MODIFICATIONS OF SODIUM CHANNEL GATING IN *MYXICOLA* GIANT AXONS BY DEUTERIUM OXIDE, TEMPERATURE, AND INTERNAL CATIONS

C. L. SCHAUF AND J. O. BULLOCK, *Departments of Physiology and Neurological Sciences, Rush University, Chicago, Illinois 60612 U.S.A.*

ABSTRACT In dialyzed *Myxicola* axons substitution of heavy water (D_2O) externally and internally slows both sodium and potassium kinetics and decreases the maximum conductances. Furthermore, this effect is strongly temperature dependent, the magnitude of the slowing produced by D_2O substitution decreasing with increasing temperature over the range 3–14°C with a Q_{10} of ~ 0.71. The relatively small magnitude of the D_2O effect, combined with its strong temperature dependence, suggests that the rate limiting process producing a conducting channel involves appreciable local changes in solvent structure. Maximum conductances in the presence of D_2O were decreased by ~ 30%, while the voltage dependences of both g_{Na} and g_K were not appreciably changed. In contrast to the effects of heavy water substitution on the ionic currents, membrane asymmetry currents were not altered by D_2O, suggesting that gating charge movement may preceed by several steps the final transformation of the Na^+ channel to a conducting state. In *Myxicola* axons the effect of temperature alone on asymmetry current kinetics can be well described via a simple temporal expansion equivalent to a Q_{10} of 2.2, which is somewhat less than the Q_{10} of G_{Na} activation. The integral of membrane asymmetry current, representing maximum charge movement, is however not appreciably altered by temperature.

INTRODUCTION

Intramembrane charge movements have been investigated in a variety of systems (Armstrong and Bezanilla, 1974; Keynes and Rojas, 1976; Neumcke et al., 1976; Meves and Vogel, 1977; Bullock and Schauf, 1978, 1979) with the general conclusion that although they may be related to the sodium channel gating process, such charge movements do not correspond very closely with the kinetics of hypothetical Hodgkin-Huxley "*m*" particles (Almers, 1978). Because the exact relationship between gating currents and those molecular events constituting the "opening" of the Na^+ channel remains unclear, it is of extreme importance to try to define experimental procedures capable of differentially affecting one phenomena or the other. Recently, both temperature (Bezanilla and Taylor, 1978) and heavy water substitution (Meves, 1974) have been suggested as having such effects and this study represents an attempt to both confirm and significantly extend such observations in *Myxicola*.

The result of D_2O substitution has been intensively studied in a variety of biological systems (Katz and Crespi, 1970) and there exist well developed theoretical approaches to understanding such isotope effects (Melander, 1960; Nemethy and Scheraga, 1964; Laidler, 1969; Thornton and Thornton, 1970). Since one can in principle distinguish kinetic and equilibrium effects involving any of the exchangeable hydrogens of membrane components from effects of

D_2O substitution involving alterations in solvent properties, careful examination of isotope effects in biological membranes can serve as a useful probe of molecular processes.

METHODS

Methods for the simultaneous voltage-clamp and internal dialysis of *Myxicola* giant axons have been previously described together with a detailed justification for the procedures employed for acquisition and analysis of asymmetry current data (Bullock and Schauf, 1978, 1979). Briefly, asymmetry currents are derived by adding the total displacement currents obtained during exactly equal but opposite voltage pulses from a holding potential of −100 mV. This can be done because the charge-voltage relation is nearly linear between −100 and −200 mV in *Myxicola* and thus the equal and opposite procedure yields identical records as those obtained by a divided pulse protocol. Charge displaced is computed from the relation $Q=I_0\tau$, where I_0 is the zero time intercept of an extrapolation of the falling phase, rather than by a direct integration. We feel the latter is uncertain because of the large contribution to the integral of the first 20–30μs during which the membrane voltage may still be settling and the possibility that the rising phase (Armstrong and Bezanilla, 1974) may originate from charge movement occuring during the hyperpolarizing pulse and thus have a different physical origin than the falling phase.

When potassium currents were measured, the internal solution was composed of 450 mM K

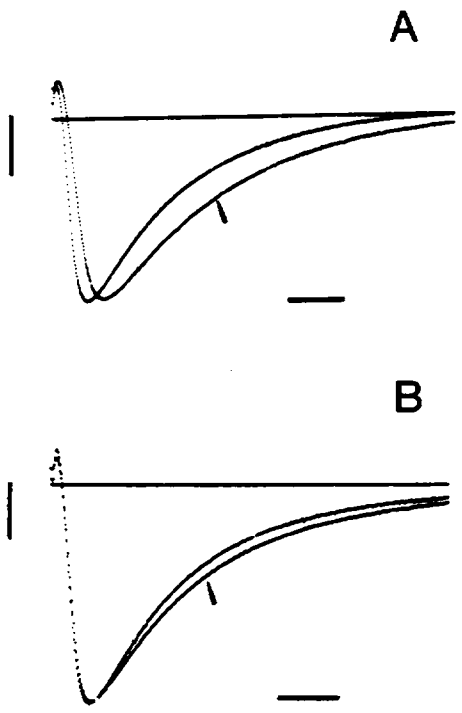


FIGURE 1 Membrane sodium currents at 4°C (*A*) and at 13°C (*B*) in an axon dialyzed with 600 mM Cs^+. In each set of records at a particular temperature the curve indicated by a pointer was obtained 30 min after D_2O substitution, both externally and internally. The D_2O records have been scaled vertically so that their peak amplitudes are equal to the amplitude originally measured using H_2O solutions. Membrane potential was −10 mV in all cases. The current calibration is 0.4 mA/square centimeter. Note, however, that the time scales are different, being 1.0 ms in *A* and 0.4 ms in *B*. Inward current is downward and the horizontal line corresponds to zero current. Records were leak corrected by adding currents obtained during equal hyperpolarizing pulses. Holding potential −80 mV.

glutamate, 50 mM KF, and 30 mM K_2HPO_4. Otherwise, the dialysis solutions generally contained 600 mM Cs^+ (with 480 mM glutamate, 50 mM F^-, and 1 mM Hepes). In some cases a fraction of the Cs glutamate was replaced by NaF or Na glutamate. The external solution was either K^+-free artificial sea water (ASW) (Na^+ current measurements) or Tris sea water containing 10^{-6} M tetrodotoxin (TTX) (asymmetry current or K^+ current measurements). Compositions were as given previously (Schauf et al., 1977). Internal and external pH's were adjusted to 7.3 ± 0.1 and 7.8 ± 0.1, respectively. Temperature was controlled to within 0.5°C over a range from 0 to 16°C.

In heavy water experiments both the internal and external solutions were prepared with reagent (Sigma Chemical Co., St. Louis, Mo.) containing 99.8% deuterium oxide (D_2O). Axons were compensated for series resistance and the adequacy of such compensation tested by methods previously described (Schauf et al., 1977). As the specific conductance of electrolyte solutions in D_2O is lower than in H_2O, it was necessary to increase the series resistance compensation during such measurements according to the tabulated values for electrical conductivities in heavy water (Tronstad and Stokland, 1937).

Some comment should be made concerning the measurements made with high internal [Na^+]. With Cs^+ dialysis the leakage current is lower than in intact axons (Bullock and Schauf, 1978) and for most purposes one can correct for its effect on I_{Na} measurements by adding the currents obtained during equal hyperpolarizing pulses. However, if one is interested in establishing the presence of some small residual I_{Na}, the small leak current rectification becomes significant, and could result in an underestimate of the residual I_{Na} for inward currents. On the other hand, application of TTX requires some time and if the leak is increasing would result in an overestimate of residual I_{Na} for inward (and an underestimate for outward) currents. Consequently we tried to combine both procedures by recording membrane current using both depolarizing and equal hyperpolarizing pulses before and after application of TTX, and scaling the TTX records to take into account any changes in the hyperpolarizing leakage currents. Nevertheless, we feel the procedure still leaves a residual uncertainty of 3–5% (see discussion of Fig. 9).

As in previous studies, ionic conductances were calculated from the relation $G_i = I_i/(V - E_i)$, where I_i is the measured current and E_i the corresponding equilibrium potential. Although this procedure makes no allowance for any changes in channel conductance from ion redistribution, it seems to provide an adequate description of the data with little potential for error in *Myxicola* (Goldman and Schauf, 1973).

RESULTS

D_2O on Sodium Currents

Substitution of heavy water both externally and internally slows both sodium and potassium kinetics and decreases the maximum channel conductances. Furthermore, this effect is temperature dependent in that the magnitude of the slowing produced by D_2O decreases with increasing temperature over the range 3–14°C. These results are illustrated in Figs. 1–3.

In Fig. 1 we have provided records of membrane sodium currents at 4°C and 13°C obtained from an axon dialyzed with 600 mM Cs^+ and bathed in K^+-free ASW both before and 30 min after D_2O substitution. The D_2O records were significantly decreased in amplitude compared to those in H_2O and therefore have been scaled by a factor of 1.3 so that the peak amplitudes were similar to aid visual comparison (see legend). Clearly both the time-course of Na^+ activation and inactivation are slowed in the presence of D_2O and this effect is smaller in the records obtained at 13°C. Fig. 2 illustrates this result in another way. Here the time-to-peak (t_{pk}) inward current is plotted as a function of membrane potential for four axons, each at two temperatures, before and after D_2O substitution. At the lower temperatures (circles) t_{pk} is 40–60% larger at all potentials in the D_2O solutions while at higher temperature t_{pk} is only increased by 10–20% in D_2O. Alternatively, one can observe that in H_2O the values of t_{pk} are

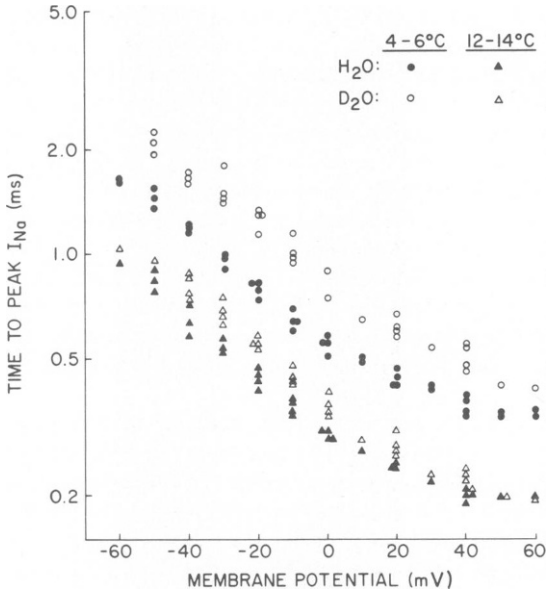


FIGURE 2 Time-to-peak inward current (t_{pk}) as a function of membrane potential, temperature, and D_2O substitution. Filled circles (●) describe the initial measurements of t_{pk} at 4–6°C in H_2O; filled triangles (▲) are measurements of t_{pk} at 12–14°C in H_2O; open circles (○) are measurements of t_{pk} at 4–6°C 30 min ofter D_2O substitution; and open triangles (△) represent measurements in D_2O at 12–14°C. Holding potentials were −80 mV in all cases. Composite data from four axons.

decreased by a factor of ~2.0 for a 7.5°C average increase in temperature, corresponding to a Q_{10} of 2.5, which is similar to that observed earlier (Schauf, 1973). In D_2O, however, the decrease in t_{pk} corresponds to a Q_{10} of ~3.0.

To compare these data with measures of Na^+ inactivation and K^+ activation, the ratio of t_{pk} (D_2O):t_{pk} (H_2O) was calculated for a particular axon for each potential used and the values of this ratio averaged over all voltages (thus ignoring any possible voltage dependence—see below). This was done in each axon for all temperatures at which measurements were made and the means (±SE) plotted in part *A* of Fig. 3. The solid line represents the best fit to the original data points. Time constants of sodium inactivation during a maintained depolarization were also determined in the same axons by using methods previously described (Schauf and Davis, 1975) and the ratios τ_h (D_2O):τ_h (H_2O) calculated, averaged, and plotted as the middle graph (*B*) in Fig. 3. In separate experiments using K^+ as the internal cation and external Tris sea water, time constants of K^+ activation were determined (Goldman and Schauf, 1973) and the ratio τ_n (D_2O):τ_n (H_2O) plotted as a function of temperature in the lower part of Fig. 3. Although we have not provided figures of the raw data for τ_h (V) and τ_n (V) to conserve space, we have listed in Table 1 typical experimental determinations of these parameters at a single voltage. As with measurements of t_{pk} (V) the major uncertainty in the D_2O:H_2O ratio arises from the variation in τ_h and τ_n obtained over the 1–2-h period needed for measurements at 4–6 temperatures and for two complete solvent exchanges by the dialysis procedure (Bullock and Schauf, 1978).

Considering the scatter in the data the linear fits in Fig. 3 cannot be viewed as appreciably

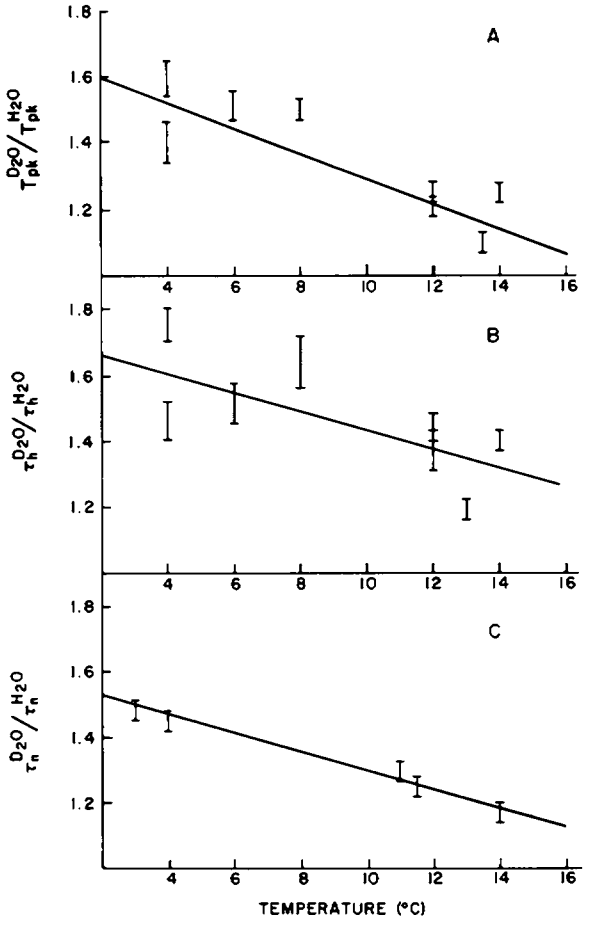


FIGURE 3 Effects of heavy water substitution on the kinetics of sodium activation and inactivation and K^+ activation in *Myxicola*. The upper graph (*A*) plots the value of time-to-peak inward current in D_2O to that in H_2O as a function of temperature. Each set of error bars represents the SE of the $t_{pk}(D_2O):t_{pk}(H_2O)$ determinations in a particular axon at a fixed temperature averaged over all membrane potentials. These were plotted instead of the raw data points to simplify the presentation; however, the solid line represents the best fit to the unaveraged data. Composite data from four axons. The middle and lower graphs are comparably derived but present data concerning D_2O effects on sodium inactivation (*B*) and K^+ activation (*C*). The solid lines again represent best fits to unaveraged data.

different. In *A* the best least squares fit to the data is given by:

$$t_{pk}(D_2O):t_{pk}(H_2O) = 1.68 - 0.038T$$

in *B* by

$$\tau_h(D_2O):\tau_h(H_2O) = 1.72 - 0.027T$$

and in *C* by

$$\tau_n(D_2O):\tau_n(H_2O) = 1.59 - 0.029T.$$

TABLE I
KINETIC PARAMETERS OF IONIC CONDUCTANCES IN D_2O AND H_2O*

Parameter	Solvent	T	Range	$\frac{D_2O}{H_2O}$ Ratio
		°C	*ms*	
τ_h^i	H_2O	4–6	1.34–1.70	—
		12–14	0.65–0.79	—
	D_2O	4–6	2.10–2.90	1.45–1.71
		12–14	0.88–1.14	1.18–1.44
τ_n	H_2O	2–4	2.8–5.0	—
		11–12	1.1–2.1	—
	D_2O	2–4	4.0–7.6	1.42–1.53
		11–12	1.35–2.9	1.26–1.34

*Membrane potential = 0 mV (τ_h^i) or +20 mV (τ_n).

It should be pointed out that there may be, in addition to the obvious temperature dependence of the D_2O effect, a slight voltage dependence. If instead of performing a simple linear regression on the ratios of t_{pk} (D_2O):t_{pk} (H_2O) as a function of temperature irrespective of membrane potential, a multiple linear regression is attempted, then the overall data is best described by the relation

$$t_{pk}\,(D_2O):t_{pk}\,(H_2O) = 1.67 - 0.038T - 0.0015V,$$

where V is the membrane potential in millivolts. Similarly, for the τ_h (D_2O):τ_h (H_2O) ratios, a multiple linear regression produces the relation

$$\tau_h\,(D_2O):\tau_h\,(H_2O) = 1.72 - 0.029T - 0.004V.$$

Both of these suggest a tendency for the D_2O effect to become slightly smaller at more depolarized potentials. However, because of the scatter in the data, the correlation coefficients in both cases are relatively small (~0.4) and this observation should be considered quite tentative.

Heavy water substitution clearly seems to affect the maximum conductances (see discussion of Fig. 1). In four axons in which the maximum conductances were determined before, during, and after D_2O substitution to minimize any uncertainty due to axon deterioration, the relative decrease in $\bar{G}_{Na}$ in D_2O was 27 ± 7% and the decrease in $\bar{G}_K$ was 32 ± 3% (Table II).

TABLE II
D_2O EFFECTS ON STEADY-STATE PARAMETERS IN *MYXICOLA*

Solution	G_{Na}^{Max}*	$V_{1/2}^{Na}$	$\bar{G}_K$*	$V_{1/2}^{K}$
		mV		*mV*
H_2O	1.0	−31 ± 4	1.0	20 ± 2
D_2O	0.73 ± 0.07	−30 ± 2	0.68 ± 0.03	22 ± 2

*Normalized to values in H_2O.
Temperature 4–6°C.

Thus, there seems to be a nearly equal effect of D_2O on the maximum conductances of both channels within the limits of accuracy of the present experiments.

In contrast to the effects of D_2O on the sodium activation and inactivation time constants, the voltage dependence of G_{Na} was not significantly affected (Table II). In four experiments in H_2O the voltage at which G_{Na} (V) was half its maximum value ($V_{1/2}^{Na}$) was -31 ± 4 mV, while in D_2O the value of $V_{1/2}^{Na}$ averaged -30 ± 2 mV. Values for the voltage at which the K^+ conductance was half-maximum ($V_{1/2}^{Na}$) were 20 ± 2 mV in H_2O and 22 ± 2 mV in D_2O, again showing there to be no isotope effect on the steady-state voltage dependence. The voltage-dependence of steady-state sodium inactivation was not examined in these experiments.

D_2O on Asymmetry Currents

In contrast to the effects of heavy water substitution on the ionic conductances, membrane asymmetry currents were largely unaffected by D_2O. Fig. 4 shows records of membrane asymmetry currents obtained at 4°C by averaging the total displacement currents recorded during sixteen 80 mV depolarizations and those obtained during 16 exactly paired hyperpolarizations (holding potential of -100 mV) and adding the result, first in H_2O, then 30 min after D_2O substitution. There is no significant difference in either the amplitude of the ON response or the time-course of the falling phase between the D_2O and H_2O records.

Table III further summarizes the asymmetry current results. The raw values for τ_{on} and τ_{off} were similar to those previously measured (Bullock and Schauf, 1978) with τ_{on} at a potential of 0–10 mV being in the range 235–400 μs in H_2O and 263–415 μs in D_2O (three axons). Ranges for τ_{off} at -100 mV were 157–217 μs in H_2O and 176–220 μs in D_2O. When the ratio of measurments in D_2O and H_2O in a particular axon were determined at each potential and averaged, the ratio of all 'ON' response time constants was 1.06 ± 0.03 and the average ratio for 'OFF' time constants was 1.07 ± 0.03. These differences are not significant, as the experimental variation in τ_{on} and τ_{off} in records obtained in a given axon at different times is at least 10% (Schauf et al., 1977). There was in addition no significant change in the voltage dependence of charge movement or in the maximum amount of charge displaced.

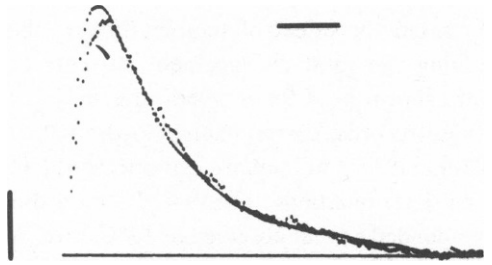


FIGURE 4 Effect of D_2O substitution on membrane asymmetry current ON responses in *Myxicola*. The axon was held at -100 mV and the total displacement currents in response to 16 exactly matched depolarizing and hyperpolarizing pulses 80 mV in amplitude averaged and summed to produce the records shown. The record indicated by the small arrow was obtained using D_2O substitution while the other was obtained in H_2O. Note the smoother appearance of the H_2O record, which has been processed via a data smoothing program of the signal averager. This was done to aid visual discrimination between the two curves. Current and time scales are 12 μA/square centimeter and 250 μs, respectively. Temperature 4°C. The horizontal line represents the best visual fit to the residual nonlinear leakage current at times in excess of 2 ms. Outward current is upward.

TABLE III

D_2O EFFECTS ON MEMBRANE ASYMMETRY CURRENTS IN *MYXICOLA*

Axon	$\frac{\tau_{on}(D_2O)^*}{\tau_{on}(H_2O)}$	$\frac{\tau_{off}(D_2O)^*}{\tau_{off}(H_2O)}$	$\frac{Q_{max}(D_2O)‡}{Q_{max}(H_2O)}$	$V^Q_{1/2}(D_2O)$§	$V^Q_{1/2}(H_2O)$§
				mV	*mV*
78M9	0.99 ± 0.06	0.98 ± 0.06	1.02	−33	−33
78M10	1.07 ± 0.04	1.11 ± 0.04	0.94	−37	−39
78M12	1.05 ± 0.05	0.99 ± 0.02	1.05	−35	−36

*Mean ± SE of measurements taken at potentials from −60 to +20 mV. Time constants determined by a least squares fit to the asymmetry current data ignoring the rising phase.

‡Q_{max} refers to the maximum charge moved in nanocoulombs per square centimeter.

§$V^Q_{1/2}$ is the membrane potential at which $Q = \frac{1}{2} Q_{max}$.

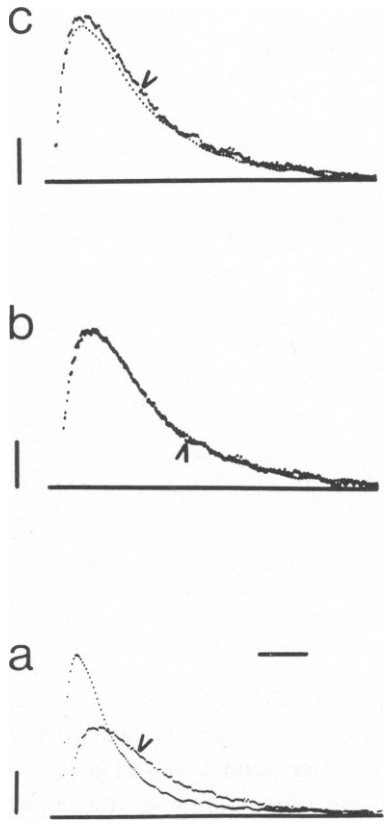
It should be noted that we did observe some tendency for the rising phase of membrane asymmetry current to be prolonged during D_2O substitution (Fig. 4), although the effect varied substantially from one experiment to another. Generally the maximum of the asymmetry current ON response was reached 20–40 μs later in the presence of D_2O with a concommitant decrease in the slope of the rising phase. The corresponding delay in the OFF response was 15–20 μs. However, as the data recording system has a resolution of only 10 μs, it was not possible to define this effect any more clearly.

Temperature on Asymmetry Currents

In squid giant axons the falling phase of the asymmetry current ON response cannot be generally described as a simple exponential process (Armstrong and Bezanilla, 1977, Bezanilla and Taylor, 1978). Analysis of the temperature dependence of membrane asymmetry currents is complex since different temperature coefficients appear to be necessary to fit different portions of the ON response transient (Bezanilla and Taylor, 1978). In *Myxicola* axons dialyzed with Cs glutamate, the ON response is in most cases more nearly a single exponential (Bullock and Schauf, 1978), making it interesting to attempt to determine the temperature dependence of these responses.

In Fig. 5 we have illustrated the effect of temperature on the ON response. The ON responses obtained by adding the total displacement currents for 16 pairs of equal and opposite 100-mV pulses are shown in *A* for temperatures of 4°C (indicated by arrow) and 12°C. The areas under these records, corresponding to the total charge moved, were 12.3 nC/square centimeter (4°C) and 11.3 nC/square centimeter (12°C), values equal within the experimental errors of such determinations. The record obtained at 4°C was then scaled so that the peak current corresponded to that observed at 12°C, after which the 12°C record was expanded temporally by a factor of 2.0 and the combined result photographed as shown in *B*. The arrow again marks the 4°C record. In *C* the amplitude scaling of the expanded 12°C record was slightly reduced so as to better separate the two curves for purposes of visual comparison. Note that in all these records the baselines were fit by eye to the ON response at the end of a 4-ms pulse with only the first portion shown for clarity. They are thus corrected for the small nonlinear leakage current that remains even in Cs^+-dialyzed axons.

A similar procedure was followed to illustrate the effects of temperature on the OFF response. The records in part *A* of Fig. 6 were obtained at 0.5°C (indicated by arrow) and 7°C,



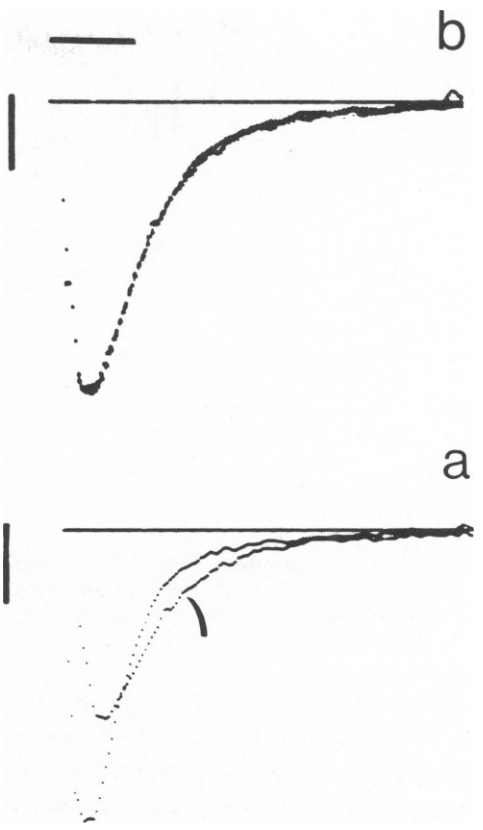


FIGURE 5

FIGURE 6

FIGURE 5 Effects of temperature on asymmetry current ON responses. In *A* is shown the asymmetry currents at 4°C (arrow) and 12°C (no arrow) obtained using 16 depolarizing and 16 equal hyperpolarizing pulses. Holding potential was −100 mV, and pulse size was 100 mV. Current and time scales are 12 μA/square centimeter and 250 μs, respectively. In *B* the records from *A* have been scaled and the 12°C record temporally expanded so as to superimpose as nearly as possible. The smoother-looking curve is the 12°C record while the 4°C record is indicated by the arrow. Same calibrations as in *A*. The records in *C* are identical to those in *B* except that the 4°C record was deliberately made slightly larger than the 12°C record to facilitate comparison. The horizontal lines are the best visual fit to the residual nonlinear leakage current at times >2 ms. Outward current is upward.

FIGURE 6 Same as *A* and *B* in Fig. 5, except these are now OFF responses at 0.5°C (indicated by arrow) and 7.0°C. Same calibrations as in Fig. 5 and inward current is downward. The horizontal line now represents zero current. Note that the pulse duration was 0.5 ms to eliminate the effects of charge immobilization, and consequently the 7°C record has a slightly larger area due to the fact at 0.5°C the 500-μs pulse duration was insufficient to move all the charge.

scaled to one another, and the 7°C record temporally expanded by a factor of 1.9 to produce the result shown in *B*. Pulses of short duration (≤0.5 ms) were used to avoid the introduction of the slow component of the OFF response, which is a consequence of immobilization (Bullock and Schauf, 1979). In this case the baseline shown represents true zero current.

It is clear from both figures that the effects of temperature on asymmetry currents in *Myxicola* can be completely described by a simple temporal expansion. In a few axons, it

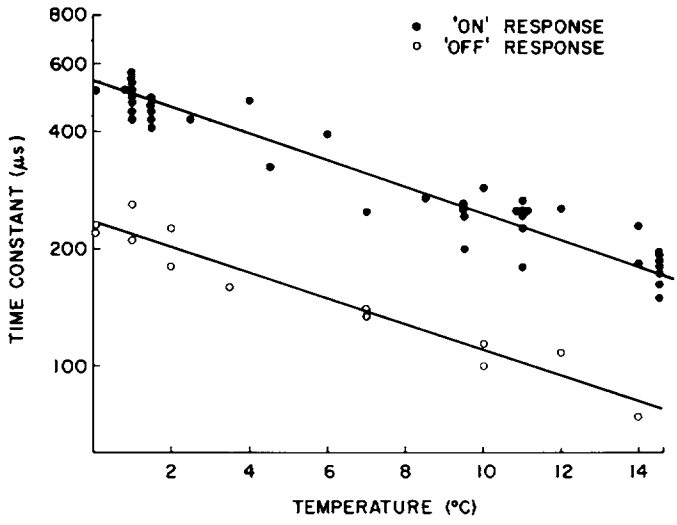


FIGURE 7 Asymmetry current time constants (τ_{on}—filled circles; τ_{off}—open circles) as a function of temperature. The solid lines were determined by calculating the least squares fit to the τ_{on} data and translating this line vertically to visually fit the τ_{off} data. The Q_{10} corresponding to this line is 2.20.

appears that the falling phase of the ON response may not be a single exponential (indeed, this may be the case in Fig. 5). However, the magnitude of other components compared to baseline noise was always too small to allow any sort of quantitative estimate of this effect. In any case, even if more than one component was present, the superposition of the results demonstrate that the temperature dependence is essentially identical for the entire response.

The overall data are summarized in Fig. 7. The ON and OFF response time constants were determined by performing a least-squares exponential fit to the falling phase of the asymmetry current records and were plotted as a function of temperature. In addition, the total charge moved was determined from the zero time extrapolated asymmetry current (I_o) and time constant via the relation $Q = I_o\tau$ (Table IV). The upper solid line in Fig. 7 represents the best fit to the ON response data and corresponds to a Q_{10} of 2.2. The lower line has the same slope and has simply been translated so as to provide a good visual fit to the somewhat sparser OFF response data. Note that the Q_{10} previously determined for sodium activation in *Myxicola* was 2.64 ± 0.2 (Schauf, 1973), significantly larger than the asymmetry current result but in reasonable agreement with the Q_{10} derived in the present studies from the temperature dependence of time-to-peak I_{Na} (Fig. 2).

In contrast to these results, neither the total charge moved during a large depolarizing pulse, nor the voltage dependence of charge movement are appreciably sensitive to changes in temperature (Table IV). All observed variations are well within the limits of experimental uncertainty.

Incomplete Inactivation with Elevated Internal Na^+

Although Na^+ inactivation is nearly complete even at large depolarizations in axons dialyzed with Cs glutamate (Bullock and Schauf, 1979), incomplete inactivation can be induced by elevation of internal [Na^+]. These results are illustrated in Fig. 8. In part *A* the axon was

TABLE IV
TEMPERATURE EFFECTS ON MEMBRANE CHARGE IN *MYXICOLA*

Axon	T	Q_{max}	$V^Q_{1/2}$
	°C	*nC/cm²*	*mV*
78M3	4.0	12.3	N.M.
	12.0	11.3	N.M.
78M5	2.5	13.8	−37
	7.0	13.8	−34
	11.0	13.6	−36
	14.0	12.5	−39
78M6	1.5	10.6	−36
	6.0	13.2	N.M.
	10.0	12.1	−35
	14.0	13.6	N.M.
78M15	0.5	11.8	N.M.
	7.0	11.3	N.M.

N.M. = not measured.

dialyzed with 600 mM Cs^+ and bathed in ASW. In part *B* records were obtained in the same axon at identical potentials after changing the internal solution to 500 mM Na F (containing 100 mM Cs glutamate) and the external solution to Tris sea water. Clearly the inward currents with Cs^+ dialysis inactivate nearly completely while the outward currents with Na^+ dialysis show a residual noninactivating component of Na^+ conductance. Although it is not illustrated, analysis of the time-course of Na^+ activation and inactivation shows that those channels that do inactivate do so with the same time-course as before elevation of $[Na^+]_i$ and that activation is unchanged.

The presence of a residual noninactivating component of sodium conductance appears to depend only on internal sodium being present and not on the nature of the internal anion (Table V). Thus a comparable increase in $I^{\infty}_{Na}/I^{pk}_{Na}$ is observed with either NaF or Na glutamate (78M33) but not with CsF. The effect does not require outward current flow, since

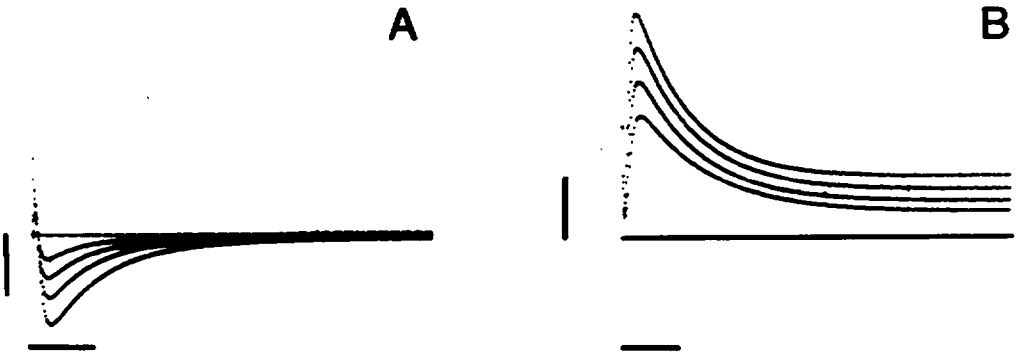


FIGURE 8 Sodium currents measured during depolarizations to +40, +50, +60, and +70 mV in an axon dialyzed first with 600 mM Cs glutamate (*A*), then with 500 mM NaF + 100 mM Cs glutamate. (*B*) Current and time scales are 0.4 mA/square centimeter and 0.5 ms, respectively. Inward current is downward. The sodium currents were corrected for leakage current by repetition of protocols in 10–6 M TTX and subtraction of corresponding records.

TABLE V

DEPENDENCE OF STEADY-STATE Na^+ CONDUCTANCE ON INTERNAL COMPOSITION

Axon	$[Cs^+]_i$	$[Na^+]_i$	$[F^-]_i$‡	$[Na^+]_o$	$\frac{I_{Na}^{\infty *}}{I_{Na}^{k}}$
	mM	*mM*	*mM*	*mM*	
78M23	600	0	50	530	<0.03
	300	300	50	530	0.22
78M25	600	0	50	530	<0.02
	120	480	400	530	0.24
	120	480	400	0	0.22
78M27	600	0	50	530	<0.015
	120	480	400	0	0.29
78M31	600	0	50	530	<0.01
	550	50	50	530	0.21
78M32	600	0	50	530	<0.02
	100	500	500	0	0.30
78M33	600	0	50	530	<0.03
	100	500	50	0	0.25
	100	500	500	0	0.23

*Ratio of I_{Na} at 10 ms to peak I_{Na} at a potential of +40 mV.
‡Remainder of anion is composed of glutamate.

with 50 mM Na^+ internally (78M31) there was a comparable residual noninactivating component for inward currents. Finally, it appears that so long as the internal Na^+ is 50 mM or more, residual component is present and does not increase very much with increasing Na^+ concentration (compare 78M31 to other data).

As a function of membrane potential (Fig. 9) the amount of residual I_{Na} varies from 4–6% at negative voltages to 25–30% at large positive potentials. However, because of the time elapsed between recordings of I_{Na} and leakage current, and the leakage current rectification that exists even in the absence of internal K^+, it is not completely certain whether a residual I_{Na} of 4–6% represents a true incomplete I_{Na} inactivation or simply an experimental baseline error. Attempts to resolve the issue by using tail currents were unsatisfactory because of the small magnitude of the signals.

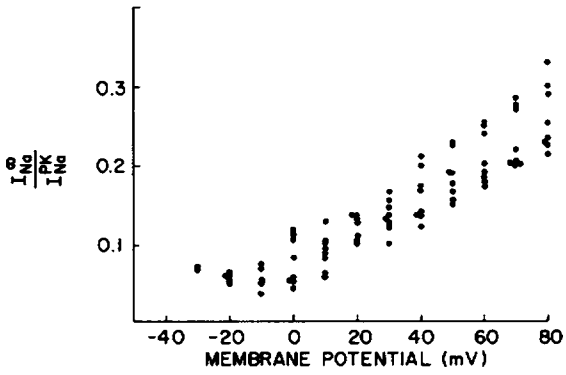


FIGURE 9 Residual Na^+ current as a function of membrane potential in axons dialyzed with high internal Na^+. Residual I_{Na} is expressed as a fraction of peak current.

An attempt was made to record membrane asymmetry currents in TTX-poisoned axons dialyzed with high [Na^+] solutions; however, this was unsuccessful. Although 10^{-6} M TTX will completely block inward I_{Na} in axons dialyzed with Cs^+ and Na^+, there is a very small amount (<0.5%) of residual outward current at large potentials that interferes with asymmetry current measurements during the later part of the falling phase of the ON response. In particular, this Na^+ current combines with the asymmetry current to produce a record that is clearly multiexponential, but this contamination is impossible to separate from components of the true charge movement. Consequently, we do not know whether incomplete inactivation of I_{Na} is associated with any alteration in membrane asymmetry current.

DISCUSSION

In earlier experiments using voltage-clamped squid giant axons perfused with 275 mM KF, 50 mM tetraethylammonium chloride, and sucrose, Meves (1974) observed that while heavy water substitution increased τ_m by 40% and τ_h by 86%, D_2O did not affect the temporal behavior of membrane asymmetry currents. The results described here both confirm and extend these findings. The increase in τ_m observed at 4°C is in good agreement with the 40–60% increase we obtained, and we also could detect no significant effect on τ_{on} or τ_{off} for the asymmetry currents. At higher temperatures, however, we found that D_2O substitution produces significantly smaller effects on the ionic current kinetics. Meves (1974) also reported a 22% decline in $\bar{G}_{Na}$ in D_2O, while we obtained an average decrease of 27%. In contrast to the kinetic effects of D_2O substitution, there was no significant effect on the voltage dependence of G_{Na} or G_K, a feature not discussed by Meves (1974).

Viewed most directly, the differential effect of D_2O substitution on ionic currents supports the concept that the initial charge movements responsible for the voltage dependence of the Na^+ channel are linked to the ultimate conductance change in a highly complex and perhaps indirect manner. This separation of gating movement from the time-course of transitions to the conducting state was previously inferred from the fact that the kinetics of charge movement do not bear a simple relationship to the activation kinetics of g_{Na} (Neumcke et al., 1976; Bullock and Schauf, 1978), but the D_2O results in addition may provide some clue as to underlying physical mechanisms.

There was a tendency for the rising phase of membrane asymmetry current to be prolonged in D_2O. Whether this rising phase is an artifact produced by the experimental procedures used for separating sodium gating from total displacement current, or whether it represents a lag in the response of the gating charge to changes in electric field is unclear (Bullock and Schauf, 1978). However, the experimental suggestion of an isotope effect confined to the rising phase, together with the differential effect of zinc ions on charge movement (Meves, 1976), suggests that the origin of the rising phase may be mechanistically distinct and worthy of continued investigation with better temporal resolution.

The effects of heavy water substitution have been studied in a wide variety of biological systems (Katz and Crespi, 1970). Unfortunately, interpretation of such results is often difficult because of the existence of a number of distinct physical possibilities. Many of the hydrogen atoms of membrane constituents will exchange with solvent deuterium and such isotopic substitution may change the rate or equilibrium constants for chemical conversions involving bonds to the substituted atoms or adjacent bonds (primary and secondary isotope

effects). In particular, the ratio of the equilibrium constant of the unsubstituted system (K) to that of the deuterium substituted system (K^*) is generally given by:

$$K{:}K^* = I \exp(\Delta E_o/RT),$$

where ΔE_0 is the isotopically induced difference in the separation of the zero point vibrational energies of products and reactants and I is an inertial 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).

For example, the fairly strong C—D and C—H bonds have $\Delta E_0 \sim 1$ kcal/mole. Assuming a large molecule for which I would be near unity, the maximum $K{:}K^*$ ratio will be ~7.0. The temperature dependence of this effect may be calculated directly so that between 5 and 15°C the value of $K{:}K^*$ varies by a factor (Q_{10}) of 0.93. The differences in zero point energies for hydrogen and deuterium "hydrogen" bonds is much smaller (perhaps only 0.14 kcal/mole [Nemethy and Scheraga, 1964]) and the expected Q_{10} therefore is essentially unity.

In addition to the above, important isotope effects are associated with the behavior of liquid H_2O and D_2O as solvents (solvent isotope effects). While the differences in the magnitude of many physical properties, including dipole moment, dielectric constant, hydrogen bond length, and molecular dimensions, are small (Nemethy and Scheraga, 1964), the viscosity, melting point, and heat capacity at a particular temperature are all significantly higher in D_2O. These differences in solvent properties can all be understood as results of a higher degree of structural order in liquid D_2O due to more extensive intermolecular hydrogen bonding. The significant characteristic of this behavior for the present study is that breakdown of structural order with increasing temperature occurs more rapidly in D_2O than in H_2O, so that at higher temperatures the two solvents become similar (Heppolette and Robertson, 1960). The temperature dependence of these properties is thus much greater than that expected from the magnitude of the isotope effect itself.

We have observed that at temperatures in the vicinity of the temperature of maximum density of H_2O, heavy water substitution produces a mild slowing of the ionic current kinetics ($K{:}K^* \sim 1.5$). The marked temperature dependence ($Q_{10} = 0.71$) of this slowing, if it were interpreted as a primary or secondary (nonsolvent) isotope effect, would correspond to a value of ΔE_0 of approximately 4 kcal/mole. Not only is such a difference in zero point energy unreasonable even for a primary effect involving the strongest covalent bonds, but even if several bonds were broken simultaneously, the magnitude of $K{:}K^*$ predicted assuming a ΔE_0 of 4 kcal/mole would be approximately 10^3 times that actually observed.

The fact that the temperature dependence is large, while the effect itself is relatively small, strongly suggests that we are dealing with a process in which the structural ordering of the solvent is of primary importance (Laughton and Robertson, 1969). For example, the activation energy of a rate-influencing step for channel opening may be higher in D_2O because a transition state of the channel involves an energetically unfavorable interaction with the solvent. This would also be consistent with the observation that the steady-state voltage dependence of the ionic conductance shows no isotope effect. If this is true, it would clearly be of interest to try to determine whether significant differences in isotope effects and temperature dependence existed for the separate kinetic processes of Na^+ activation, Na^+ inactivation, and K^+ activation. Unfortunately, the scatter in our data precludes such a detailed

analysis at this time. Finally, the observed decreases in $\bar{G}_{Na}$ and $\bar{G}_K$ may be due to a solvent induced change in channel structure, a change in hydration of the permeant ions, either in bulk solution or within the channel, or simply a decrease in channel population. Further studies of this effect, and in particular its temperature dependence, may help discriminate among such possibilities.

In their examination of the temperature dependence of membrane asymmetry currents in squid axons, Bezanilla and Taylor (1978) reported a small increase in total charge movement with increasing temperature (~1.3% per degree), but could not fit the ON response at different temperatures by any single temporal expansion. The early portion of the ON response could be described by using a scaling factor of 1.45 for a 10°C temperature change, but at later times a scaling factor of 2.2 was required. Kimura and Meves (1977), on the other hand did not analyze the behavior of τ_{on} and τ_{off}, but reported a large Q_{10} (~2) for total charge movement between 0 and 12°C in squid axons.

The data described here supports the relative insensitivity of Q_{max} to temperature seen by Bezanilla and Taylor (1978), while the Q_{10} of 2.2 for τ_{on} and τ_{off} we observed is not too different from their result. In particular, we agree that the temperature dependence of the kinetics of charge movement is significantly less than the temperature dependence of the ionic conductance itself. However, in contrast to the squid data, the effects of temperature on I_g in *Myxicola* can be well described via a simple temporal expansion, implying the existence of either a single process or multiple processes with comparable activation energies. Our previous inability to consistently detect a significant slow component in the ON response (Bullock and Schauf, 1978), combined with the absence of a maintained I_{Na} at large voltages in Cs^+-dialyzed *Myxicola* compared to squid (Bezanilla and Armstrong, 1977), might possibly account for our inability to observe a complex temperature dependence of the asymmetry current.

Finally, in spite of the fact that we could not accurately measure asymmetry currents in *Myxciola* axons dialyzed with high Na^+ solutions due to a very small outward I_{Na}, even in the presence of TTX, the ability to induce a noninactivating component of sodium conductance suggests that a second conducting state similar to that hypothesized for squid (Armstrong and Bezanilla, 1977) may be present in this preparation, but simply not be occupied under the same set of conditions. Again, such observations suggest that a variety of cation/anion/channel interactions may occur that complicate study of the molecular mechanisms involved in channel gating and lead to difficulty when different preparations are to be compared or a single preparation is examined under different conditions.

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REFERENCES

ALMERS, W. 1978. Gating currents and charge movements in excitable membranes. *Rev. Physiol. Biochem. Pharm.* **82**:97–190.

ARMSTRONG, C. M., and F. BEZANILLA. 1974. Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. Gen. Physiol.* **63**:533–552.

ARMSTRONG, C. M., and F. BEZANILLA. 1977. Inactivation of the sodium channel. II. Gating current experiments. *J. Gen. Physiol.* **70**:567–590.

BEZANILLA, F., and C. M. ARMSTRONG. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* **70**:549–566.

BENZANILLA, F., and R. E. TAYLOR. 1978. Temperature effects on gating currents in the squid giant axon. *Biophys. J.* **23**:479–484.

BULLOCK, J. O., and C. L. SCHAUF. 1978. Combined voltage-clamp and dialysis of *Myxicola* axons: Behavior of membrane asymmetry currents. *J. Physiol. (Lond.).* **278**:309–324.

BULLOCK, J. O., and C. L. SCHAUF. 1979. Immobilization of intramembrane charge in *Myxicola* giant axons. *J. Physiol. (Lond.).* **286**:157–171.

GOLDMAN, L., and C. L. SCHAUF. 1973. Quantitative description of sodium and potassium currents and computed action potentials in *Myxicola* giant axons. *J. Gen. Physiol.* **61**:261–284.

HEPPOLETTE, R. L., and R. E. ROBERTSON. 1960. The temperature dependence of the solvent isotope effect. *J. Am. Chem. Soc.* **83**:1834–1838.

KATZ, J. L., and H. L. CRESPI. 1970. Isotope effects in biological systems. *In* Isotope Effects in Chemical Reactions. C. J. Collins and N. S. Bowman, editors. Van Nostrand Reinhold Company, New York. 286–363.

KEYNES, R. D., and E. ROJAS. 1976. The temporal and steady-state relationships between activation of the sodium conductance and movement of the gating particles in squid giant axons. *J. Physiol. (Lond.).* **255**:157–189.

KIMURA, J. E., and H. MEVES. 1977. Effect of temperature on the asymmetrical charge movement in squid giant axon. *J. Physiol. (Lond.).* **271**:14p.

LAIDLER, K. J. 1969. Theories of Chemical Reaction Rates. McGraw-Hill Book Company, New York.

LAUGHTON, P. M., and R. E. ROBERTSON. 1969. Solvent isotope effects for equilibria and reactions. *In* Solute-Solvent Interactions. J. F. Coetzee and C. D. Ritchie, editors. Marcel Dekker Inc., New York. 402.

MELANDER, L. 1960. Isotope Effects on Reaction Rates. Ronald Press, New York.

MEVES, H. 1974. The effect of holding potential on the asymmetry currents in squid giant axons. *J. Physiol. (Lond.).* **243**:847–867.

MEVES, H. 1976. The effect of zinc on the late displacement current in squid giant axons. *J. Physiol. (Lond.).* **254**:787–801.

MEVES, H., and W. VOGEL. 1977. Inactivation of the asymmetrical displacement current in giant axons of *Loligo forbesi*. *J. Physiol. (Lond.).* **267**:377–393.

NEMETHY, G., and H. A. SCHERAGA. 1964. Structure of water and hydropholic bonding in proteins IV. The thermodynamic properties of liquid deuterium oxide. *J. Chem. Phys.* **41**:680–687.

NEUMCKE, B., W. NONNER, and R. STÄMFLI. 1976. Asymmetrical displacement current and its relation with activation of sodium current in the membrane of frog myelinated nerve. *Pflügers Arch. Eur. J. Physiol.* **363**:193–203.

SCHAUF, C. L. 1973. Temperature dependence of ionic current kinetics in *Myxicola* giant axons. *J. Physiol. (Lond.).* **235**:197–205.

SCHAUF, C. L. and F. A. DAVIS. 1975. Further studies of activation-inactivation coupling in *Myxicola* axons: insensitivity to changes in calcium concentration. *Biophys. J.* **15**:1111–1116.

SCHAUF, C. L., J. O. BULLOCK, and T. L. PENCEK. 1977. Characteristics of sodium tail currents in *Myxicola* axons: comparison with membrane asymmetry currents. *Biophys. J.* **19**:7–28.

THORNTON, E. L., E. R. THORNTON. 1970. Origin and interpretation of isotope effects. *In* Isotope Effects in Chemical Reactions. C. J. Collins and N. S. Bowman, editors. Van Nostrand Reinhold Company, New York. 286–363.

TRONSTAD, L., and K. STOKLAND. 1937. Electrical conductivities and ion mobilities in heavy water. *K. Nor. Vidensk. Selsk. Forh.* **10**:141–144.