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Sodium Flux Ratio in Voltage-clamped Squid Giant Axons

TED BEGENISICH and DAVID BUSATH

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ABSTRACT The sodium flux ratio across the axolemma of internally perfused, voltage-clamped giant axons of Loligo pealei has been measured at various membrane potentials. The flux ratio exponent obtained from these measurements was about unity and independent of membrane voltage over the 50 mV range from about -20 to +30 mV. These results, combined with previous measurements of ion permeation through sodium channels, show that the sodium channel behaves like a multi-ion pore with two ion binding sites that are rarely simultaneously occupied by sodium.

INTRODUCTION

Excitable membranes contain several types of ionic channels through which several millions of ions per second can pass. Yet these same channels can select among ions of various charges, sizes, and hydrogen bonding capacities. Historically, the permeation of ions through these pores has been described by free diffusion models in which the passive movement of an ion is independent of the presence of other ions. One criterion of such models is that the ionic current should increase linearly with permeant ion concentration. However, recent experiments have shown that the ionic currents through sodium channels in nerve saturate as the concentration of permeant ions is raised (Hille, 1975; Mozhayeva et al., 1977; Begenisich and Cahalan, 1980 b). In potassium channels of giant axons the unidirectional potassium efflux is inhibited by external potassium (Hodgkin and Keynes, 1955; Begenisich and De Weer, 1977), also a violation of independence.

Another criterion of independent ion movement is that the relationship between efflux, $m_e$, and influx, $m_i$, should obey the Ussing (1949) flux-ratio equation:

$$\frac{m_e}{m_i} = \frac{[Na]_i}{[Na]_o} \exp \left( \frac{V_m F}{RT} \right) = \exp \left( \frac{[Na]_o}{[Na]_i} \right) \frac{F}{R T},$$

where $[Na]_i$ and $[Na]_o$ are the internal and external ion activities of, in this example, Na; $V_m$ is the membrane potential; $R$, $T$, and $F$ have their usual thermodynamic meanings; $V_{Na} = (RT/F) \ln [Na]_o/[Na]_i$ is the sodium equilibrium potential. The movement of potassium ions through potassium...
channels of giant axons cannot be described by this equation. Rather, as first discovered by Hodgkin and Keynes (1955), the data are consistent with Eq. 1 only if the right-hand side is raised to a power \( n' \), called the flux ratio exponent:

\[
\frac{m_e}{m_i} = \exp (n'[V_m - V_K]F/RT).
\]

Hodgkin and Keynes (1955) found \( n' \) values near 2.5 for Sepia axons, and in squid axons, \( n' \) is between 1.5 and 3.3, depending upon membrane potential and internal potassium concentration (Begenisich and De Weer, 1980). A simple interpretation of these results is that the potassium channel of nerve is a multi-ion pore with three or more binding sites for potassium ions (Heckmann, 1972; Hille and Schwarz, 1979).

Although the ionic currents through sodium channels of nerve have been shown not to obey independence criteria, flux-ratio measurements have not been made. In this paper we present the results of our measurements of flux ratio and \( n' \) for sodium fluxes through sodium channels of squid giant axons. In contrast to the large values in potassium channels, \( n' \) is only slightly, if at all, greater than unity and is independent of membrane potential from about -20 to about +30 mV. These results, combined with previous measurements of ion permeation through sodium channels (Begenisich and Cahalan, 1980 a and 1980 b), suggest that the sodium channel acts like a multi-ion pore, with two binding sites for Na ions, and that both sites are rarely simultaneously occupied by Na under physiological conditions.

**Methods**

These experiments were performed on well-cleaned giant axons of Loligo pealei obtained from the Arrive Alive Biological Supply Company, Long Island, New York. Animals were delivered in three ways: decapitated with the mantles shipped in ice-cold seawater, live by air freight, and live via truck. Live animals were maintained in a 400-gal marine aquarium for as long as 3 d. Axons from fresh mantles were indistinguishable from those of fresh-killed squid. However, axons from mantles that had been stored for several hours often had to be discarded. The diameter of the axons used in this study ranged from 400 to 700 \( \mu \)m, with an average of 518 \( \mu \)m. The resting potential in our standard K-free seawater and Na-free internal solution (Table I) averaged -65 mV.

The internal perfusion and voltage-clamp techniques were similar to those used by Begenisich and Lynch (1974), with modifications of the chamber to allow the measurement of both influx and efflux of radioabeled tracers as described by Begenisich and De Weer (1980). Briefly, the chamber consisted of a 3-mm-long (~30-\( \mu \)-vol) central chamber and two "guard" regions separated by petroleum jelly seals. The electrical resistance between the central and guard compartments with an axon in place was between 100 and 600 k\( \Omega \). Tests with a small differential current electrode showed that the Na currents were strikingly uniform in the central region and equal to the currents in the guard regions near the seals. A series resistance compensation of 4–6 \( \Omega \) cm\(^2\) typically was used.

1 Bean, B. P. Sodium channel inactivation in the crayfish giant axon: must channels open before inactivating? In preparation.
The voltages reported are all corrected for the junction potential between the 0.56 M KCl internal electrode and the internal perfusion solutions. These were measured on two separate occasions for each of the internal solutions used.

The artificial seawater (ASW) used in these experiments contained (mM) NaCl, 440; CaCl2, 10; MgCl2, 50; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) buffer, 5 (pH 7.4-7.5; 940-970 mosmol/kg). The internal solutions used are listed in Table I. The pH of these solutions was 7.2-7.5 and osmolarity was 940-980 mosmol/kg. Because we were interested in the movement of Na ions through sodium channels, potassium channel currents were eliminated by using one of the K-free internal solutions listed in Table I. Of the external and internal cations, only sodium is significantly permeant in the sodium channel. All experiments were performed at 10°C.

**Influx Measurements**

Unidirectional 22Na influx was measured with ASW containing ~250 µCi/ml 22Na. This solution flowed through the central portion of the chamber at 2-3 µl/min; the guard regions were washed with "cold" ASW at 0.2 ml/min. Samples (1-µl) of the central chamber were obtained approximately every 5 min to monitor tracer specific activity. The solution in the central compartment also contained 1 mM phenol red, so that any leak across the seals could be detected and remedied. The internal perfusate was collected by flowing isotonic sucrose at 2 ml/min across the cut end of the axon and into a fraction collector. Typically, 1-min, but occasionally 2-min samples, were collected. The 22Na content was determined by gamma counting.

**Efflux Measurements**

For the efflux experiments, the axon was perfused at ~2 µl/min with a K-free solution (see Table I) containing ~250 µCi/ml 22Na. The central and guard compartments were washed independently with ASW at 1-2 ml/min and 0.1-0.2 ml/min, respectively. The outflow from the central compartment was directed to a fraction collector, and the 22Na content was determined as described above.

**Electrical Measurements**

Membrane current in the central region was sampled by a 12-bit A/D converter controlled by a Data General Corp. (Westboro, Mass.) Nova 3/12 computer and stored on magnetic disk for further analysis. The membrane potential was maintained at ~65 or ~77 mV and, for periods of 4-6 min, depolarized every 100 or 200 ms to, usually, ~27, ~17, ~7, +23, or +33 mV. The duration of these pulses was 4, 5, or 10 ms: in all cases, long enough for the inactivation of Na channels to reach steady state. Four digitized records of membrane current were stored for each minute of stimulation. Leakage currents were usually obtained by repeating the protocol after 1 µM tetrodotoxin (TTX) had been added to the ASW. In a few experiments a linear

---

**TABLE I**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Na</th>
<th>K</th>
<th>TMA</th>
<th>Cs</th>
<th>F</th>
<th>Glutamate</th>
<th>Glycine</th>
<th>Phosphate</th>
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<tr>
<td>K SIS</td>
<td>350</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>50</td>
<td>270</td>
<td>390</td>
</tr>
<tr>
<td>200 Na SIS</td>
<td>200</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>100</td>
<td>50</td>
<td>690</td>
</tr>
<tr>
<td>Na/Cs SIS</td>
<td>50</td>
<td>--</td>
<td>--</td>
<td>150</td>
<td>--</td>
<td>150</td>
<td>150</td>
<td>690</td>
</tr>
<tr>
<td>Na/TMA SIS</td>
<td>50</td>
<td>150</td>
<td></td>
<td></td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
leakage current was assumed. The leakage-corrected current records were then integrated over the pulse length, and all the integrals during the stimulation period were averaged to yield a measure of sodium current (or net flux).

We often observed that for our largest depolarizations a reversible progressive reduction of sodium current occurred during the stimulation period. This was probably the result of the slow inactivation processes in these channels (Adelman and Palti, 1969; Rudy, 1978). We were able to minimize, eliminate, or even reverse this decline by preceding the depolarizing pulse with a 20- to 30-mV hyperpolarizing conditioning pulse lasting 20-50 ms. This procedure also served to ensure that all the inactivation produced by one depolarizing pulse was removed before the next was delivered.

**Flux-Ratio Exponent**

In these experiments the flux-ratio exponent, \( n' \), was determined from the following equation:

\[
 n' = \frac{RT}{(V_m - V_{Na})F} \ln \left( \frac{m_e}{m_i} \right).
\]  

The membrane potential, \( V_m \), was set by the voltage-clamp circuit, and \( V_{Na} \) was measured as the reversal potential for sodium current. Except in one experiment, both unidirectional sodium fluxes were not obtained. Rather, net sodium flux (from the integral of sodium current) and one unidirectional \( ^{22}\text{Na} \) flux were measured, the remaining unidirectional flux was computed from these measured values. We call these the efflux and influx methods, denoting the \( ^{22}\text{Na} \) flux actually measured.

A requirement for the validity of these methods is that the membrane area through which sodium current and \( ^{22}\text{Na} \) flux are measured be identical. Although we attempted to confine the radioactive tracer to the central compartment, where current was measured, the areas would not be the same if, for instance, a leak in the petroleum jelly seal allowed tracer to contaminate the guard region. In some cases, leaks adjacent to the axon could be seen to contaminate the solution near the axon in one of the guard regions. These leaks were generally repaired successfully before the experiments. Samples of the fluid in the guard regions contained <0.1% of the specific activity of the central pool.

A quantitative test of the equality of these areas can, in principle, be made at potentials sufficiently negative that efflux makes a negligible contribution to net current. For example, with \( n' \) equal to unity (which is approximately our measured value), efflux is only 1% of the net flux at \(-60\) mV. However, this potential is too negative to activate an appreciable amount of sodium current. Instead, we typically used pulses to \(-27\) or \(-7\) mV and determined the ratio of net flux to influx, which is expected to be (for \( n' = 1 \)) 0.96 and 0.94, respectively. In 10 experiments the measured influx had to be multiplied by an average of \( 1.06 \pm 0.10 \) (SEM, \( n = 10 \)) to meet these expectations. We call this number the scale factor. (If \( n' > 1 \), the scale factor would be closer to unity.) That is, the areas for isotopic flux and ionic current agreed to within 6%. The large standard error in the scale factor arises chiefly from three experiments (axons SQDDM, SQDDN, SQDDP) carried out before modifications were made in the experimental chamber to improve the petroleum jelly seals. The remaining seven experiments yielded an average value of \( 1.04 \pm 0.05 \).

For the membrane potentials used here, influx and current are of the same magnitude. Therefore, calculation of the efflux as the difference between these two numbers is sensitive to even small differences in isotope and current membrane areas. Consequently, at least two potentials were used for all axons for which the influx
method was employed: one to allow determination of the scale factor, the other for determining flux ratio and \( n' \). No scaling was applied with the efflux method; the results would not be changed appreciably if the average factor of 1.04–1.06 were used.

**Sources of Error**

In contrast to similar experiments on potassium channels (Begenisich and De Weer, 1980; Mullins et al., 1962), accumulation or depletion of ionic concentrations in the periaxonal space is expected to be insignificant. The inactivation of Na channels limits the net flux in or out of this space to rather small values.

The inactivation of these channels also limits the error produced by the "tail" currents that flow briefly upon repolarization. Typically, the amount of current during the tail was 1–2% of that during the test pulse: too small to cause significant errors in our estimates of \( n' \) with the efflux method but perhaps large enough to slightly underestimate \( n' \) determined with the influx method at negative potentials.

Errors in the measurement of membrane potential could arise from electrode drift or variability in junction potentials. These errors would contribute equally to \( V_m \) and \( V_{Na} \), however, and therefore do not effect the calculated \( n' \). We estimate errors in the difference, \( V_m - V_{Na} \), to be <2 mV.

The sources and magnitudes of the errors for the influx and efflux methods are probably different. The relatively good agreement of results with these two techniques supports the validity of these methods.

**RESULTS**

**Efflux Method**

Fig. 1 A shows an example of the measurement of \( ^{22} \text{Na} \) efflux. For about the first 10 min the membrane potential was maintained at −67 mV, and the measured flux during this period reached a resting level of ~15 pmol/cm\(^2\) s. Then, for the next 5 min, the membrane potential was stepped every 100 ms to +23 mV for 4 ms. The efflux increased to a relatively constant level of ~75 pmol/cm\(^2\) s and after the stimulation period returned to about the same low resting level. Because there were 10 pulses per second, the extra flux per pulse was 6 pmol/cm\(^2\) [(75 − 15)/10]. Another stimulation period, to a membrane potential of −7 mV, is also shown. (This period was inadvertently interrupted for ~1 min, which produced a corresponding dip in the flux record.) The \( ^{22} \text{Na} \) efflux per pulse during this period was 1.7 pmol/cm\(^2\). At the time indicated by the arrow, TTX was added to the ASW bathing the axon, resulting in a slight decrease in resting efflux. More significantly, no increase in \( ^{22} \text{Na} \) efflux occurred when the pulse periods were repeated, demonstrating that all of the measured extra flux was through sodium channels.

The inset in Fig. 1 A shows the sodium current records obtained during each stimulation period. These currents were fairly constant for the 5-min stimulation period, especially when the relatively high stimulation rate is considered. The integrals of the sodium currents were averaged and converted to flux units. These values represent net flux and were 35.5 and 23.8 pmol/cm\(^2\) per pulse for the +23 and −7 mV voltages, respectively. The calculated influxes for these potentials are 41.5 and 25.5 pmol/cm\(^2\) per pulse.

The calculation of \( n' \) requires a value for the sodium equilibrium potential, \( V_{Na} \). An example of the measurement of \( V_{Na} \) is shown in Fig. 1 B for the axon...
Axon SQDEU

![Graph A](image)

![Graph B](image)

**Graph A**
- Y-axis: Na Efflux (pmol/cm²)
- X-axis: Minutes
- Key conditions: +23 mV, -7 mV, +23 mV - 7 mV

**Graph B**
- Y-axis: \( I_m (\text{pA/cm}^2) \)
- X-axis: \( V_m (\text{mV}) \)
- Data points at: -80, 0, 100
illustrated in Fig. 1 A. The zero-current voltage is \( V_{Na} \) and is 60.5 mV in this example. The average value of \( V_{Na} \) for the efflux experiments was 53.9 ± 2.6 (SEM, \( n = 7 \)) mV; the average value for the influx experiments was 54.6 ± 1.2 (SEM, \( n = 9 \)). These values are close to the expected value of 51.2 mV calculated with activity coefficients of 0.68 and 0.74 for the external and internal solutions, respectively (Robinson and Stokes, 1965; see also Begenisich and Cahalan [1980 a]).

### Table II

<table>
<thead>
<tr>
<th>Axon</th>
<th>( V_m )</th>
<th>( V_{Na} )</th>
<th>( m_s )</th>
<th>( m_{Net} )</th>
<th>( m_i )</th>
<th>( m_s/m_i )</th>
<th>( n' )</th>
</tr>
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<tbody>
<tr>
<td>SQDDT</td>
<td>-7</td>
<td>58</td>
<td>2.88</td>
<td>28.9</td>
<td>31.8</td>
<td>0.091</td>
<td>0.91</td>
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<tr>
<td></td>
<td>33</td>
<td>8.6</td>
<td>11.0</td>
<td>19.6</td>
<td>0.44</td>
<td>0.81</td>
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<td>SQDDU</td>
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<td>58</td>
<td>1.88</td>
<td>26.2</td>
<td>28.1</td>
<td>0.067</td>
<td>1.01</td>
</tr>
<tr>
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<td>33</td>
<td>5.23</td>
<td>9.46</td>
<td>14.7</td>
<td>0.36</td>
<td>1.0</td>
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<tr>
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<td>-7</td>
<td>56</td>
<td>3.22</td>
<td>43.8</td>
<td>47.0</td>
<td>0.069</td>
<td>1.04</td>
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<tr>
<td></td>
<td>23</td>
<td>9.9</td>
<td>47.4</td>
<td>57.3</td>
<td>0.173</td>
<td>1.3</td>
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<td>SQDDZ</td>
<td>-7</td>
<td>40</td>
<td>1.06</td>
<td>8.9</td>
<td>9.96</td>
<td>0.106</td>
<td>1.17</td>
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<td>SQDEA</td>
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<td>52</td>
<td>2.14</td>
<td>18.0</td>
<td>20.1</td>
<td>0.106</td>
<td>0.93</td>
</tr>
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<td></td>
<td>23</td>
<td>9.64</td>
<td>17.9</td>
<td>27.5</td>
<td>0.33</td>
<td>0.89</td>
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<td>SQDET</td>
<td>-7</td>
<td>53</td>
<td>2.63</td>
<td>27.8</td>
<td>30.4</td>
<td>0.086</td>
<td>1.0</td>
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<td></td>
<td>23</td>
<td>10.2</td>
<td>27.4</td>
<td>37.6</td>
<td>0.27</td>
<td>1.07</td>
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<td>60.5</td>
<td>6.0</td>
<td>35.5</td>
<td>41.5</td>
<td>0.145</td>
<td>1.26</td>
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<tr>
<td>-7</td>
<td>1.7</td>
<td>23.8</td>
<td>25.5</td>
<td>0.067</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean±SEM (\( n \)) | -7 | 53.9±2.6(7) | 1.01±0.03 (7) |
| 23,33 | 1.06±0.08 (6) |

With the fluxes and sodium equilibrium potential determined as described above, we calculated for the experiments of Fig. 1 \( n' \) values of 1.26 and 0.98 at membrane potentials of 23 and -7 mV, respectively. The results of this and six other efflux experiments, all with Na/Cs SIS are summarized in Table II. The mean value of \( n' \) is 1.01 ± 0.03 (\( n = 7 \)) at -7 mV and 1.06 ± 0.08 (\( n = 6 \)) at 23 and 33 mV. These are not significantly different (\( P > 0.2 \)), indicating that \( n' \) has little or no voltage dependence over this voltage range. The overall average is 1.03 ± 0.04 (\( n = 13 \)).

**Figure 1.** (A) Sodium efflux from axon with membrane potential held at -67 mV. During intervals marked by arrows, 4-ms depolarizations to voltage indicated preceded by 30-ms hyperpolarizations to -97 mV were imposed 10 times per second. At the arrow, the ASW was replaced with ASW + 1 \( \mu \)M TTX. **(Inset)** Current records obtained during stimulation periods. The bottom two are original records in TTX ASW for depolarizations to +23 and -7 mV. The top two are the result of subtracting the bottom two records (scaled by factors of 0.95 and 0.74 to 0.80, respectively, to compensate for increased leak conductance) from the original records in ASW for depolarizations to +23 and -7 mV. Calibration bars, 1 ms × 1 mA/cm². **(B)** Peak sodium current vs. voltage used to interpolate reversal potential Na/Cs SIS.
Influx Method

An example of an influx experiment is shown in Fig. 2 A. In this experiment two stimulation rates (10 and 5 Hz) were used for pulses to -7 mV. The measured influx at the low frequency was about half that at the high frequency, as expected. The average influx value for these two periods was 62 pmol/cm² per pulse. After a brief rest period the axon was pulsed (at 5 Hz) to 33 mV, and the influx per pulse reached a value of 48 pmol/cm². Again, the addition of TTX reduced the resting level, and no extra influx occurred during the stimulation periods. There is a bump of flux at ~40 min because the voltage clamp was off during this period, and the fiber was probably somewhat depolarized.

The net flux per pulse for membrane potentials of -7 and +33 mV was found by integrating the sodium current records, yielding values of 41.9 and 22.9 pmol/cm², respectively. A scale factor of 0.74 was calculated from the -7 mV level and applied to the measured influx at +33 mV. The calculated efflux was then 12.4 pmol/cm². With these values and a $V'_{Na}$ of 55 mV, $n'$ at 33 mV was calculated to be 1.17.

Another example of an influx experiment is shown in Fig. 2 B. The measured influxes in this case increased slowly during the stimulation periods and then decreased slowly after the stimulation had ceased: no apparent steady state is reached. This abnormally sluggish response resulted from an abnormally slow flow of internal solution. The $^{22}$Na influx here was obtained by integrating the flux records, yielding values of 19, 25, and 16.7 pmol/cm² per pulse for membrane voltages of -27, -7, and 23 mV. The corresponding net fluxes were 20, 24.6, 13.1 pmol/cm², respectively. A scale factor of 1.1 was determined from the -27-mV values, and at -7 and 23 mV the $n'$ values were calculated to be 0.96 and 1.1.

A summary of the results of our influx experiments is shown in Table II. The average $n'$ value for all influx experiments (except SQDEO) is 0.97 ± 0.04 ($n$ = 13), which is not significantly different ($P > 0.2$) from the value of 1.03 obtained with the efflux method. Also, in agreement with the efflux method, voltage has no significant effect on $n'$.

Two types of internal solutions with 50 mM Na were used in the experiments of Table II: Na/Cs SIS and Na/tetramethylammonium (TMA) SIS. The $n'$ values with TMA are a bit lower than, but generally consistent with, those from axons with Cs. One of these axons (SQDER) was treated with the
proteolytic enzyme papain, which removed the inactivation process. The value of $n'$ for this fiber was 0.83, a little smaller than the average but within the range of $n'$ values from axons with intact inactivation.

We attempted to determine $n'$ values at elevated internal Na levels. In four of five attempts with 200 Na SIS, resting influx levels were too large and unstable for reliable estimates of $n'$ to be made. The one experiment (SQDEO) in which $n'$ could be measured yielded a value of 0.8, which is at least similar to the values with 50 mM Na.

In one experiment (SQDDW), first $^{22}$Na efflux and then $^{22}$Na influx were measured. Because of the length of time necessary to wash residual $^{22}$Na from the interior of the axon, an hour elapsed between the efflux and influx measurements. Even after this long time, there was still some residual radioactivity in the axon, so that the stimulated influx was superimposed on a large

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**FIGURE 3.** Ratio of calculated efflux to influx (influx method) or efflux to calculated influx (efflux method) plotted semilogarithmically against electric driving force. Straight lines represent values expected if $n'$ is constant with voltage at 0.8, 1.0, and 1.2. Symbols are measured values for all experiments reported in Tables I and II with SIS as indicated.
<table>
<thead>
<tr>
<th>Axon</th>
<th>SIS</th>
<th>$V_m$</th>
<th>$V_m$</th>
<th>$m_i$ raw</th>
<th>$m_i$</th>
<th>SF</th>
<th>$m_i$ (scaled)</th>
<th>$m_o$</th>
<th>$m_o/m_i$</th>
<th>$n'$</th>
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</thead>
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<tr>
<td>SQDDM</td>
<td>Na/Cs</td>
<td>33</td>
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<td>48.3</td>
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<td>1.14</td>
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<td>Na/Cs</td>
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<td>0.72</td>
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<td>Na/Cs</td>
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<td>Na/Cs</td>
<td>23</td>
<td>56</td>
<td>25.0</td>
<td>24.6</td>
<td>1.1</td>
<td>27.5</td>
<td>2.85</td>
<td>0.104</td>
<td>0.96</td>
</tr>
<tr>
<td>SQDEO</td>
<td>200 Na</td>
<td>23</td>
<td>52</td>
<td>16.7</td>
<td>13.1</td>
<td>1.3</td>
<td>18.3</td>
<td>3.2</td>
<td>0.29</td>
<td>1.10</td>
</tr>
<tr>
<td>SQDEQ</td>
<td>Na/TMA</td>
<td>+8</td>
<td>56</td>
<td>15.5</td>
<td>11.9</td>
<td>0.94</td>
<td>14.6</td>
<td>2.67</td>
<td>0.18</td>
<td>0.87</td>
</tr>
<tr>
<td>SQDEQ</td>
<td>Na/TMA</td>
<td>+13</td>
<td>61</td>
<td>14.6</td>
<td>40</td>
<td>1.2</td>
<td>50.0</td>
<td>10.0</td>
<td>0.20</td>
<td>0.83</td>
</tr>
<tr>
<td>SQDES</td>
<td>Na/TMA</td>
<td>+13</td>
<td>61</td>
<td>16.8</td>
<td>16.2</td>
<td>1.04</td>
<td>17.5</td>
<td>1.27</td>
<td>0.073</td>
<td>0.93</td>
</tr>
<tr>
<td>SQDFD</td>
<td>Na/Cs</td>
<td>-7</td>
<td>52</td>
<td>8.2</td>
<td>7.8</td>
<td>1.04</td>
<td>8.5</td>
<td>0.73</td>
<td>0.085</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean±SEM(n) Na/Cs</td>
<td>-7</td>
<td>53.8±1.2(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.96±0.02(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SEM(n) Na/TMA</td>
<td>-17, -22</td>
<td>56.3±2.6(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.85±0.08(2)</td>
<td></td>
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</tr>
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</table>
background level. During this period the Na currents declined by 43%. The $n'$ values determined with the efflux method were 1.04 and 1.30 at -7 and 23 mV, respectively. With the influx technique, a value of 0.93 at 23 mV was found. Considering the difficulties of attempting both methods on the same axon, the agreement between $n'$ values is not unreasonable.

A graphical representation of the data in Tables II and III is shown in Fig. 3. Here the flux ratio ($m_o/m_i$) is plotted vs. $V_m - V_{Na}$ on a semilog scale. For this type of plot Eq. 2 predicts a straight line with the slope determined by $n'$. The data scatter about the $n' = 1$ line and fit fairly well within the $n' = 0.8$ and $n' = 1.2$ lines. A nonconstant $n'$ value would be expected to produce a systematic deviation from the linear relationship between flux ratio and $V_m - V_{Na}$ shown here. A linear least squares fit to the logarithm of the flux ratio gives $n' = 0.96 \pm 0.02$. This is very similar to the value of 1.01 obtained from a nonlinear least squares fit to the data. Both are similar to the mean of all measurements of $n'$ listed in Tables I and II, $1.00 \pm 0.03$ ($n = 26$).

**DISCUSSION**

The results of this study show that the sodium flux ratio in sodium channels of squid giant axons with approximately physiological Na concentrations follows the unmodified Ussing (1949) flux-ratio equation, i.e., $n' = 1$. Furthermore, $n'$ shows no detectable membrane voltage dependence over the 55 mV range from -22 to +33 mV. Increasing internal Na to 200 mM or exchanging TMA for Cs are without significant effect on $n'$. This value seems little affected by treatment of the axons with the proteolytic enzyme papain. These results are in contrast to those on K channels in *Loligo* and *Sepia* axons, where $n'$ is between 2 and 3 under physiological conditions and may be a function of $V_m$ and internal K concentration.

A value of unity for $n'$ is incompatible with a pore that transports ions with a strict "knock-on" mechanism (Hodgkin and Keynes, 1955; Hladky and Harris, 1967; Armstrong, 1975). For example, a one-ion pore that obeyed knock-on kinetics would give an $n' = 2$ (Hodgkin and Keynes, 1955; Begenisich, 1979). As Begenisich and Cahalan (1979) point out, the current through such a pore would also fail to saturate, which is incompatible with their observations (Begenisich and Cahalan, 1980 b).

There are at least four general types of pores for which $n'$ values of unity are expected: (a) pores through which Na ions pass independently of each other, (b) one-ion saturating pores (Läuger, 1973; Begenisich and Cahalan, 1979), (c) multi-ion pores with a single large energy barrier (Hille and Schwarz, 1978), and (d) multi-ion pores with almost all sites empty or almost all sites filled (Heckmann, 1972; Hille and Schwarz, 1978). For the sodium channel in squid axons, the first possibility is eliminated by the observation of sodium current saturation with increasing internal Na concentration (Begenisich and Cahalan, 1980 b). Pores of the second and third type can show voltage-dependent but not concentration-dependent permeability ratios (Hille and Schwarz, 1978) as found in these fibers (Chandler and Meves, 1965; Cahalan and Begenisich, 1976; Begenisich and Cahalan, 1980 a), so the sodium channel appears to act as a multi-ion pore without a large central barrier. The finding
that the half-saturating concentration of internal Na is on the order of 600 mM (Begenisich and Cahalan, 1980 b) suggests that, with the low concentration (50 mM) of internal Na used here, the pores are empty, or nearly so, most of the time.

Begenisich and Cahalan (1980 a and 1980 b) estimated from electrical measurements the potential energy barriers that a sodium ion faces as it traverses the membrane. The measurements of unidirectional fluxes or the flux ratio constitute an independent test of these barriers. The three-barrier, two-site model of Begenisich and Cahalan (1980 a and 1980 b) predicts $n'$ values of 1.07 and 1.04 at -20 and +30 mV, respectively, for ASW and 50 mM Na, and 1.11 and 1.16 for 200 mM Na, at these same voltages. These predictions are consistent with our measured values. So the sodium channel appears to act as a multi-ion pore with two sites that are rarely simultaneously filled.

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