SOLVENT SUBSTITUTION AS A PROBE OF CHANNEL GATING IN MYXICOLA

Effects of D₂O on Kinetic Properties of Drugs that Occlude Channels

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ABSTRACT The effects of solvent substitution on the steady-state and kinetic properties of drugs (gallamine triethiodide) and ions (nonyltriethylammonium and Ba⁺⁺) known to occlude Na⁺ and K⁺ channels have been examined and compared with the effects of D₂O on unmodified channels. In general, we observed large isotope effects on the kinetics of occlusion at temperatures of 5°C, but only minor effects at 15°C, consistent with processes involving significant solvent interaction. Steady-state behavior was not affected. In the case of gallamine, where a dual effect on $I_{Na}$ is evident, although both processes were D₂O sensitive, only the occlusion phase had a significant temperature dependence.

INTRODUCTION

The effects of making an isotopic substitution in a chemical system can often provide considerable insight into the reaction processes occurring in it. In particular, we have previously shown that studies of the effects of heavy water (D₂O) substitution on the kinetics of ion channels at different temperatures can be used to distinguish those features of Na⁺ and K⁺ channel gating that may involve a significant interaction with the surrounding solvent from those that appear to be solvent independent (Schauf and Bullock, 1979). Furthermore, differential effects of solvent substitution on various components of channel gating (e.g., Na⁺ activation vs. Na⁺ inactivation) enable us to place constraints on the kinds of physical models and/or reaction sequences that may be capable of explaining existing voltage-clamp data (Schauf and Bullock, 1981).

Activation and inactivation of sodium channels in excitable membranes do not seem to be completely independent. Experimental evidence for this view has been obtained both from studies of the kinetics of sodium currents (Goldman and Schauf, 1972, 1973; Schauf et al., 1976b; Bezanilla and Armstrong, 1977) and from the time-course of membrane asymmetry currents and their variation with increasing pulse duration (Armstrong and Bezanilla, 1977; Bullock and Schauf, 1979). However, the nature of the "coupling" between activation and inactivation is unclear and may be more complicated than that expected if a channel is obliged to open before becoming inactivated (Horn et al., 1981). Nevertheless, a detailed kinetic model for inactivation has been developed based in part on the existence of a physiological "inactivating particle," which can enter and block the sodium channel from the interior of the cell (Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979).

As a result, there has been considerable interest in substances that can block sodium currents from the inside of a nerve fiber in ways consistent with a voltage- and/or time-dependent occlusion, and thus mimic physiological inactivation. Compounds with this effect include anesthetics (Strichartz, 1973; Cahalan, 1978; Cahalan and Almers, 1979a), methylstrychnine (Cahalan and Almers, 1979b), pancuronium (Yeh and Narahashi, 1977), polyglycyl arginine amide (Eaton et al., 1980), alkylguanidines (Kirsch et al., 1980; Morello et al., 1980), and gallamine triethiodide (Schauf and Smith, 1981). It might be expected that drugs that enter and occlude Na⁺ channels would have kinetics that are strongly influenced by solvent changes. Thus, a comparison between solvent effects on drug interaction with the Na⁺ channel and physiological inactivation might serve as a useful means of testing channel occlusion as a model for inactivation.

Voltage- and time-dependent occlusion can be observed in K⁺ channels without the complication of a concomitant inactivation. Such effects are produced by Cs⁺ (Adelman and French, 1978; Bezanilla and Armstrong, 1972), Ba⁺⁺ (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980), and tetraethylamonium and its various derivatives such as nonyltriethylammonium (Armstrong, 1966, 1969, 1971; Armstrong and Hille, 1972). Again, solvent substitution would be expected to have significant effects on the rate of block by such compounds.

The present study represents an initial attempt to evaluate the effects of D₂O substitution on occlusion of both Na⁺ and K⁺ channels by drugs and ions. It is hoped that
such data can be ultimately used to more precisely define the molecular processes occurring not only during channel occlusion, but also under normal physiological conditions.

**METHODS**

*Mycisola* giant axons were dialyzed and voltage-clamped by methods that have been previously described (Bullock and Schauf, 1978). When potassium currents were measured, the internal solution was composed of 450 mM K+ glutamate, 50 mM KF, 30 mM K2HPO4, and 1 mM Hepes (except for the Ba2+ experiments; see below). For all the gallamine experiments the dialyzate contained 600 mM Cs+ glutamate and 1 mM Hepes. The external solution was artificial seawater (ASW) containing 430 mM NaCl, 50 mM MgCl2, 10 mM CaCl2, and 20 mM Tris. Both external and internal solutions were adjusted to pH 7.3 ± 0.1. For experiments in high external [K+], the Na+ concentration was reduced accordingly. In some experiments external [Na+] was reduced by equi-molar substitution of Tris. Tetrodotoxin (10⁻⁴ M) was used when necessary to block Na+ currents. Series resistance was routinely compensated and leakage and capacity currents eliminated by appropriate analog circuitry (Schauf and Bullock, 1980). Axons were recompensated after solvent substitution to allow for the lower equivalent conductivity of electrolyte solutions in D2O (Swain and Evans, 1966). Temperature was 5.0 ± 0.5°C unless otherwise specified.

Deuterium oxide (99.8% D2O) was obtained from Sigma Chemical Co., St. Louis, Mo. and redistilled before use. Ion equilibria differ in D2O because the self-ionization of D2O is an order of magnitude smaller than for H2O. As a consequence, pD does not equal pH, and pH readings for glass electrodes must be corrected via the expression pD = pH + 0.41 (Katz and Crespi, 1970). Thus, for example, an ASW solution in D2O must show a pH of 6.9 to have a pD = 7.3. It is also known that D2O behaves as a stronger acid than H2O, thus effectively raising the pKₐ of titratable weak acids by ~0.5 pH unit (Covington and Jones, 1968).

Fortunately, extensive data concerning the effect of pD changes on both the voltage-dependent ionic conductances and rate constants are already available in *Mycisola* (Schauf and Davis, 1976). Both the maximum sodium and potassium conductances (gₙa and gₚₚ) are reversibly depressed at low pH but the pKₐ’s are 4.8 and 4.4, respectively. The voltage dependence of gₙa and the time constants for Na+ activation are also fairly insensitive to mild acidic conditions. In particular, at neutral pH and a membrane potential of 0 mV, a decrease of 0.5 pH unit causes no more than a 5% decrease in maximum sodium conductance, a shift of gₙa(V) of 0.5 mV, and a shift in Na+ kinetics of ~2 mV. Such effects are negligible.

The effect of D2O on drug occlusion of Na+ channels was studied using gallamine triethiodide (Sigma Chemical Co.), a nondepolarizing neuromuscular blocking agent that, when applied internally, modifies Na+ channels in a manner similar to pancuronium (Schauf and Smith, 1981). At concentrations of ~0.1 mM, gallamine alters Na+ channel kinetics and causes a striking increase in the magnitude of Na+ tail currents after long duration pulses. However, at low concentrations such effects can take as long as an hour to reach a steady state similar to that seen after 5–10 min exposure to higher concentrations of gallamine. We feel this is primarily the result of the dialysis procedure. Although salt concentrations rapidly equilibrate (Bullock and Schauf, 1978), the dialysis tubing retards the movement of larger molecules such as gallamine (mol wt = 892). We found that dialysis with 5–10 mM gallamine produced an optimally rapid effect that would subsequently remain stable for the duration of our experiments.

Heavy water effects on K+ channel occlusion were examined in tetrodotoxin (TTX)-treated axons, using both nonlyttriethylammonium (C₄) and Ba2+.-C₄ was synthesized for us by Polysciences Inc., Warrington, Pa., and was applied using the normal F- containing dialyzate. Barium complexes with F-, and thus in these studies only glutamate was used as the internal anion (Cl-) solutions could not be used because in *Mycisola* they produce a steady increase in leakage current and deterioration of both Iₚₚ and Iₚₚ. Even so, Ba2+ concentrations as high as those used by Armstrong and Taylor (1980) were required to demonstrate an effect. Either glutamate also complexes with Ba2+ or the dialysis procedure failed to remove some internal constituent that buffers the divalent cation concentration. In any case, we do not know the true concentration of Ba2+ near the interior of the cell membrane.

All experiments involving D2O substitution were done with bracketing controls in H2O, and the data are generally presented in the form of ratios of time constants in D2O to the average of those determined during the bracketing H2O runs. Data are given as means ± standard errors. In general, four to six axons were examined in each part of this study, with data being obtained at 5–10 different membrane potentials in each axon. Since the D2O effects were not appreciably voltage dependent, ratios were usually averaged over all voltages. Thus the tests of significance referred to in the text generally had n’s of 20–60.

**RESULTS**

**Gallamine-modified Na+ Channels in H2O**

Gallamine triethiodide does not affect gₚ in *Mycisola* and does not alter the Na+ channel when applied externally, even at high concentrations. However, when added internally, the decline in Iₚₚ during step depolarizations is altered, and as the membrane potential is made more positive, the majority of Na+ channels fail to inactivate (complete details are to be found in Schauf and Smith, 1981). Fig. 1A shows that for depolarizations to potentials more negative than —10 mV, the sole effect of gallamine is to slow inactivation. However, inactivation is still complete, and thus for long pulses (15 ms; not illustrated) no Na+ tail currents are observed. At potentials more positive than —10 mV, the rate of Na+ inactivation in the presence of gallamine is biphasic, with an initial decrease that is voltage dependent and faster than inactivation in untreated axons (we have defined this time constant as rₜₚ), followed by a slower decline (time constant rₜₚ). This latter decline is not only slower than normal inactivation, but seems nearly independent of membrane potential, and has a time constant comparable to that measured for gallamine-modified channels at potentials below —10 mV. As the potential increases, the fraction of channels showing a rapid shut-off increases and simultaneously the magnitude of the Na+ tail conductance following 15-ms pulses increases by a comparable amount (open triangles in Fig. 1B). At large positive potentials ~70–80% of Na+ channels fail to inactivate. The gallamine-induced Na+ tail currents exhibit a pronounced "hook," and decline more slowly than the Na+ tail currents which are measured in control axons after short pulses (Fig. 1B; inset).

At potentials positive to Eₚₚₚ, a third effect of gallamine is seen. Outward Na+ currents are inhibited more strongly by gallamine than expected from the voltage dependence of rₜₚ. When Eₚₚₚ is changed by increasing internal [Na+] (Fig. 1B), rₜₚ(V) is invariant but peak outward currents are more strongly inhibited than inward currents. Channels that
have not become activated behave normally in the presence of gallamine as determined by measuring both steady-state prepulse inactivation (h<sub>i</sub>) curves (filled and open circles in Fig. 1 B), and the time constants for recovery from and development of inactivation at potentials more negative than -30 mV (not shown). Gallamine does not alter the time-course of Na<sup>+</sup> activation.

Thus gallamine has a dual effect on the Na<sup>+</sup> channel (Schauf and Smith, 1981). On the one hand it acts in a manner similar to pancuronium (Yeh and Narahashi, 1977), another neuromuscular blocking agent, by occluding Na<sup>+</sup> channels in a voltage-dependent fashion and preventing them from inactivating. On repolarization, the Na<sup>+</sup> channel cannot close until gallamine first dissociates, resulting in slow, hooked Na<sup>+</sup> tail currents. However, in contrast to pancuronium, gallamine and Na<sup>+</sup> are apparently able to compete for occupancy of a binding site. A second, possibly independent, effect of gallamine is to slow the rate of inactivation of nonoccluded Na<sup>+</sup> channels without preventing their ultimate inactivation.

**Effects of Temperature on Gallamine-modified Channels**

In gallamine-modified Na<sup>+</sup> channels at 5°C, τ<sub>1</sub> varies from 0.44 ms at 0 mV to 0.15 ms at +100 mV, whereas values for τ<sub>2</sub> scatter between 2.7 and 3.2 ms with no resolvable voltage dependence (Schauf and Smith, 1981). The time constant for gallamine tail currents varies from 0.2 ms at -120 mV to 2.0 ms at 0 mV (Schauf and Smith, 1981). For V > -10 mV, where inactivation during a maintained depolarization is biphasic and good double exponential fits can be obtained, we found in the present study that the Q<sub>10</sub>'s in H<sub>2</sub>O ranged from 2.2 to 2.6 for both τ<sub>1</sub> and τ<sub>2</sub>. At voltages below -10 mV, a comparable Q<sub>10</sub> was seen for τ<sub>2</sub>. These are similar to the Q<sub>10</sub> of 2.56 ± 0.02 previously determined for inactivation during a maintained depolarization in untreated Myxicola axons (Schauf, 1973). In contrast, the average Q<sub>10</sub> for gallamine tail currents in H<sub>2</sub>O was 3.62 ± 0.48 (range 2.65-4.8). This was significantly larger (P < 0.002) than the Q<sub>10</sub>'s in...
H₂O for the biphasic inactivation of gallamine-modified Na⁺ channels. It was also larger than the Q₁₀ for tail currents in untreated axons.

Solvent Substitution and Gallamine

As shown previously (Schauf and Bullock, 1980), D₂O slows the rate of Na⁺ activation. In order to have an estimate for this effect in gallamine-treated axons, we measured the time at which the rate of rise of Iₙa was a maximum (tₘₐₓ). Since Na⁺ channels must open before they can be occluded (Schauf and Smith, 1981), this should give a reasonably uncontaminated measure of the rate of Na⁺ activation. That this is the case was shown by the fact that gallamine dialysis did not significantly alter tₘₐₓ at any potential. Values for tₘₐₓ in gallamine-treated axons were determined as a function of membrane potential in H₂O and D₂O, and the ratio tₘₐₓ(D₂O)/tₘₐₓ(H₂O) calculated at each potential and subsequently averaged over all potentials (since the D₂O-induced slowing of Iₙa is not appreciably voltage dependent; Schauf and Bullock, 1979). At 5°C the mean increase in tₘₐₓ in D₂O was to 1.42 ± 0.09 times the values in H₂O, whereas at 14°C the ratio was 1.10 ± 0.07, giving a Q₁₀ of 0.75 (Table IV). These values are comparable to those previously obtained in untreated axons (Schauf and Bullock, 1980).

The effects of D₂O on the decline of Iₙa during step depolarizations in gallamine-treated axons were then examined at 5 and at 13–14°C. For each of six axons, the decline in Iₙa at voltages between -30 and +50 mV was fit by the sum of two exponentials both in D₂O and for the bracketing H₂O runs. At each voltage the time constants in D₂O were divided by the average of those obtained in H₂O. As in normal axons, there was no significant voltage dependence of the D₂O effects, and thus the time constant ratios were averaged over all membrane potentials examined. The rapid, voltage-dependent occlusion of Na⁺ channels for V > -10 mV was slowed by D₂O substitution with an average τₕ₁(D₂O)/τₕ₁(H₂O) ratio (Table IV) of 1.42 ± 0.15 at 5°C and 0.94 ± 0.43 at 13°C, giving a Q₁₀ of 0.60. The voltage-independent, slow decline in Iₙa that is seen both immediately after the rapid gallamine occlusion at V > -10 mV, and in isolation for V < -10 mV, was also slowed by D₂O. However, the ratio of τₕ₂(D₂O)/τₕ₂(H₂O) averaged 2.00 ± 0.20 at 5°C and 1.88 ± 0.09 at 13°C (Table IV). Although the magnitude of the D₂O effect on τₕ₂ was larger at 5°C, its temperature dependence was lower (Q₁₀ = 0.92). These differences between the D₂O effects on τₕ₁ and τₕ₂, as well as between the results on normal and gallamine-modified channels, were all statistically significant (P < 0.005).

Gallamine-induced Na⁺ tail currents following long pulses are also significantly slowed by D₂O substitution at all voltages (Fig. 2). The average ratio of tail time constants in gallamine-treated axons in D₂O compared with H₂O was 1.69 ± 0.08 at 5°C and 1.32 ± 0.06 at 13.5°C, giving a Q₁₀ of 0.75 (Table IV). Again, there was no dependence of these ratios on membrane potential. This is a particularly interesting result because, as we show in the next section, Na⁺ tail currents in untreated axons are not sensitive to D₂O substitution.

At V > -10 mV, gallamine causes a substantial fraction of Na⁺ channels to fail to inactivate (Schauf and Smith, 1981). However, substitution of D₂O had no effect on the fraction of channels that remained conducting in the steady state at any membrane potential.

D₂O Insensitivity of Na⁺ Tails in Untreated Axons

Fig. 3 shows sodium currents in a normal axon in H₂O and D₂O for a depolarizing pulse short enough so that repolarization occurred when gₙa was large. Since D₂O decreases maximum sodium conductance by ~25% (Schauf and Bullock, 1980) the currents in D₂O were scaled for comparison. In these experiments [Na⁺] was reduced to 86 mM (20% of normal) to eliminate possible artifacts from residual uncompensated series resistance (Schauf et al., 1977). The duration of the depolarizing pulse was made longer in D₂O because the rates of activation and inactivation are slowed, and we wished to record tail currents in both cases at comparable times after the
occurrence of maximum inward current. In Fig. 3 the time to peak inward current was increased from 0.60 to 0.91 ms, and the time constant for inactivation of conducting channels \((r_i)\) was increased from 1.50 to 2.40 ms. In contrast, solvent substitution produced little or no change in the rate at which open channels closed after repolarization.

Sodium tail currents in *Myxicola* cannot be described by a single exponential (Schauf et al., 1977; Goldman and Hahn, 1978). Both fast and slow components exist, and

Figure 3 The effects of solvent substitution on sodium repolarization (tail) currents in *Myxicola*. The capacitative transient and linear leak current were removed by adding the response to an exactly matched hyperpolarizing pulse. In part a, currents are recorded in H2O and D2O during a depolarization to 0 mV, with the latter (indicated by the arrows) scaled so that the maximum inward currents appear equal. In part b, the D2O record (arrows) has been shifted to the left so that the repolarization times coincide, and the time scale is expanded to aid visual comparison. The horizontal lines represent zero current. The vertical scale is 0.075 mA/cm². The time scale is 0.5 ms in part a and 0.25 ms in part b. The temperature was 5°C, and the holding potential was −80 mV.

### Table 1

<table>
<thead>
<tr>
<th>(V_H) (mV)</th>
<th>(r_{iH2O}) (μs)</th>
<th>(r_{iD2O}) (μs)</th>
<th>(I_{rH2O}/I_{rH2O})</th>
<th>(r_{iD2O}) (μs)</th>
<th>(I_{rD2O}/I_{rD2O})</th>
<th>(I_{D2O}/I_{H2O})</th>
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<tr>
<td>−80(2°C)</td>
<td>195</td>
<td>—</td>
<td>195(1.0)</td>
<td>—</td>
<td>1.69</td>
<td></td>
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<tr>
<td>−100(5°C)</td>
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<td>340</td>
<td>86(1.13)</td>
<td>320(1.08)</td>
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<tr>
<td>−60(5°C)</td>
<td>120</td>
<td>380</td>
<td>97(0.92)</td>
<td>320(0.89)</td>
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<td>1.66</td>
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<td>104</td>
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<td>450(0.69)</td>
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<td>550(0.96)</td>
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<td>−60(5°C)</td>
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<td>700</td>
<td>119(1.07)</td>
<td>490(0.62)</td>
<td>0.11</td>
<td>1.52</td>
</tr>
</tbody>
</table>

*The holding potential is given in column 1. The numbers in parentheses in columns 5 and 6 are the ratios of the time constants in D2O to those in H2O as calculated from the data in columns 2, 3, 5, and 6. In the first four experiments there were no bracketing data in H2O, whereas in the remainder the bracketing H2O data were used to calculate the D2O effect. Data from six axons is included. Columns 4 and 7 contain values for the relative coefficients (expressed as a ratio) of the slow and fast components in H2O and D2O, respectively. The last column gives the ratios of time to peak inward current in D2O compared with H2O and is a measure of the basic solvent effect. A two-exponential fit to the data for 2 ms following repolarization was used because three exponentials gave no better fit, and because the lability of the slow components was sufficient to render any more detailed analysis suspect.*

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the relative contribution of each is a function of membrane potential, but not of the absolute magnitude of inward current (Schauf et al., 1977). Nevertheless, it is clear that at least the fast component of Na\(^+\) tail current was quite unaffected by D\(_2\)O. The slower components of Na\(^+\) tail currents can be relatively labile during the course of an experiment. In particular, the decrease in the magnitude of the slow components in Fig. 3b in the D\(_2\)O solution is probably not significant since comparable changes often occurred simply with the passage of time in H\(_2\)O (in the absence of any change in the magnitude of inward current).

The complete results of D\(_2\)O substitution on tail currents in six axons are given in Table I. We assumed a two-component decline in \(I_{Na}\) for the tail currents with time constants \(\tau_f\) and \(\tau_s\). The value of Na\(^+\) tail currents extrapolated to \(t = 0\) for the fast and slow components are termed \(I_{Na}^f\) and \(I_{Na}^s\). The ratio of time to peak \(I_{Na}\) in D\(_2\)O to that in H\(_2\)O averaged over all voltages was 1.50 \(\pm\) 0.04 at 5°C, whereas the ratio of the time constants for the fast component of the Na\(^+\) tail current was 1.01 \(\pm\) 0.03. As can be seen from the tabulated data, there was some tendency for the slow component to become faster as the experiment progressed. If anything, D\(_2\)O accelerated the slow component(s).

It should, of course, be noted that the isotope effects on Na\(^+\) activation and deactivation are measured at very different membrane potentials. Unfortunately, there is little choice since measurements of activation require depolarizations to potentials of at least \(-35\) mV, but in this range the repolarization currents are in part governed by the kinetics of inactivation (Schauf et al., 1977; Goldman and Hahn, 1978). Our previous data concerning the effects of isotope substitution on Na\(^+\) activation kinetics suggest that the D\(_2\)O effect may increase, rather than decrease, very slightly as the membrane potential becomes more negative over the range +60 to \(-60\) mV (Schauf and Bullock, 1979). Furthermore, the voltage dependence of the steady-state conductance was not detectably altered by D\(_2\)O over the same voltage range. Thus, a voltage-dependent D\(_2\)O effect that was smaller at \(-80\) mV does not seem a likely explanation for the insensitivity of repolarization currents to solvent substitution.

The measurement of Na\(^+\) repolarization currents is, of course, relatively difficult. Tail currents are sensitive to series resistance compensation and the presence of any spatial nonuniformity. We minimized such problems by the use of low sodium solutions and by verifying that the kinetics did not change when the absolute magnitude of membrane current is varied (Schauf et al., 1977). Still, some uncertainty remains. However, these considerations are not relevant here, since the experimental observation is that D\(_2\)O was without a measurable effect on repolarization currents, even though activation was significantly delayed.

C\(_4\) Kinetics in Myxicola Axons

The effects of internal C\(_4\) in Myxicola giant axons qualitatively resemble those seen previously in squid axons (Armstrong, 1969, 1971) and are summarized in Fig. 4. In contrast to normal K\(^+\) currents, which monotonically increase with time to a voltage-dependent steady-state level, K\(^+\) currents in the presence of internal C\(_4\) increased to a peak value and subsequently decreased as a single exponential. As C\(_4\) concentration increased, the peak K\(^+\)

![Figure 4](image-url)  
Figure 4 Effects of C\(_4\) on K\(^+\) currents in Myxicola. Membrane currents are shown for step depolarizations of 30–180 mV (10-mV increments; holding potential, \(-80\) mV) in an axon bathed in K\(^+\)-free ASW plus 10\(^{-4}\) M TTX. Top to bottom are control records, and records with 0.1 mM C\(_4\) and 1.0 mM C\(_4\) dialysis. Calibrations are 3 ms and 0.75 mA/cm\(^2\). Temperature, 5°C.
currents decreased, and inactivation was accelerated and became more complete. However, $C_b$ did not affect the rate of activation of $K^+$ channels. In $K^+$-free ASW the fraction of $K^+$ current blocked at equilibrium [$J(V)$] increased with increasing $C_b$ concentrations and averaged 0.70 $\pm$ 0.02 with 0.1 mM $C_b$. However, $J(V)$ was not voltage dependent over the range of $-10$ to $+100$ mV where reliable measurements could be obtained. Values for the time constant for the decline in $I_K$ to its equilibrium value [$\tau_{C_b}$] averaged 17.4 $\pm$ 1.6 ms at 0 mV and 6.9 $\pm$ 0.7 ms at $+100$ mV (Table II). There was little or no effect of external $K^+$ concentration (0-200 mM) on the rate or steady-state level of $C_b$ block in *Myxicola*.

In experiments in which a small conditioning depolarization of variable duration precedes a fixed test pulse to $+160$ mV, the maximum current during the test step decreased exponentially with increasing pulse duration after an initial delay (cf. Armstrong, 1969). This allowed us to define the fraction of $g_K$ that is inactivated at potentials more negative than 0 mV in a way analogous to the definition of prepulse inactivation of the sodium conductance. Values for what we will call $\tau_{C_b}^\text{ref}$ averaged 45 ms at $-40$ mV, 34 ms at $-20$ mV, 17 ms at 0 mV, and 13.5 ms at $+20$ mV. These time constants were comparable to the values of $\tau_{C_b}$ measured during maintained depolarizations at the same potentials. A similar protocol in which a pulse to $+160$ mV (lasting long enough to allow complete $K^+$ inactivation) was followed by a return to the holding potential of $-80$ mV, then after a variable interval by a fixed test pulse, allowed calculation of the recovery time constant ($\tau_{C_b}^\text{rec}$). This averaged 180 ms at $-80$ mV.

### Temperature and $C_b$ Kinetics

Because they have not been previously reported in detail, the effects of temperature on $C_b$ kinetics have been provided along with the solvent substitution data in Table II. With increasing temperature $\tau_{C_b}$ decreased with a $Q_{10}$ of 3.02 $\pm$ 0.10 (compare column 2 with column 5) in axons in which $K^+$ activation itself had a $Q_{10}$ of 2.40 $\pm$ 0.08. Thus, the temperature dependence of $C_b$ block was greater than the temperature dependence of $g_K(t)$. The time constant for recovery from $C_b$ inactivation had a $Q_{10}$ of 1.70 (not shown), significantly less than the foregoing. Steady-state inhibition of $g_K$ decreased substantially at higher temperatures (Fig. 5), with the average steady-state $K^+$ current being 30% of maximum at 5°C, and 55% of maximum at 14°C. This implies a larger activation energy for inactivation by $C_b$ than for recovery.

### Solvent Substitution and $C_b$ Kinetics

The effects of $D_2O$ substitution on a $C_b$-treated axon are shown in Fig. 5. At 5°C, $D_2O$ decreased the rate of $K^+$ inactivation at all voltages. At 14°C, however, $D_2O$ had no appreciable effect on $\tau_{C_b}$. At neither temperature did $D_2O$ substitution change the steady-state level of $C_b$ block. These results are presented quantitatively in Table II for three axons at two different temperatures. Only kinetic data are given since, as we noted, $J(V)$ was unchanged by $D_2O$. At 5°C, $\tau_{C_b}$ is increased by $D_2O$, and the degree of slowing was independent of membrane potential. On the average (including data at all voltages) $\tau_{C_b}$ was increased by 74 $\pm$ 7%. At 14°C, the $D_2O$ induced slowing averaged 14 $\pm$ 5% in all axons, yielding a $Q_{10}$ of 0.61 for the solvent substitution effect.

The time constant for the decrease in peak potassium current as a function of prepulse duration ($\tau_{C_b}^\text{ref}$) was also examined in $D_2O$ (three axons; data not shown). $D_2O$ substitution increased $\tau_{C_b}^\text{ref}$ by 120 $\pm$ 17% at 5°C, a significantly greater effect than seen for maintained depolarizations. In a single axon the time constant of recovery from $C_b$ block was increased by 64% at 5°C and was unaffected by $D_2O$ at 14°C.

In the $C_b$-treated axons, $I_K(t)$ records were differentiated to determine the time at which $dI_K(t)/dt$ was maximum, and thus provide a measurement of the rate of $K^+$ activation. In these axons $D_2O$ slowed $K^+$ activation by 46 $\pm$ 5% (comparable to that seen previously; Schauf and Bullock, 1980), which is significantly less than the $D_2O$

### Table II

<table>
<thead>
<tr>
<th>$V_m$ (mV)</th>
<th>$\tau_{H_2O}^{C_b}$ (ms)</th>
<th>$\tau_{D_2O}^{C_b}$ (ms)</th>
<th>$D_2O/H_2O$ ratio</th>
<th>$\tau_{H_2O}^{C_b}$ (ms)</th>
<th>$\tau_{D_2O}^{C_b}$ (ms)</th>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>17.4 $\pm$ 1.6</td>
<td>30.0 $\pm$ 1.9</td>
<td>1.72 $\pm$ 0.18</td>
<td>6.2 $\pm$ 0.4</td>
<td>7.1 $\pm$ 0.5</td>
<td>1.14 $\pm$ 0.08</td>
</tr>
<tr>
<td>20</td>
<td>14.0 $\pm$ 0.09</td>
<td>24.5 $\pm$ 2.4</td>
<td>1.75 $\pm$ 0.14</td>
<td>5.0 $\pm$ 0.6</td>
<td>5.0 $\pm$ 0.4</td>
<td>0.98 $\pm$ 0.08</td>
</tr>
<tr>
<td>40</td>
<td>11.5 $\pm$ 1.0</td>
<td>20.0 $\pm$ 2.1</td>
<td>1.74 $\pm$ 0.11</td>
<td>3.9 $\pm$ 0.5</td>
<td>4.1 $\pm$ 0.7</td>
<td>1.04 $\pm$ 0.08</td>
</tr>
<tr>
<td>60</td>
<td>9.9 $\pm$ 0.8</td>
<td>17.0 $\pm$ 0.9</td>
<td>1.72 $\pm$ 0.17</td>
<td>3.4 $\pm$ 0.3</td>
<td>3.8 $\pm$ 0.5</td>
<td>1.12 $\pm$ 0.07</td>
</tr>
<tr>
<td>80</td>
<td>7.9 $\pm$ 0.08</td>
<td>13.4 $\pm$ 1.4</td>
<td>1.70 $\pm$ 0.14</td>
<td>2.7 $\pm$ 0.4</td>
<td>3.0 $\pm$ 0.4</td>
<td>1.10 $\pm$ 0.07</td>
</tr>
<tr>
<td>100</td>
<td>6.9 $\pm$ 0.7</td>
<td>11.7 $\pm$ 1.1</td>
<td>1.70 $\pm$ 0.21</td>
<td>2.6 $\pm$ 0.4</td>
<td>2.7 $\pm$ 0.3</td>
<td>1.05 $\pm$ 0.08</td>
</tr>
</tbody>
</table>

*Only data for $\tau_{C_b}$ are shown because $D_2O$ did not change $J(V)$. These data are averages from three axons in which $\tau_{C_b}$ was determined at both 5 and 14°C. The overall averages referred to in the text include not only these data but also data at other voltages and from axons examined at only a single temperature.

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effect on $\tau_C$, ($P < 0.01$). The $Q_{10}$ for $D_2O$ slowing of $K^+$ activation was 0.75, compared with the $Q_{10}$ of 0.61 for $\tau_C$, ($P < 0.007$).

**Ba**$^{++}$ Kinetics in *Myxicola*

Fig. 6 illustrates the interaction of internal Ba$^{++}$ with $K^+$ channels in *Myxicola* axons. In contrast to $C_9$-modified axons, for voltages more negative than +20 mV, little effect of Ba$^{++}$ was seen in 50 or 100 mM external $K^+$. However, for $V > +20$ mV, $K^+$ currents inactivated in the presence of Ba$^{++}$, and furthermore, unlike $C_9$, the steady-state level of $K^+$ inactivation was strongly voltage dependent. In fact, the steady-state level of Ba$^{++}$ block increased sufficiently rapidly with voltage that the current-voltage relation exhibited a negative slope conductance. The Ba$^{++}$ block was antagonized by increasing external $[K^+]$. In $K^+$-free solutions the steady-state $I(V)$ curve was decreased at most voltages and peaked at +25 mV, whereas at $[K^+]_o = 100$ mM there was no change until $V \geq 35$ mV and the peak was at +45 mV. The rate of decline of $I_k$ in Ba$^{++}$-treated axons also was decreased with increasing $[K^+]$. These effects qualitatively resemble those reported previously in squid axons (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980). The results of a more quantitative analysis of such data are given in Table III along with the solvent substitution data for several different membrane potentials. Internal Ba$^{++}$ only slightly delays $K^+$ activation. As in the $C_9$ experiments, the time of maximum $dI_k/dt$ was determined after Ba$^{++}$ dialysis as a function of membrane potential for untreated axons. In general, a 10% slowing was observed in the presence of Ba$^{++}$. At +100 mV the respective values were 0.86 ± 0.05 and 0.99 ± 0.08 ms. These values were unaffected by

### Table III

**EFFECTS OF SOLVENT SUBSTITUTION ON Ba$^{++}$ KINETICS IN MYXICOLA**

<table>
<thead>
<tr>
<th>$V_m$</th>
<th>$F(V)^*$</th>
<th>$t_{in}^{H_2O}$</th>
<th>$t_{in}^{D_2O}$</th>
<th>Ratio ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$mV$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiments with $[K^+]_o = 0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+20</td>
<td>0.47 ± 0.05</td>
<td>25.2 ± 0.4</td>
<td>37.2 ± 4.4</td>
<td>1.48</td>
</tr>
<tr>
<td>+40</td>
<td>0.65 ± 0.04</td>
<td>18.0 ± 1.1</td>
<td>28.0 ± 3.6</td>
<td>1.56</td>
</tr>
<tr>
<td>+60</td>
<td>0.77 ± 0.03</td>
<td>15.5 ± 0.0</td>
<td>26.6 ± 3.5</td>
<td>1.72</td>
</tr>
<tr>
<td>+80</td>
<td>0.82 ± 0.03</td>
<td>12.2 ± 1.1</td>
<td>18.9 ± 2.5</td>
<td>1.55</td>
</tr>
<tr>
<td>+100</td>
<td>0.90 ± 0.02</td>
<td>10.1 ± 0.4</td>
<td>16.0 ± 2.9</td>
<td>1.58</td>
</tr>
<tr>
<td>Experiments with $[K^+]_o = 215$ mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+20</td>
<td>&lt;0.02</td>
<td>NR</td>
<td>NR</td>
<td>-</td>
</tr>
<tr>
<td>+40</td>
<td>0.02 ± 0.02</td>
<td>NR</td>
<td>NR</td>
<td>-</td>
</tr>
<tr>
<td>+60</td>
<td>0.20 ± 0.05</td>
<td>33.0 ± 4.7</td>
<td>37.0 ± 4.9</td>
<td>1.12</td>
</tr>
<tr>
<td>+80</td>
<td>0.47 ± 0.06</td>
<td>23.0 ± 5.6</td>
<td>24.0 ± 4.4</td>
<td>1.04</td>
</tr>
<tr>
<td>+100</td>
<td>0.65 ± 0.07</td>
<td>16.5 ± 4.4</td>
<td>19.7 ± 3.9</td>
<td>1.19</td>
</tr>
</tbody>
</table>

*Values of $F(V)$ in $H_2O$ are given. Those in $D_2O$ were not significantly different.

$\frac{\text{Ratio}}{\text{NR, not resolvable.}}$
Effects of barium on K⁺ currents in *Myxicola*. Membrane currents are shown for depolarizations of 30–180 mV (10-mV increments; holding potential, −80 mV) before and during dialysis with 5 mM Ba⁺⁺, and for two different external K⁺ concentrations. Calibrations are 0.75 mA/cm² (upper records) or 0.4 mA/cm² (lower Ba⁺⁺ records) and 3 ms. Temperature, 5°C.

Solvent Substitution and Ba⁺⁺ Kinetics

In axons bathed in K⁺-free ASW, substitution of D₂O slowed the rate of K⁺ inactivation in the presence of internal Ba⁺⁺ (Table III). The ratio of τ_Ba(D₂O)/τ_Ba(H₂O) was voltage independent in K⁺-free ASW and averaged 1.56 ± 0.14 at 5°C. The isotope effect on K⁺ activation in the same axons was determined by measuring the time of maximum dI/K(t)/dt and the ratio t_{max}(D₂O)/t_{max}(H₂O) averaged 1.51 ± 0.13.

At higher [K⁺] the behavior was very different, however. Although the ratio t_{max}(D₂O)/t_{max}(H₂O) describing the isotope effect on K⁺ activation was unchanged, averaging 1.56 ± 0.14 in 215 mM K⁺, the ratio τ_Ba(D₂O)/τ_Ba(H₂O) was only 1.12 ± 0.08. Thus, increased external K⁺ seems to antagonize not only the rate and degree of Ba⁺⁺ block, but also the magnitude of the isotope effect itself. Data at higher temperatures unfortunately were not available.

**DISCUSSION**

The overall results are summarized in Table IV. The effects of solvent substitution on various parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D₂O/H₂O ratio at 5°C</th>
<th>D₂O/H₂O ratio at 13–14°C</th>
<th>Q₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated axons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺ Activation</td>
<td>1.47 ± 0.02</td>
<td>1.18 ± 0.03</td>
<td>0.72</td>
</tr>
<tr>
<td>τᵣ Na⁺</td>
<td>1.52 ± 0.03</td>
<td>1.37 ± 0.03</td>
<td>0.84</td>
</tr>
<tr>
<td>τᵣ Na⁺ tails</td>
<td>2.61 ± 0.21</td>
<td>1.43 ± 0.05</td>
<td>0.47</td>
</tr>
<tr>
<td>Sodium current</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Gating current</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>K⁺ activation</td>
<td>1.40 ± 0.03</td>
<td>1.17 ± 0.02</td>
<td>0.79</td>
</tr>
<tr>
<td>Gallamine-treated axons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺ activation</td>
<td>1.42 ± 0.09</td>
<td>1.10 ± 0.07</td>
<td>0.75</td>
</tr>
<tr>
<td>τᵣ Na⁺</td>
<td>1.42 ± 0.15</td>
<td>0.94 ± 0.43</td>
<td>0.60</td>
</tr>
<tr>
<td>τᵣ Na⁺ tails</td>
<td>2.00 ± 0.20</td>
<td>1.88 ± 0.09</td>
<td>0.92</td>
</tr>
<tr>
<td>Cg-treated axons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K⁺ activation</td>
<td>1.46 ± 0.05</td>
<td>1.16 ± 0.08</td>
<td>0.75</td>
</tr>
<tr>
<td>τᵣ Na⁺</td>
<td>1.74 ± 0.07</td>
<td>1.14 ± 0.05</td>
<td>0.61</td>
</tr>
<tr>
<td>τᵣ Na⁺ tails</td>
<td>2.20 ± 0.17</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>f(V)</td>
<td>No effect</td>
<td>No effect</td>
<td>—</td>
</tr>
<tr>
<td>Ba⁺⁺-treated axons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K⁺ activation</td>
<td>1.51 ± 0.13</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>τᵣ (0 K⁺)</td>
<td>1.56 ± 0.14</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>τᵣ (215 K⁺)</td>
<td>1.12 ± 0.08</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>f(V)</td>
<td>No effect</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
describing channel gating are given at a temperature of 5°C along with the $Q_{10}$ derived from the temperature dependence of the isotope effects. This is done both for untreated axons (data taken from Schauf and Bullock, 1980, 1981), and axons in which gating has been modified by the agents examined in this study. It is noteworthy that measurements of solvent effects on Na$^+$ and K$^+$ activation were remarkably consistent over the course of this and prior studies.

The framework for interpretation of isotope effects is detailed elsewhere (Schauf and Bullock, 1979). Briefly, hydrogen atoms of membrane constituents may in the first instance exchange with solvent deuterium and change the rate or equilibrium constants for reactions involving bonds to the substituted atoms or adjacent bonds (primary and secondary isotope effects). The ratio of the rate constant of an unsubstituted system ($K$) to that of the deuterium-substituted system ($K^*$) is generally given by: 

$$ \frac{K}{K^*} = 1 \exp \left( \frac{\Delta E_0}{RT} \right), $$

where $\Delta E_0$ is the isotopically induced difference in the separation of the zero-point energies of products and reactants and $I$ is a term (between 1 and 2) involving the ratio of the masses and moments of inertia of both systems (Melander, 1960; Laidler, 1969; Thornton and Thornton, 1970). The general characteristic of these effects is that an appreciable temperature dependence is only evident if the kinetic effects themselves are very large. For example, a $K/K^*$ ratio of 10 (typical of a deuterium-hydrogen exchange in a strong covalent bond) gives a $Q_{10}$ of ~0.9.

However, important effects are also associated with the behavior of liquid H$_2$O and D$_2$O as solvents. The viscosity, melting point, and heat capacity are all significantly higher in D$_2$O as a result of more extensive intermolecular hydrogen bonding. It is significant that solvent structure breaks down more rapidly with temperature in D$_2$O than in H$_2$O (Heppolette and Robertson, 1960). The temperature dependence of solvent-dependent properties is experimentally observed to be much greater than that predicted from the $K/K^*$ ratio assuming a hydrogen-deuterium exchange. At 5°C D$_2$O produces an ~50% slowing of the ionic current kinetics in normal Myxicola axons, but the effects disappear with increasing temperature with $Q_{10}$'s of 0.6–0.8 (Schauf and Bullock, 1980). A primary or secondary isotope effect with this temperature dependence should yield a $K/K^*$ ratio of the order of 1,000. Thus we have suggested that changes in structural properties of the solvent may be the most important factor determining the magnitude of these kinetic effects.

The two phases of Na$^+$ inactivation in the presence of gallamine, probably representing a voltage-dependent occlusion combined with some other alteration in channel kinetics, are affected very differently by D$_2$O. The fast decline ($\tau_{f2}$) is moderately slowed by D$_2$O at 5°C, but this effect disappears almost completely at 14°C ($Q_{10} = 0.6$), which suggests that changes in solvent structure may be most important for occlusion. The slower phase of Na$^+$ inactivation ($\tau_{s2}$) is more strongly affected by D$_2$O at 5°C, and this marked slowing persists at high temperatures. The temperature dependence of the D$_2$O effect on $\tau_{s2}$ could therefore be explained by a primary or secondary isotope effect on membrane macromolecules in which solvent interaction plays no significant role.

Tail currents in gallamine-treated axons are slowed by D$_2$O in a way consistent with a moderate solvent effect ($Q_{10}$ of 0.75). In contrast, Na$^+$ tail currents in untreated axons are insensitive to solvent substitution, despite the fact that Na$^+$ activation and inactivation are markedly slowed by D$_2$O. Thus, although the creation of an open channel normally involves appreciable changes in solvent order, deactivation is solvent independent. This differential effect may well be an important clue to understanding the underlying mechanisms of channel gating. At a minimum, the rate constant(s) for the transition from closed to open channels need to be D$_2$O sensitive, whereas those for the transition from open to closed channels need not be. This would be possible if only the last of a sequence of steps were reversed, and this particular step was solvent insensitive. However, such an interpretation is complicated by the fact that D$_2$O slows activation of Na$^+$ channels with no effect on the intramembrane charge movement which is presumably associated with transitions among preceding closed states (Schauf and Bullock, 1979, 1981). Perhaps the process by which a channel closes upon repolarization is not simply the reverse of that by which it opened.

Nonyltriethylammonium produces K$^+$ inactivation in Myxicola axons in much the same fashion as in squid axons and frog node (Armstrong, 1969, 1971; Armstrong and Hille, 1972), except that the voltage dependence of $\tau_c$ in Myxicola is only about half as large. Inactivation by C$_9$ seems to require opening of the K$^+$ channel, and recovery is delayed by hyperpolarization. Interestingly, recovery from C$_9$ block at ~80 mV seems to have a lower activation energy than the initial binding step. Substitution of D$_2$O has the same sort of effect on C$_9$ block during step depolarizations as it does on the rapid phase of Na$^+$ inactivation in gallamine-treated axons. The increase in $\tau_c$, produced by D$_2$O at 5°C is moderate and disappears almost completely with increasing temperature, which suggests a strong solvent interaction. It is well established that in contrast to kinetic effects, under equilibrium conditions free energy differences are less sensitive to solvation because of the high degree of entropy-enthalpy compensation present in such systems. This is particularly true of hydrophobic bond strengths, which differ very little in H$_2$O and D$_2$O (Kresheck et al., 1973; BenNaim et al., 1973; Oakenfull and Fenwick, 1975). Such an insensitivity was observed when D$_2$O effects on the steady-state properties of the Na$^+$ channel were examined (Schauf and Bullock, 1979), and thus it is not surprising that the steady-state binding of C$_9$ was also D$_2$O insensitive.

Armstrong (1969) has suggested that the tetraalkylammonium (TAA) binding site contains a hydrophobic

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component because of the alkyl chain length dependence of the binding constant. In the present study C₈ binding was found to decrease with increasing temperature, corresponding to an enthalpy change of approximately −8 kcal/mol. Hydrophobic bonding contributes positive terms to the enthalpy, so if present it is evidently overwhelmed by other forces. According to the studies of Kirshnan and Friedman (1969), the structural contribution to the enthalpy of desolvation of alkyl groups ranges from +2.5 kcal/mol for ethyl to +8.5 kcal/mol for amy1. Thus if the hydrophobic and negative contributions are simply additive, the temperature dependence of TAA derivatives of differing chain lengths may be quite different. Such experiments may be useful in further characterizing the TAA binding site.

Barium effects in Myxocola again differ from those seen in squid axons only in the magnitude of the voltage dependence of τBa. Nevertheless, the voltage dependence of the Ba⁺⁺ effect was sufficient to produce the same sort of crossover of K⁺ currents with increasing steady-state block seen with increasing depolarizations in squid axons. Solvent substitution slows the rate of Ba⁺⁺ inactivation at 5°C, but we were unable to obtain data on temperature dependence. The effect of Ba⁺⁺ is decreased by external potassium, and it is interesting that the D₂O effect is also antagonized. Perhaps more than a simple competition for binding sites is involved.

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