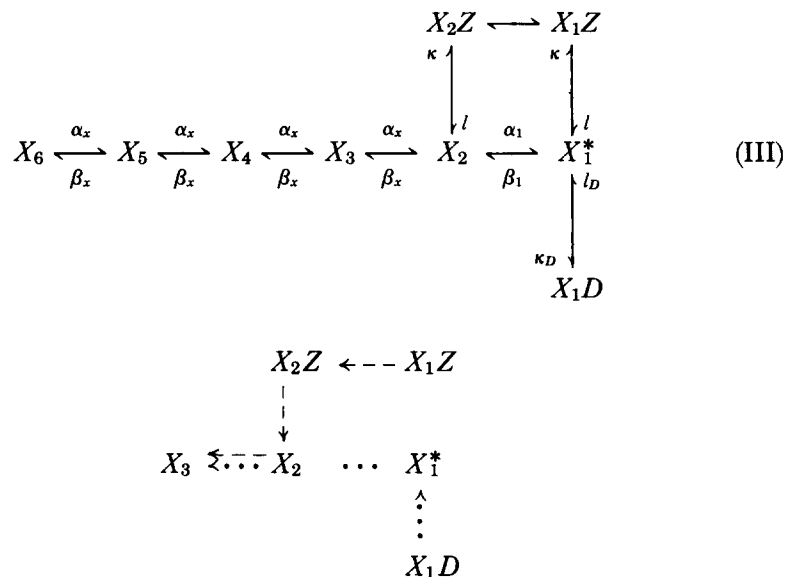
FIGURE 4. Sodium channels conduct during recovery from azure A block. Tails recorded at -80 mV with and without azure A (0.25 mM) after 1- and 30-ms pulses to 0 mV (A) or $+80$ mV (B). After the longer step in azure A, the initial tail current is almost zero, and increases in magnitude for ~ 200 μ s as azure A molecules come out of the channels. The traces cross, which proves that at least some of the azure A-blocked channels are not inactivated: if they were, they would not conduct during recovery. AU219C, HP -80 mV, 8° C. $1/4$ Na SW//200 TMA.

Dye Block and Inactivation Are Competitive

In contrast to channels inactivated by the normal mechanism, dye-blocked channels in a pronase-treated axon were shown above to conduct during recovery from block. The same is true for dye-blocked channels even when inactivation is intact, as shown in Fig. 4. Part A illustrates tails after 1- and 30-ms pulses to 0 mV in the presence and absence of 0.25 mM azure A internally. With no dye there is a small inward current at the end of the activating pulse, generated by the 10–20% of the channels that have not inactivated. The current jumps momentarily when the driving force is in-

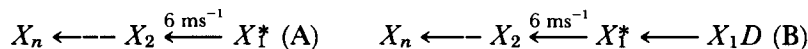
creased on stepping back to -80 mV, and current then decays quickly to negligible amplitude after 1 ms. In the presence of azure A the current both before and after the step back to -80 mV is near zero: essentially all of the channels are either dye blocked or inactivated. Current increases as azure A comes out of the channels and then decays slowly. 1 ms after the step there is still appreciable current. The same general description holds for tails recorded after an activating pulse to $+80$ mV, as shown in part B of Fig. 4. In particular, there is substantial current flowing 1 ms after the step back to -80 mV, at a time when there is no current in the absence of dye.

The current seen 1 ms after stepping to -80 mV shows that dye-blocked channels do not inactivate normally. If they did, there would be no current, as in the control traces. A state diagram that is compatible with the evidence is the following:



The diagram is identical to scheme II except for state X_1D , which is the dye-blocked state. The dominant recovery paths for inactivated channels and for dye-blocked channels are given respectively by the dashed and dotted lines below: the dye-blocked channels reflux through the open state, whereas the inactivated channels bypass it.

It is difficult to be sure whether all of the dye-blocked channels reflux through the open state, because it is not certain how the channels are distributed between X_1Z and X_1D at the end of the pulses in Fig. 4. One can get an estimate of the fraction of all channels that pass through X_1^* by integrating the area under the current tails, with the following rationale. The time integral or charge in the ionic current tails is the same for the following state diagrams:



This is true also for gating charge, but only charge carried by ions is considered in the following. For A, the tail is exponential when all channels start in X_1^* and migrate to X_n . The area under the tail is the product of $X_1^*(t = 0)$ and the time constant is $\frac{1}{6}$ ms. For B, starting with all channels in X_1D , the tail has an initial amplitude of zero and a rounded time course, like the dye trace in Fig. 4. The area under the tail is nonetheless exactly the same as for A, provided the X_1^* -to- X_2 transition has the same rate constant in the two cases.

Table II compares the charge carried by I_{Na} tails in a number of different conditions, in an attempt to quantitate the fraction of the channels that reflux

TABLE II
CHARGE CARRIED BY I_{Na} TAILS WITH AND WITHOUT AZURE A

Experiment	V_{pulse}	Duration	Dye	I_{Na}	Q_{obs}	Q_{exp}	Q_{excess}
	<i>mV</i>	<i>ms</i>	<i>mM</i>	$\mu A/cm^2$	$e/\mu m^2$	$e/\mu m^2$	$e/\mu m^2$
JL179B	0	1	0	772	12,490	12,490	0
		10	0	215	3,184	3,478	-294
		10	0.25	68	4,208	1,100	3,108
	80	0.5	0	866	13,354	13,354	0
		10	0	469	7,116	7,232	-116
		10	0.25	53	7,033	817	6,216
AU219C	0	1	0	379	5,137	5,137	0
		15	0	74	727	1,003	-276
		15	0.25	29	3,313	393	2,920
	80	0.5	0	533	6,908	6,908	6,908
		15	0	268	3,169	3,348	-179
		15	0.25	16	5,369	207	5,161
JL209B (after pronase)	0	2	0	285	3,203	3,203	0
		15	0	230	2,335	2,584	-249
		15	0.1	32	2,268	360	1,908
		0.5	0	379	5,315	5,315	0
		15	0	405	7,186	5,680	1,506
		15	0.1	14	4,536	191	4,345

Experiments were performed in 1/4 Na SW//200 TMA.

I_{Na} : initial amplitude of the current tail after step back to -80 mV, after a step to V_{pulse} for the specified duration.

Q_{obs} : integrated ionic charge movement in the tail. The gating charge has been subtracted out.

Q_{exp} : charge movement expected if the tail decayed with the same kinetics as a short (0.5- or 1-ms) pulse.

$Q_{excess} = Q_{obs} - Q_{exp}$.

through the open state after losing the dye molecule blocking them. Gating charge has been subtracted out. The table lists pulse duration and voltage, I_{Na} amplitude just after the step back to -80 mV, and the charge carried by the tail (Q_{obs}), obtained by integrating for 7 ms after the step back. The "expected" charge is calculated from the product

$$Q_{exp} = [I_{Na}(\text{long pulse})/I_{Na}(\text{short pulse})]Q_{obs}(\text{short pulse}),$$

where I_{Na} is the initial amplitude of the tail in question. That is, the expected charge is that which would be carried by ions through the channels open at the beginning of the step back if they closed with the same kinetics as after a

short pulse. The “excess” charge, Q_{excess} , is the difference between Q_{obs} and Q_{exp} . It is the charge carried through channels that reflux through the open state after the exit of a dye molecule. For a long pulse (no dye), the observed charge is almost completely accounted for by the decay of the channels open at the end of the activating pulse, and the “excess” charge is almost zero. This is another reflection of the point made above, that channels do not conduct during recovery from inactivation.

When azure A is present in the axon, the charge carried by the tail (Q_{obs}) is much larger than expected from initial I_{Na} amplitude, which is close to zero. Q_{obs} of the largest tails (+80 mV, 0.5 ms, no dye) gives a lower limit of the “maximum” charge, that which would flow in a tail that began with all Na channels open. Using this as a reference, the excess charge after the long pulse to 0 mV is 23–42% of the maximum (experiments JL179B and AU219C), which suggests that somewhat less than half of all the channels reflux through the open state. After a pulse to +80 mV the excess is 47–75% of the maximum. From these numbers it seems that many but possibly not all of the channels are dye blocked after the large step and reflux through the open state when the dye molecules come out. What happens to the rest of the channels? It could be argued that at the end of the activating pulse almost half of the channels are inactivated and the remainder are dye blocked. All of the dye-blocked channels then reflux through the open state, and none of the inactivated channels do. Continuing the argument, after the smaller pulse (0 mV), more of the channels are inactivated and fewer are dye blocked, because the dye competes less effectively for the channels at this voltage. The excess charge is thus smaller.

This hypothesis can be tested by removing inactivation with pronase, after which all of the channels that are not conducting at pulse end must be dye blocked. For a large pulse that activates and dye-blocks all of the channels, the excess charge should be 100%, i.e., all of the Na channels should be dye blocked and should reflux through the open state if the hypothesis is correct. As shown in the table, the excess charge in the one experiment (JL209B) that tested this was still substantially less than the maximum charge, particularly if the maximum charge is taken as $7,186 \text{ e}/\mu\text{m}^2$ (+80 mV, 15 ms). Thus, it is suggested that many of the dye-blocked channels did not reflux through the open state. A discussion of what may happen to the remainder of the channels is given below (Discussion). A final point to note in Fig. 4B is that azure A not only blocks Na tail currents, but also reduces the initial amplitude of $I_{\text{g OFF}}$ almost to zero. Generally, $I_{\text{g OFF}}$ is comparable in magnitude to $I_{\text{g ON}}$ and would be easily visible had it not been severely diminished, thus reducing the initial amplitude of the tail current virtually to zero.

Azure A Comes Out of the Channels Quickly in the Absence of TTX

The time course of the tails in the presence of azure A, described above, gives an indication that the dyes come out of the channels quickly on repolarization to -70 or -80 mV. An accurate measure of the time course can be obtained using a two-pulse procedure, in which the first pulse blocks the channels with

dye and a second pulse measures the fraction of the channels that have recovered in the interval between pulses. The protocol is illustrated in the inset of Fig. 5. The channels were either inactivated or dye blocked at the end of the first pulse, and the membrane potential was then returned to -80 mV. The degree of recovery during the rest interval at -80 mV was determined by measuring the current in the second pulse. The results are plotted semilogarithmically in Figs. 5A and B. The ordinate is the normalized fraction of blocked or inactivated channels, and the abscissa is the recovery interval. In all cases the recovery measurements are reasonably well fitted by a straight

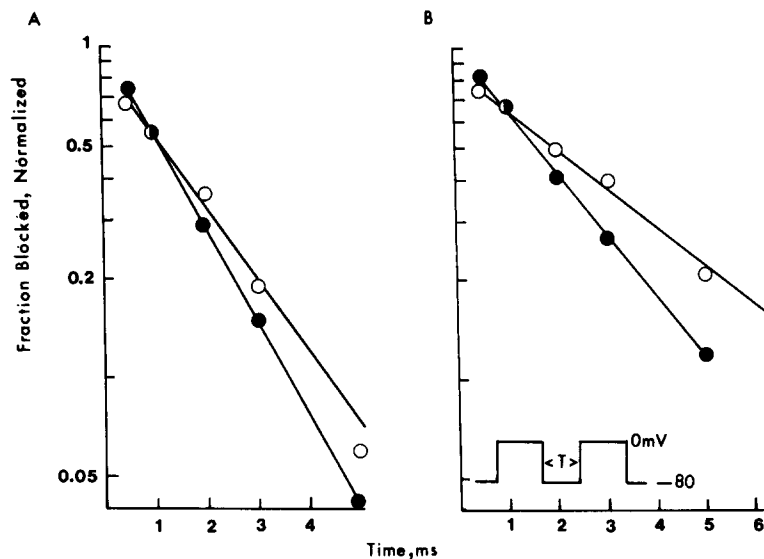


FIGURE 5. Recovery from azure A block is slightly faster than recovery from inactivation. Channels were inactivated or dye blocked by a first pulse, and recovery was monitored by measuring and plotting current in a second pulse applied after a test interval T , which is the abscissa. The ordinate is the normalized fraction blocked. A. JL179A, HP -80 mV, 8°C . $1/4$ Na SW//200 TMA + 0.125 mM azure A. B. JL179B, HP -80 mV, 8°C . $1/4$ Na SW//200 TMA + 0.25 mM azure A.

line. The time constants for recovery from inactivation were 2.1 and 3.7 ms in the two experiments, using different axons. Conditions were identical, and we cannot explain the rather large differences in recovery time constant. Similar variability has been observed previously (Armstrong and Bezanilla, 1977).

In both experiments recovery in the presence of azure A was significantly faster, with time constants of 1.55 and 2.25 ms. It is hard to be sure how many of the channels were azure A blocked and how many were inactivated at the end of the prepulse, but it seems clear that recovery from azure A block is somewhat faster (in the absence of TTX) than recovery from inactivation. As shown below, the opposite seems to be true in the presence of TTX.

Thionin Does Not Affect I_g ON

Azure A does not affect the part of I_g ON that is visible in the presence of low-Na concentrations (see Figs. 1 and 2). The same holds true with the dye thionin even when all Na is removed (no TTX), as illustrated in Fig. 6A. Gating currents are shown after pulses to 0, 40, and 80 mV in the presence and absence of dye. The traces are almost identical and the effects of the dye, if any, must be very small.

If the interval between pulses is so reduced that thionin cannot clear from the channels, I_g ON is reduced as shown in Fig. 7. A two-pulse protocol was used, as diagrammed in the inset. The traces begin at the arrow (inset) and show I_g ON in the second or test pulse. The interval (in milliseconds) between the end of the first, activating pulse, and the test pulse is indicated near each

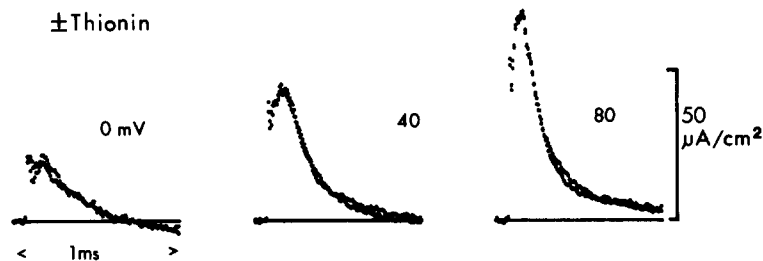


FIGURE 6. After a long rest interval, thionin does not affect I_g ON in the absence of TTX. Gating current traces are shown for three depolarizations in sodium-free medium, with and without thionin (0.5 mM) in the internal medium. Compare Figs. 1 and 2, in which azure A did not noticeably affect I_g ON in sodium-containing medium. The results are quite different in TTX (see Figs. 10 and 11A). AU239B, HP -80 mV, 8°C . Tris SW//200 TMA + 0.5 mM thionin.

trace. I_g ON is quite small if only 0.3 ms is allowed for its dissociation from the channels, and it returns to almost full amplitude after 5 ms.

Thionin Reduces and Slows I_g OFF

Thionin has a profound effect on I_g OFF, as shown by comparing Figs. 8A (control records) and B (thionin). No TTX was present in either case, so there is a possibility that I_g may be somewhat contaminated by ionic current through the Na channels. The OFF/ON ratio for the 0.5-ms control tail (obtained by comparing the area under the tail with ON charge movement up to 0.5 ms) is 1.27, which suggests that there is some contamination that increases the ratio above the expected ratio of ≤ 1 . As pulse length increases there is a reduction in the amplitude of I_g OFF, and the 10-ms tail has a slow component that is not present in the 0.5-ms tail. Both changes have been shown to be related to the effects of inactivation on I_g OFF (Armstrong and Bezanilla, 1977; Nonner, 1980; but see Meves and Vogel, 1977). After 10 ms there remains, despite inactivation, a substantial current that reaches maxi-

mum amplitude $40 \mu\text{s}$ after the step back to -80 mV . This current is composed of the inactivation-resistant component of $I_g \text{ OFF}$ generated by the inactivated channels, as well as gating current from the population of channels that have not inactivated.

Thionin reduces the OFF current particularly after the longer steps, and current is very small immediately after the step back. Current magnitude increases to a peak $\sim 300 \mu\text{s}$ after the step back, and then decays slowly. Overall, the time course strongly resembles that shown in Figs. 2 and 4 for I_{Na} . One interpretation is that presence of a dye molecule in a channel

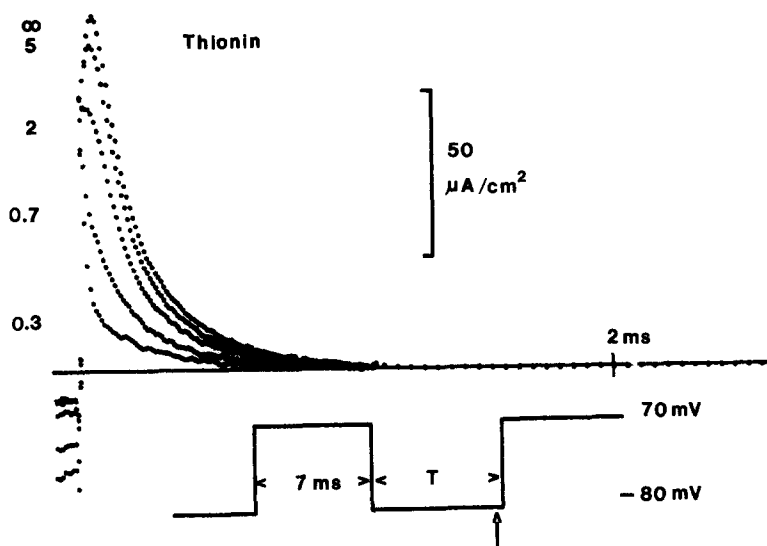


FIGURE 7. Thionin-blocked channels generate little ON gating current. Channels were thionin blocked by pulsing to 70 mV , and V was then returned to -80 mV for a period T . The current traces show $I_g \text{ ON}$ generated by the second pulse. Gating current is very small after a 0.3-ms recovery period, and is almost fully recovered after 5 ms . AU221A, HP -80 mV , 8°C . Tris SW//200 TMA + 0.5 mM thionin.

immobilizes all of that channel's gating charge, reducing $I_g \text{ OFF}$ from that channel to zero. When the dye comes out, the gating charge is free to move and close the channel, and total $I_g \text{ OFF}$ has a hooked time course that reflects the departure of dye from the channels.

A significant point is that thionin affects virtually all of the gating current, even the inactivation-resistant part. The next section shows that the time course of this reduction of I_g parallels the time course of drug block of the Na channels, and the two findings together show that most "gating current" is Na channel related.

Thionin Immobilizes Gating Charge as It Blocks Channels

Fig. 8C illustrates the reduction of I_{Na} plus I_g by thionin and is taken from the same experiment as part B of the figure. The pulse protocol was identical, and

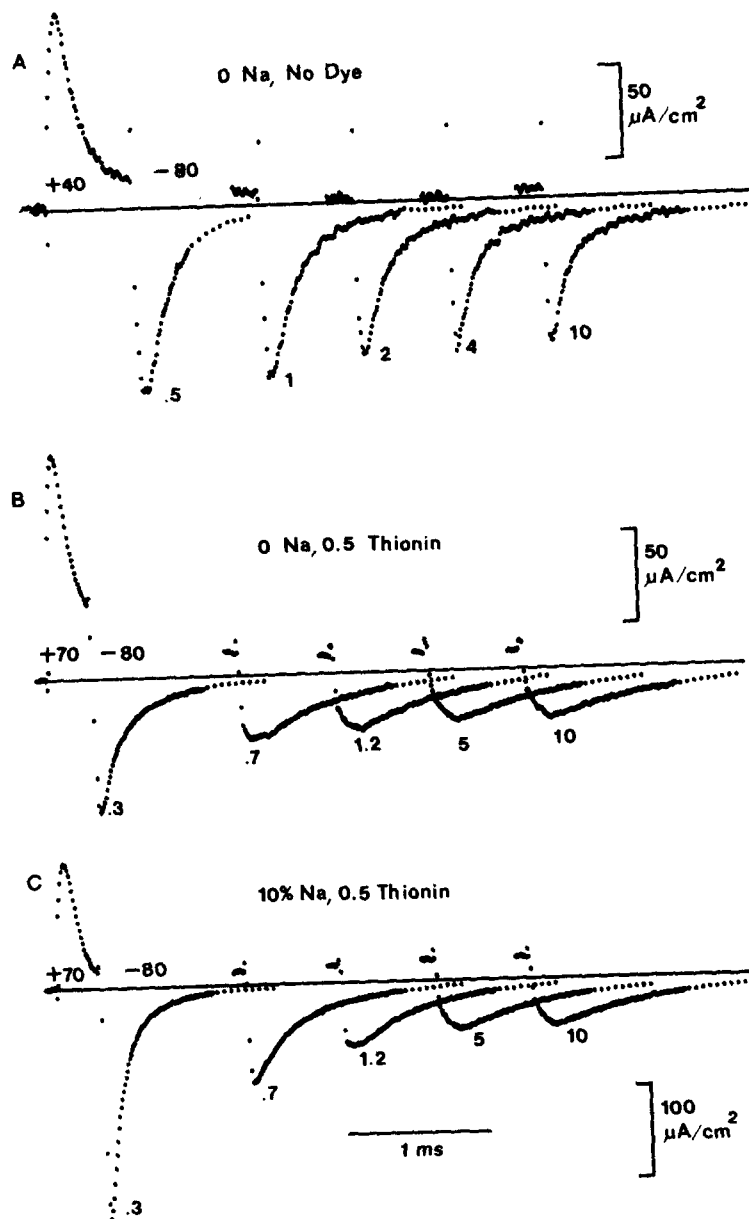


FIGURE 8. Dye blocks both I_g and I_{Na} during channel closing. A, B. Membrane current, mostly I_g , is shown in the absence of TTX without (A) and with (B) dye. The fibers were depolarized to the voltage indicated, and then returned to -80 mV after the interval noted next to each tail. Dye changes the time course of the tails, and reduces initial amplitude to near zero after steps longer than 1 ms. B, C. Currents in the presence of dye without (B) and with (C) external sodium. The outward peak is gating current in both cases. The inward current in B is largely gating current, whereas in C it is a mixture of gating and sodium current. Dye reduces the initial amplitude of both ionic and gating current to near zero. A. SE058B, HP -80 mV, 8°C . 10 Ca Tris SW// 200 TMA. B. AU221A, HP -80 mV, 8°C . Tris SW// 200 TMA + 0.5 mM thionin. C. AU221A, HP -80 mV, 8°C . $1/10$ Na SW// 200 TMA + 0.5 mM thionin.

the two sets of traces differ only in the Na content of the external medium: 0 Na (Tris SW) in B, and $\frac{1}{4}$ Na SW in C. The effect of increasing pulse duration on I_g (part B) is almost identical to the effects on I_{Na} plus I_g (part C).

This is shown more clearly in Fig. 9, where the early peak amplitudes of the tails are plotted as a function of pulse duration (see figure legend for details). The two time courses are almost exactly the same, and almost all of I_g is affected. The immobilization of the OFF gating charge by dye thus follows precisely the time course of entry of dye into the Na channels. This gives the strongest evidence presented to date that virtually all of the recorded "gating current" is in fact related to Na channel gating.

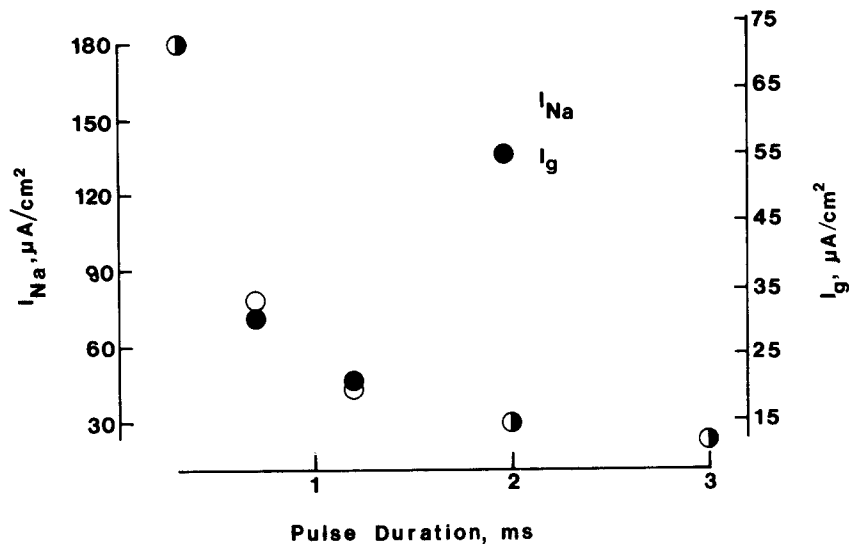


FIGURE 9. Dye blocks I_{Na} and I_g with the same time course. Peak amplitudes of I_{Na} and I_g from Fig. 8 are plotted as a function of pulse duration.

I_g ON, I_g OFF, and TTX

When TTX is added to the external medium, I_g ON is reduced in amplitude by the dye (thionin) and its time course is speeded, as shown in Fig. 10A. A possible interpretation is that the affinity of Na channels for thionin is increased by TTX, and that a fraction of the channels remain thionin occupied at the holding potential (see below).

I_g OFF is more drastically affected, and is almost eliminated after a 10-ms pulse to +70 mV. As can be seen, the reduction is more pronounced as pulse duration increases, and after 10 ms there remains only a small, slow component with a rounded time course. In general, the tails resemble those recorded in the absence of TTX (Fig. 8), but current amplitude is depressed more profoundly and the time course is slower.

I_g OFF decreases with pulse duration at almost the same rate as in the absence of TTX (compare Fig. 10 with Fig. 8B). The presence of TTX thus

does not greatly alter the rate of entry of dye into the channels, although there could be a slowing by as much as a factor of two. As shown in the next section, the effect on the dissociation rate of dye from the channels is much more profound.

Dyes Leave the Channels Slowly in the Presence of TTX

The I_g tails in the presence of thionin suggest that the dye dissociates slowly from the channels on repolarization when TTX is present. The rate of dye removal in TTX was measured by using the two-pulse procedure, and monitoring the total charge movement in the second pulse after most of the channels were dye blocked by the first pulse. Typical traces are shown in Fig. 11A. As labeled, the traces give I_g ON with no prepulse and with a prepulse

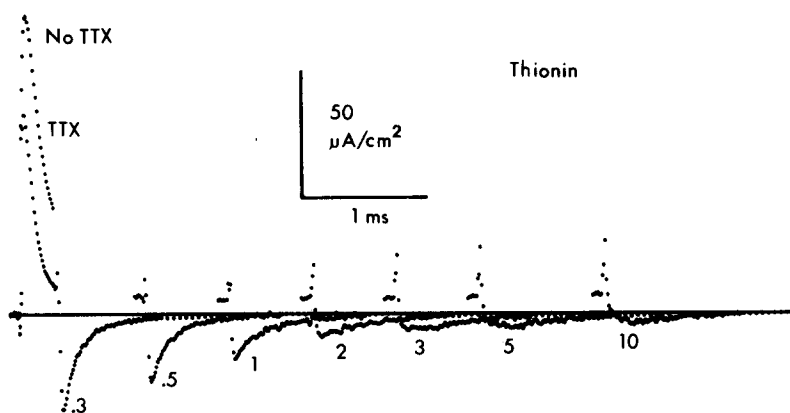


FIGURE 10. Dye reduces I_g ON and almost eliminates I_g OFF after long pulses. The fiber was depolarized to 70 mV, producing the outward current peaks, and then returned to -80 mV after the interval noted. AU221A, HP -80 mV, 8°C . Tris SW + TTX//200 TMA.

followed by the specified recovery interval (in milliseconds) at -80 mV. After a 0.7-ms recovery interval there is almost no charge movement during the test step, and it increases gradually as the recovery interval is lengthened. Total charge movement in the second pulse is plotted semilogarithmically as a function of recovery interval in Fig. 11B. Recovery has a complex time course, requiring at least two exponentials for an approximate fit, with the constants of 8 and 80 ms. Both are longer than recovery in the absence of TTX, which is illustrated (from the same experiment) by the other curve, which has a time constant of 2.2 ms (see also Fig. 5).

Thus the presence of TTX in the channel increases the affinity of the dye for the channel by decreasing the rate of dissociation. This effect is interpreted in the Discussion.

DISCUSSION

Dyes of the methylene blue family share with a number of other compounds the ability to simulate inactivation when applied internally in squid axons.

Perhaps the most important result of the present paper is the completeness of the block of what has been called the $I_{g\text{ OFF}}$ or OFF gating current. The dyes reduce the initial amplitude of this current almost to zero. Furthermore, the time courses of $I_{g\text{ OFF}}$ reduction and I_{Na} block are identical. These two facts combine to give the strongest evidence yet produced that virtually all of what has been called gating current is indeed associated with gating of Na channels.

There are a wide variety of other substances that simulate inactivation, including derivatives of tetraethylammonium ion, *N*-methyl strychnine, pancuronium, quaternary local anesthetics, and acridine orange (which, like the methylene blue dyes, contains a triple ring). All of these substances are positively charged, and all act from the inside. There are possibly lessons to be

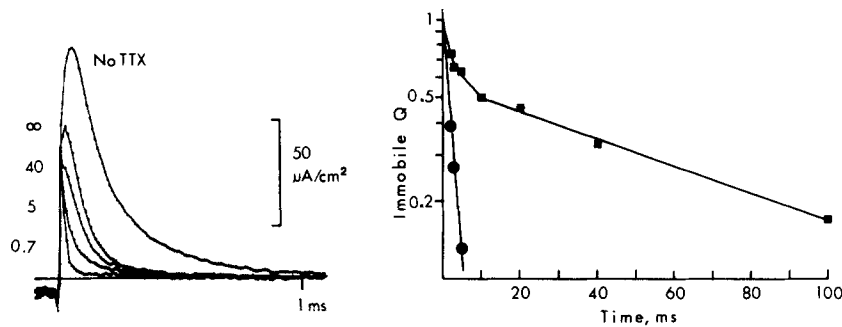


FIGURE 11. TTX slows the dissociation of dye from the channels. A. Channels were blocked during a first pulse to 70 mV, and voltage was returned to -80 mV. The traces show the current in a second pulse to 70 mV after the interval noted. For short recovery intervals in TTX, gating current is almost eliminated, except for a fast spike. TTX reduces $I_{g\text{ ON}}$ even with long recovery intervals, as was noted in Fig. 10. B. The fraction of immobilized charge is plotted as a function of recovery interval in the presence and absence of TTX. AU221A, HP -80 mV, 8°C . Tris SW + TTX//200 TMA.

drawn from the structures of these compounds and the slight differences in their action. The only clear conclusion at the moment is that the inner end of the channel, which accepts all of them, is not very selective.

The gating current effects of at least three of these compounds have been examined. All suppress OFF gating current, and do not interfere with $I_{g\text{ ON}}$, provided the interpulse interval is long and no TTX is present (Yeh and Armstrong, 1978; Cahalan and Almers, 1979). This presumably means that none of the channels are blocked at rest: if they were, $I_{g\text{ ON}}$ would be reduced. In the presence of TTX, both *N*-methyl strychnine (Cahalan and Almers, 1979) and methylene blue dyes (Figs. 10 and 11) reduce $I_{g\text{ ON}}$. Presumably this means that some of the channels are blocked at the holding potential, even after a long interval of rest. A reasonable explanation (Cahalan and Almers, 1979) is that TTX prevents external cations from displacing the inactivation simulator from the channel.

The artificial inactivators are similar in their action to normal inactivation, but there are differences. The similarities are that both block conduction, and

recovery requires several milliseconds. Both inactivation and the simulators immobilize a fraction of gating charge, causing a reduction of $I_{g\text{ OFF}}$. Finally, both inactivation and inactivation simulators seem able to block the channels before they open fully, as discussed below.

The evidence in Fig. 4 clearly shows that dyes compete with inactivation rather than speeding or enhancing it. Restating this evidence, dye-blocked channels conduct when the dye molecule comes out of them. If they were inactivated, they would not conduct. The opposite has been proposed for uncharged local anesthetics, which are said to enhance inactivation (Hille, 1977).

A major difference between inactivation and the simulators is that $I_{g\text{ OFF}}$ is almost completely eliminated by (most clearly) the methylene blue dyes, whereas about one-third of the total charge remains free to move after inactivation. This difference was illustrated in Fig. 8. In terms of scheme II in the Introduction, the inactivation-resistant gating current is generated by the transition from X_1Z to X_2Z , a one-step process that has exponential kinetics. Functionally this gating current is associated with a step that closes the channels so they do not conduct during recovery from inactivation. The transition out of state X_2Z is relatively very slow, resulting in temporary immobilization of most of the charge.

Dyes immobilize most of the gating charge, presumably because the dye hinders closing of the activation gate. The dye comes out of the channels so slowly in the presence of TTX that gating current as the activation gates close is very small. In addition, dye makes impossible the X_1Z -to- X_2Z transition that occurs in normally inactivated channels.

The total suppression of gating current by dyes is associated with the second major difference between inactivation and the simulators: Na channels do not conduct during recovery from inactivation (Fig. 3), but they do conduct as they recover from dye block (Fig. 4). Dyes prevent the channels from bypassing the conducting state during recovery, as shown in the diagram of scheme III in the Results section. This scheme gives a good qualitative explanation, but there is a problem: it cannot be shown that all of the dye-blocked channels reflux through the open state, as the scheme implies. In fact as many as one-half of them may not do so. This may result from one of two possibilities: either there is a bypass route, as for normal inactivation via state X_2Z , or the method for counting the refluxing channels is not valid. The method depends on the assumption that rate constant β_1 is always the same, and it is not impossible that it speeds up a bit after dye block. The existence of a state X_1D is compatible with the evidence shown below that channels can become dye blocked before they open fully.

Attempts were made to fit scheme III to experimental traces with and without dye. To simplify, we fitted a large depolarization (to +40 mV) for which it was reasonable to assume that all the β 's are zero. Further, we chose an experiment in which most of inactivation had been destroyed by pronase: only 19% of the channels inactivated. We first fitted the control traces, setting κ_D and l_D to zero (Fig. 12A). The dye traces were then fitted, leaving all

parameters from the control fit unchanged except for κ_D and l_D , which were adjusted to give the best fit. Increasing κ_D reduces peak I_{Na} amplitude, and speeds its decay. We found that if κ_D was selected to give the correct decay rate of the current, the predicted amplitude invariably was too large. Alternatively, if peak amplitude was fitted, the decay rate was too fast. A sample fit that correctly predicts decay rate is shown in Fig. 12B, using the parameters listed in the figure legend. (No attempt was made to simulate I_g .) Peak amplitude of the fitted trace is clearly too large.

A much better fit could be obtained by reducing the total number of channels by a factor of 0.67 in the presence of dye, or, equivalently, by assuming that 33% of the channels were dye blocked at the holding potential. A sample calculation is shown in Fig. 12C. A reduced number of channels, however, is not consistent with the constancy of I_g ON in the presence of dye:

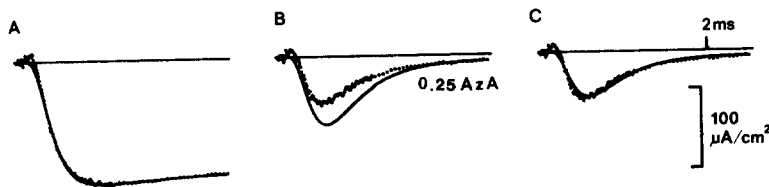


FIGURE 12. Fits of scheme III to I_{Na} from a pronased fiber, without (A) and with (B, C) dye. I_g (determined after TTX addition) has been subtracted from the traces, leaving mostly I_{Na} . The theoretical trace in B assumes that channels must open fully before they can become dye blocked. With this model, a blocking rate that reproduces the time constant of decay of current does not sufficiently reduce peak current. The theoretical trace in C assumes that 33% of the channels are dye blocked before they open. Parameters for the fitting were α_1 5.5 ms^{-1} , α_x 18 ms^{-1} , κ 0.7 ms^{-1} , κ_D 2 ms^{-1} , l_D 0.25 ms^{-1} , current with all channels conducting 180 $\mu\text{A}/\text{cm}^2$. 19% of the channels inactivate. JL209B, HP -80 mV, 8°C. 1/4 Na SW//200 TMA + 0.25 mM azure A, after pronase.

a 33% reduction of I_g ON from either deletion or dye block of channels would be easily detectable. A more reasonable explanation is that (a) all channels remain operative and none are dye blocked at rest, consistent with unchanged I_g ON; and (b) after depolarization about one-third of the channels become dye blocked before they conduct. For example, a channel might be susceptible to block in either state X_1^* or X_2 , as is postulated to be the case for normal inactivation (schemes II and III).

Note added in proof: Since submission of this paper, Greeff, Keynes, and Van Helden have suggested that the non-inactivating fraction of gating current is not associated with sodium channel gating (*Proc. R. Soc. B Biol. Sci.* 215:375-389). The evidence in the present paper clearly speaks against this possibility.

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