Potassium Flux Ratio in Voltage-clamped Squid Giant Axons

TED BEGENISICH and PAUL DE WEER

From the Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, the Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT The potassium flux ratio across the axolemma of internally perfused, voltage-clamped giant axons of Loligo pealei has been evaluated at various membrane potentials and internal potassium concentrations ([K]i). Four different methods were used: (a) independent measurement of one-way influx and efflux of 42K; (b) simultaneous measurement of net K current (IK) and 42K influx; (c) simultaneous measurement of IK and 42K efflux; and (d) measurement of potassium conductance and 42K influx at the potassium equilibrium potential. The reliability of each of these methods is discussed. The average value of the exponent n' in the Hodgkin-Keynes equation ranged from 1.5 at -4 mV and 200 mM [K]i to 3.3 at -38 mV and 350 mM [K]i and appeared to be a function of membrane potential and possibly of [K]i. It is concluded that the potassium channel of squid giant axon is a multi-ion, single-file pore with three or more sites.

In 1949 Ussing published a general relationship between efflux, M_e, and influx, M_i, for the passive, independent movement of an ion across a membrane, such that in the case of potassium for example,

\[
\frac{M_e}{M_i} = \frac{[K]_i}{[K]_o} \exp \left( \frac{V_m F}{RT} \right) = \exp \left( \frac{V_m - V_K}{F/RT} \right),
\]

where [K]i and [K]o are the internal and external ion activities, V_m is the membrane potential, R, T, and F have their usual meaning, and V_K = (RT/F) ln ([K]o/[K]i) is the potassium equilibrium potential. Hodgkin and Keynes (1955) found that the potassium efflux from Sepia giant axons subjected to long-term voltage control was inhibited by external potassium, and we (Begenisich and De Weer, 1977) obtained similar results with Loligo pealei giant axons subjected to short voltage-clamp pulses. These observations demonstrate that potassium ions do not move in an independent manner across the membranes of these cells. Indeed, to describe their flux data Hodgkin and Keynes (1955) found it necessary to raise the right-hand side of the flux-ratio equation (Eq. 1) to a power n':

\[
\frac{M_e}{M_i} = \exp \left( V_m - V_K \right) n' F/RT,
\]

J. GEN. PHYSIOL. © The Rockefeller University Press - 0022-1295/80/07/0083/16 $1.00 83
Volume 76 July 1980 83-98
where the deviation of \( n' \) from unity serves as a measure of nonindependence. Hodgkin and Keynes (1955) obtained a value of about 2.5 for \( n' \) and proposed a "long-pore" model for the K channel in which ions move in single file and cannot pass one another. Recently, a value of \( \sim 2 \) has been calculated for Rb fluxes through channels formed by Gramicidin A in artificial lipid bilayers (Shagina et al., 1978).

We have repeated, on squid giant axons, potassium flux-ratio experiments of the type first performed by Hodgkin and Keynes (1955) on cuttlefish axons. Our axons, however, were internally perfused with various potassium concentrations, and the depolarizing pulses were of short duration (30–40 ms) so as to minimize long-term inactivation of the K channels (Ehrenstein and Gilbert, 1966). We find that the value of the parameter \( n' \) in the Hodgkin-Keynes equation (Eq. 2) is a function of membrane potential and possibly of internal K concentration and ranges from \( \sim 1.5 \) at \(-4 \) mV and 200 mM \([K_i]\) to \( 3.3 \) at \(-38 \) mV and 350 mM \([K_i]\). A preliminary account of these findings has been presented (Begenisich and De Weer, 1979).

**METHODS**

Experiments were performed on well-cleaned giant axons of *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, Mass. The diameter of the axons ranged from 400 to 625 \( \mu \)m (average, 500 \( \mu \)m). The resting potential in our standard (10 mM K) artificial seawater (ASW) and with the standard internal perfusion solution (350 mM K) ranged from \(-58 \) to \(-73 \) mV (average, \(-65 \) mV). The internal perfusion and voltage-clamp techniques used were similar to those described by Begenisich and Lynch (1974), with modifications of the chamber to allow the measurement of both influx and efflux of \(^{42}\)K. The ASW always contained \( 3 \times 10^{-7} \) M tetrodotoxin (TTX) to eliminate sodium currents. The chamber consisted of a 3-mm central and two 4-mm lateral compartments (separated by 1-mm petroleum jelly seals) containing the central and guard current electrodes, respectively, of the voltage-clamp apparatus. Each compartment had its own solution inlet and outlet. The axon was positioned longitudinally across the seals. Cuts were made in the axon at both ends of the chamber; one allowed insertion of the perfusion cannula while the other admitted the "piggy-back" electrode assembly and served as outlet for the internal solution. The cut ends were isolated from the chamber proper by petroleum jelly seals and air gaps of, respectively, 2 and 3 mm.

**Efflux Measurements**

One-way efflux was measured from axons internally perfused at 1.5 \( \mu \)l/min with \(^{42}\)K-labeled solutions. Separate flows of ASW, driven by peristaltic pumps at about 1 ml/min, ran through the three chamber compartments. The outflow from the center compartment was directed to a fraction collector, and the \(^{42}\)K content of the samples was determined by liquid scintillation counting.

**Influx Measurements**

For influx measurements, \(^{42}\)K-labeled ASW was placed in the central compartment. To conserve isotope this solution flowed very slowly (1.5 \( \mu \)l/min) or not at all. Of serious concern was the necessity to confine the labeled ASW to the middle compartment where voltage-clamp currents were also being measured. For this reason the labeled ASW was colored with 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 1 ml/min across the cut end of the axon and into a fraction collector. The \(^{42}\)K content was determined as described above.

**Electrical Measurements**

Membrane current in the central pool was measured by standard techniques, except that the record was sampled every 120 or 200 \(\mu\)s by an 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 \(-68, -78,\) or \(-88\) mV and, for periods lasting 3-6 min, depolarized every 400 ms to \(-38, -28, -18,\) or \(-8\) mV for 40 ms (or, in a few experiments, every 300 ms for 30 ms). A digitized record of membrane current was stored at the beginning, middle, and end of each pulse period. The current (corrected for leak if necessary) was integrated over the length of the pulse, and the three integrals averaged to allow computation of net potassium flux, \(M_{net}\), through the same axon area across which unidirectional \(^{42}\)K flux was measured simultaneously.

We often observed, in the case of large depolarizations, that current and \(^{42}\)K efflux gradually and reversibly decreased by 10-30% during the stimulus period. Several mechanisms could possibly account for this reduction. Potassium channels of depolarized squid axons slowly inactivate with a time constant of 10-30 s (Ehrenstein and Gilbert, 1966). The pulse sequence of the largest depolarizations used here is equivalent to a time-averaged depolarization of the holding potential by 6-8 mV, enough to produce some inactivation. In addition, potassium accumulation in the periaxonal space will reduce both K currents and \(^{42}\)K efflux. Also, at these high stimulation rates, enough hydrogen ions might be produced electrolytically on the current-passing platinum electrode to acidify the perfusate; and, indeed, the internal phenol red solution occasionally turned orange. An internal pH drop from 7.5 to 7.0 would reduce K conductance by 20% (Wanke et al., 1979). This reduction of potassium current was not investigated further for several reasons: (a) the effect occurred only for the largest depolarizations; (b) the reduction was at most 15% when averaged over the test period; and (c) both K current and \(^{42}\)K flux were reduced, a circumstance which tends to leave the computed value of \(n'\) unaffected.

**Flux Ratio Exponent**

The flux ratio exponent, \(n'\), was obtained by four methods, which we call the two-flux, influx, efflux, and potassium conductance methods. In the first three methods \(n'\) is determined by solving Eq. 2 for \(n'\):

\[
\frac{RT}{(V_m - V_K) F} \ln \left( \frac{M_e}{M_i} \right).
\]

**TWO-FLUX METHOD** This is the most direct way of determining \(n'\), yet technically the most difficult. Efflux and influx of \(^{42}\)K are determined consecutively on the same axon under otherwise identical conditions. After measuring \(^{42}\)K efflux, one must remove essentially all \(^{42}\)K from the inside of the axon before proceeding with influx measurements. This creates an inevitable delay of 20-30 min between the two measurements. In principle, simultaneous measurements of influx and efflux could be made with two different isotopes but, unfortunately, a second K isotope is not conveniently available.

In the next two methods, a single one-way isotope flux is measured, and the other is computed using the isotope flux and the simultaneously determined net potassium
current. Net tracer flux and net charge flux are equal by necessity, even though, in certain models, one-way charge movements may differ from one-way transmembrane isotopically labeled ionic fluxes.

**INFLUX METHOD** From the measured $^{42}$K influx and the time integral of the potassium current, K efflux can be computed. This is, in principle, the most accurate "indirect" method for calculating the flux ratio, because efflux usually greatly exceeds influx, and any errors in determining influx do not produce significant errors in computing efflux. As will be shown below, however, the isotope influx measurements can be grossly contaminated with contributions from the "tail current" at the end of each pulse.

**EFFLUX METHOD** In this technique potassium current and $^{42}$K efflux are measured, and K influx is computed. However, at most potentials of interest, efflux and total current are of comparable magnitude (becoming equal at extreme depolarizations) and thus yield unreliable estimates of K influx. Accuracy can be improved by using two depolarizing steps, one of them sufficiently large to justify the arbitrary scaling of efflux to equal net current. (In fact, with $n' = 2$ and $(V_m - V_K) \approx 40$ mV, i.e., test pulses to $-8$ or $-18$ mV, the influx:efflux ratio is $\approx 0.01$). For the second pulse, influx is then computed by subtracting net current from (scaled) efflux. This procedure serves to correct for small differences in the axon area over which current and flux are measured and for any changes in K conductance during the flux measurements. Even with these precautions, the computed influx for one of the experiments had a negative sign and had to be discarded.

**POTASSIUM CONDUCTANCE METHOD** It has been shown (Hodgkin and Keynes, 1955; Abercrombie, 1977; Hille and Schwarz, 1978) that, at a membrane potential equal to the potassium equilibrium potential, potassium slope conductance ($g_K$) and potassium flux $M_e = M_i$ are related by $g_K = n' F^2 M_i / RT$, whence $n'$ can be calculated from a simultaneous measurement of $g_K$ and $^{42}$K influx:

$$n' = RT g_K / F^2 M_i.$$  

For this method we used the same pulse protocol as described above, except that the test pulse potential was adjusted to the voltage ($V_K$), which resulted in zero potassium current. The time course of the potassium conductance, $g_K$, during a test pulse was determined by repolarizing to the holding potential, $V_{HP}$, at various times during the pulse. The resulting tail currents allow computation of $g_K = I_{Ktail} / (V_{HP} - V_K)$. While this is, strictly speaking, the chord conductance, only a small error will result since, at the elevated external and lowered internal potassium concentrations used in this method, the instantaneous K current-voltage relation is approximately linear between $V_K$ and $V_{HP}$.

### Technical Difficulties and Sources of Error

The measurements described here are beset with difficulties and errors (see also Mullins et al., 1962), some simply technical and some unavoidable and due to the nature of the preparation.

A technical requirement for the last three methods is that the membrane area through which potassium current and $^{42}$K flux are measured be identical. In four experiments we compared the time-averaged $I_K$ with $^{42}$K efflux at depolarizations sufficiently large to render them theoretically equal to within 1-2%; their ratio was $1.05 \pm 0.09$ (SEM), evidence that the areas were reasonably well matched. Yet, for
accurate results with the efflux method, we found it necessary to correct for even slight mismatches, as already described.

Problems of interpretation also arise. Operationally, we must measure selectively both the extra current and the extra $^{42}$K flux flowing through newly opened potassium channels during the imposed voltage pulses. In Results we present pharmacological evidence that the observed extra $^{42}$K flux and extra delayed current do, indeed, take place exclusively through potassium channels. Leakage currents were generally so small as to make a negligible contribution to the time integral of the voltage-clamp current record. The exact definition of "extra" $^{42}$K flux is subtle, however. Its definition as flux above baseline assumes that baseline flux (through leakage channels, cut branches, resting Na and K channels, etc.) remains unchanged during the imposed voltage pulse. This is not a valid assumption: during a depolarizing pulse, baseline influx is expected to decrease, and baseline efflux to increase. Our baseline influx was generally several times lower than that seen during the periods of stimulation. Hence, considering that the test pulses occupy only 10% of the time, even if baseline influx were to vanish completely during each test pulse, the underestimation of extra influx, when measured simply from the baseline, would amount to no more than a few percent. We have, therefore, not made any allowance for influx baseline shifts. Baseline efflux, on the other hand, will probably rise during the test pulse. The extent of this rise is unpredictable in the absence of detailed knowledge about the potential profile across the membrane; an upper limit of e-fold per 25 mV depolarization seems reasonable. Again, considering the low baseline effluxes observed (Begenisich and De Weer [1977] and Fig. 4), the fact that the pulses occupy only 10% of the time, and our ignorance concerning the voltage dependence of baseline efflux, we chose not to apply any correction for baseline shift during $^{42}$K efflux measurements. This procedure, by slightly overestimating extra $^{42}$K efflux, may have appreciably overestimated calculated influx (efflux method), and thus have led to the somewhat lower $n'$ values obtained with the efflux method.

Unavoidable problems related to the nature of the preparation are caused mainly by two phenomena: the accumulation of potassium in the periaxonal space and the presence of a "tail current" at the end of the test pulse.

During depolarization from $V_K$, the outflow of potassium ions raises $[K^+]$ in the periaxonal space (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973; Adam, 1973); hence, average $V_K$ during the test pulse is less negative than at rest. In principle, if the thickness of the space and the permeability of its boundary are known, the time average of $[K_o]$ can be computed from the time-course of $I_K$. In the simple case in which $I_K$ is a simple rectangular pulse of duration exactly equal to $\tau$, the time constant for potassium escape from the Frankenhaeuser-Hodgkin space to the bathing medium, it can be shown that the average extra potassium concentration in the space ($[\Delta K_o]$) is related to the final extra potassium concentration in the space ($[\Delta K_o]$) as follows:

$$[\Delta K_o] = [\Delta K_o]_{fn} / (e - 1) = 0.58 [\Delta K_o]_{fn}.$$  

In the case of a current pulse much shorter than $\tau$, $[K_o]$ will be the arithmetic average of initial and final potassium concentration:

$$[\Delta K_o] = 0.50 [\Delta K_o]_{fn}.$$  

Our clamp pulses lasted 30 or 40 ms, compared with a $\tau$ value of 50-100 ms given by Frankenhaeuser and Hodgkin (1956). (In a single experiment on a Loligo pealei axon kindly performed for us by Dr. C. M. Armstrong, Department of Physiology, University of Pennsylvania, a time constant of slightly less than 50 ms was found.) In addition, the characteristic delay in the K current would also tend to lower the averaging coefficient from 0.58, the value given above for a rectangular pulse of duration $\tau$. We therefore computed $[K_o]$ simply as the arithmetic average of nominal
bathing fluid $[K]_0$ and final periaxonal $[K]_0$. The latter was computed from the magnitude of the tail current as described by Connor and Stevens (1971), a method that assumes linear instantaneous conductance over the voltage range under study. Fig. 12 of Hodgkin and Huxley (1952) shows this assumption to be valid, even when periaxonal space loading is minimal. Our tables list average $V_K$ values computed from $[K_0]$ as obtained above, and these were the values entered in Eq. 3 for the calculation of $n'$.

Accumulation of K in the periaxonal space affects efflux and influx measurements differently. Efflux will be underestimated by the amount of $^{42}K$ that reenters the fiber during the test pulse. However, at most of the test voltages and K concentrations used here, influx is small compared with efflux, and no significant error in efflux measurement is caused by this mechanism, even though, in the worst cases, time-averaged specific activity in the periaxonal space may reach 25% of that inside the axon. Influx, on the other hand, is seriously underestimated as a result of dilution of $^{42}K$ specific activity in the periaxonal space by unlabeled potassium leaving the axon. The error is larger for strong depolarizations, and its magnitude can be calculated from the average periaxonal K concentration as described above. We calculate true influx to be on the average, 1.9, 1.6, and 1.3 times larger than the measured one at $-18$, $-28$, and $-38$ mV.

A different set of problems arises from the occurrence, at the end of each test pulse, of a tail current whose time-course reflects mostly the closure of the potassium channels. This K channel current is usually inward because accumulation of K in the periaxonal space has made $V_K$ more positive than the holding potential. The magnitude of this extra inward current is larger the longer and the more depolarized the test pulse (i.e., more K accumulation in the periaxonal space), and its duration is shorter the more hyperpolarized the holding potential (i.e., faster gating kinetics). Whereas tail currents can, of course, be ignored in computing the time integral of $I_K$ during the test pulse, they will give rise to extra $^{42}K$ fluxes that cannot be separated from those in which we are interested. The effects of tail currents on $^{42}K$ influx and efflux measurements will again be different. Efflux will be underestimated because some $^{42}K$ returns to the axon during the tail. This error is very small—no worse than the ratio of areas under the tail and test pulse current traces, multiplied by the fraction of K of intracellular origin in the periaxonal space, or an underestimate of $\sim2\%$ at $-18$ mV, and less for smaller pulses. Nevertheless, small errors in $^{42}K$ efflux measurement seriously affect the influx computed by difference, as explained above. Influx of $^{42}K$, on the other hand, may be seriously contaminated by contributions from the tail current. One can, in principle, estimate the magnitude of these contributions from (a) the area under the tail curve relative to that under the test current curve (on the average, 4.4% after a pulse to $-18$ mV and 6.4% after a pulse to $-38$ mV), (b) the periaxonal K$^+$ concentration during and just after the pulse, and (c) the value of $n'$ at the prevailing test and holding potentials. We have no knowledge of $n'$ for the holding potentials ($-69$ to $-89$ mV) at which the tail currents flowed, but even with $n' = 1$, one-way influx during the tail current will be underestimated by no more than 30% if its magnitude is set equal to the time integral of the tail current itself. Thus, the absolute magnitude of $^{42}K$ influx expected during the tail current can be calculated to within 30% from the current record, regardless of the value of $n'$. (Since it is likely that $n'$ is close to 3, net tail current approximates tail $^{42}K$ influx to within a few percent.) The relative overestimation of "test pulse" $^{42}K$ influx that results from including "tail" $^{42}K$ influx depends strongly on the test voltage. For example, the error is $\sim30\%$ for small test pulses (to $-38$ mV) and as high as 300% for the largest pulses (to $-8$ mV).
From the above discussion it appears that only $^{42}\text{K}$ influx measurements are likely to be seriously affected by extracellular space and tail current problems. These complications, however, are less formidable than they might appear: (a) periaxonal space dilution and tail current contamination produce errors of opposite sign; (b) the relative magnitudes of both these errors vary in the same direction with membrane potential (i.e., they both decrease with decreasing test pulse size); and (c) the calculation of $n'$ from Eq. 3 is relatively insensitive to errors in the measurement of $^{42}\text{K}$ fluxes (for instance, when $V_m - V_K = 25 \text{ mV}$, a 100% flux error adds ±0.7 to the value of $n'$.

Because the potassium conductance method described above involves a test pulse to $V_K$, no net current flows into the periaxonal space. However, the “tail” problem remains. We do not have sufficient experience with this method to estimate the magnitude of errors in influx and efflux measurements. However, the fast kinetics of the tails and the logarithm in Eq. 3 will tend to minimize the errors in computing $n'$.

In conclusion, the four techniques for determining $n'$ described here are afflicted with errors of different origin and magnitude. The relative agreement between $n'$ values obtained by our various procedures, in spite of the errors, suggests that these values are fairly reliable, not so much because the errors are small, but because they tend to compensate for one another and because the calculation of $n'$ from Eq. 3 is comparatively insensitive to errors in the measurement of $^{42}\text{K}$ fluxes. For these reasons, and the fact that we do not have high-resolution tail current records for every single experiment performed, our tables only report raw $^{42}\text{K}$ fluxes; no corrections have been applied.

**RESULTS**

The results of our attempts to determine the value of the flux ratio exponent $n'$ in Eq. 2, by each of the four methods, are described separately below.

**Influx Method**

In this method, net current and isotope influx are measured, and efflux is computed. Fig. 1 A shows how, during the pulsing period, $^{42}\text{K}$ influx rises to a relatively stable level ~35 pmol/cm$^2$ s above resting. Current records taken near the beginning, middle, and end of the 6-min pulsing period are shown in Fig. 1 B. These records, typical for our experiments, have not been corrected for leakage current (which was very small: resting leakage resistance was 5–7 kohm·cm$^2$). Average net flux during the test pulse, computed from the time integral of the $I_K$ records, was 6,660 pmol/cm$^2$ s. Average extra $^{42}\text{K}$ influx during the pulse is 10 times the extra influx seen in Fig. 1 A (since the pulses occupy only 10% of the time), or 350 pmol/cm$^2$ s. From the magnitude of the tail currents, we derive estimates for $[\text{K}]_o$ (31 mM) and $V_K$ (~58 mV). Thus, computed efflux is 6,660 + 350 = 7,010 pmol/cm$^2$ s. These quantities, entered in Eq. 3, yield an $n'$ value of 2.45.

If our findings are to bear on the properties of “excitable” potassium channels in nerve, the measured isotope flux should demonstrably take place through such channels. The records in Fig. 1 B show, by the usual criterion (very small leakage current), that most of the net current takes place through so-called “potassium” channels. We have previously shown (Begenisich and De Weer, 1977) that extra $^{42}\text{K}$ efflux during depolarizing pulses is inhibited
to the same extent as K current is by the specific K-channel blocker 4-
aminopyridine (Meves and Pichon, 1977; Yeh et al., 1976). Fig. 2 illustrates
the effect on \(^{42}\text{K}\) influx of 3,4-diaminopyridine (DAP), another compound
that specifically reduces K conductance (Kirsch and Narahashi, 1978). In the
experiment shown, the membrane potential was held at \(-68\,\text{mV}\) for the first
6 min, pulsed during the next 5 min to \(-28\,\text{mV}\) for 30 ms every 300 ms,
pulsed for another 5 min to \(-8\,\text{mV}\) for 30 ms every 300 ms, and finally held
at \(-68\,\text{mV}\) again. After switching to an internal perfusion solution containing
100 \(\mu\text{M}\) DAP, this experimental procedure was repeated. Although the

![Figure 1](image_url)

**Figure 1.** (A) Influx of \(^{42}\text{K}\) into an axon internally perfused with 350 mM \(\text{K}^+\)
and bathed in ASW containing 20 mM \(\text{K}^+\) and 300 nM TTX. The membrane
potential was held at \(-78\,\text{mV}\) except during the pulsing period indicated, when
it was clamped to \(-28\,\text{mV}\) every 300 ms for 30 ms. Axon No. 7062; diameter,
550 \(\mu\text{m}\); temperature, 10.5\(^\circ\text{C}\). (B) Voltage-clamp records from the same axon as
in (A) taken, from top to bottom, at minutes 7, 10, and 12 on the scale of
(A). The currents at the end of the pulses are 0.82, 0.81, and 0.79 mA/cm\(^2\) (top to
bottom). The tail currents following the pulses are not shown.

unstable baseline influx makes accurate determination of both resting and
stimulated influx difficult in this case, it is clear that DAP reduces resting \(^{42}\text{K}\)
influx and essentially eliminates extra \(^{42}\text{K}\) influx during the application
of pulses to \(-28\,\text{mV}\). Shortly after initiation of pulses to \(-8\,\text{mV}\), however, \(^{42}\text{K}\)
influx increased precipitously. This often happened in axons exposed to DAP
for some time and was associated with a large irreversible increase in leakage
conductance. A similar deleterious effect of DAP has been observed by P.
Shrager.\(^1\)

\(^1\) Personal communication.
The results shown in Fig. 1 A and B are consistent with most of the measured extra $^{42}$K influx occurring through the voltage- and the time-dependent, potassium-specific ion channel. The $n'$ values computed from these and several similar experiments are summarized in Table I. Had part of the extra $^{42}$K influx in fact occurred through pathways (e.g., “leakage” channels) that do not contribute to the “late” (“potassium”) current, the calculated $n'$ values would be underestimates. Table I lists 10 axons with $K_i = 350$ mM, and two with $K_i = 200$ mM. The average values of $n'$ for these two groups of axons are listed separately. The two internal solutions produced slightly different junction potentials; hence, the absolute potentials differ somewhat as is shown. The data of Table I are also plotted in Fig. 3. For those axons with 350 mM internal $[K]$, $n'$ is strikingly voltage dependent. This trend appears even within single axons, as inspection of Table I will show. The question arises whether this trend is genuine, or whether it results from a voltage-dependent error in our measurements. In the case of five experiments in which we had high-resolution tail current records, we applied all the various $^{42}$K flux corrections discussed under Methods and found that the voltage dependence of $n'$ was still present. Our tentative conclusion is that $n'$ decreases with depolarization in the range of $-40$ to $0$ mV, but a precise description of the voltage dependence of $n'$ must await more refined experiments. In addition, the $n'$ values for axons with low internal K appear to be lower than those for axons with high $[K]$, but, again, further experiments are needed to confirm this.
Two-Flux Method

Fig. 4 shows 42K efflux and influx, measured on a single axon, at a holding potential of -78 mV and during repeated clamping to -28 mV. The apparent high baseline influx illustrates one of the problems besetting this technique: most of it represents not actual influx through the axolemma but, rather, washout of residual radioactivity from the perfusion and collection apparatus. Still, a measurable extra influx occurs throughout the period during which pulses to -28 mV were applied. The potassium current was about 20% smaller during the influx measurements than during the efflux measurements. This is not surprising considering that >30 min elapsed between the measurements. For calculation of n', the measured influx was, therefore, scaled up by ~20%, and an n' value of 3.50 was found. Table II summarizes the results of this and several other experiments in which both influx and efflux were measured. The average values of n' for three pulse potentials are in reasonable agreement with those obtained with the influx method (Table I); the correlation coeffi-

### Table I

| Axon No. | [K]o (bath) | [K]o (periaxonal) | Vm | mV | | M | M | M | M | M' | n' |
|----------|-------------|------------------|----|----|---|---|---|---|---|----|
| 6291     | 350         | 20               | -28| 31 | -58 | 4,380 | 130 | 4,510 | 34.7 | 2.90 |
| 6292     | 350         | 20               | -28| 33 | -57 | 4,820 | 110 | 4,930 | 44.0 | 3.21 |
| 6293     | 350         | 40               | -28| 47 | -48 | 3,630 | 350 | 3,980 | 11.4 | 2.98 |
| 6294     | 350         | 40               | -28| 58 | -43 | 9,020 | 620 | 9,640 | 15.5 | 1.92 |
| 7061     | 20          | 20               | -38| 51 | -46 | 6,040 | 420 | 6,460 | 15.4 | 2.39 |
| 7062     | 350         | 20               | -38| 25 | -63 | 2,760 | 100 | 2,860 | 28.6 | 3.29 |
| 7064     | 20          | 20               | -24| 31 | -58 | 6,660 | 350 | 7,010 | 20.0 | 2.45 |
| 7072     | 20          | 350               | -38| 28 | -61 | 1,710 | 110 | 1,820 | 16.5 | 2.99 |
| 7121*    | 20          | 350               | -38| 23 | -63 | 2,020 | 50  | 2,070 | 41.4 | 3.65 |
| 7122*    | 20          | 350               | -38| 37 | -54 | 5,690 | 75  | 5,765 | 76.9 | 2.96 |
| 7123*    | 20          | 20               | -38| 39 | -53 | 5,520 | 80  | 5,600 | 70.0 | 2.97 |
| 7132*    | 20          | 20               | -38| 28 | -61 | 2,670 | 55  | 2,725 | 49.5 | 4.16 |
| 7131     | 20          | 20               | -24| 34 | -55 | 3,220 | 50  | 3,270 | 65.4 | 3.79 |
| 7132*    | 20          | 20               | -24| 49 | -35 | 810 | 500 | 1,310 | 2.62 | 2.15 |
| 7132*    | 20          | 20               | -24| 64 | -38 | 2,620 | 750 | 3,380 | 4.51 | 1.54 |
| 7132*    | 20          | 20               | -24| 36 | -55 | 4,960 | 210 | 5,170 | 24.6 | 2.12 |

* Axons on which both the influx and the two-flux methods were applied (see Table II).

Conditions Value of n' (mean ± SEM) at given membrane potential Vm

| Conditions | Value of n' (mean ± SEM) |
|------------|--------------------------|
| -38 mV     | 3.26±0.32 (5) 3.09±0.28 (4) 2.68±0.28 (3) 2.26 |
| -28 mV     | 2.98 2.39 1.92 |
| -24 mV     | 2.22±0.07 (2) 1.54 |

Number of experiments is given in parentheses.
FIGURE 3. Dependence of potassium flux ratio exponent \( \eta' \) (Eq. 2) on membrane potential. Data are from Tables I (influx method; filled symbols) and II (two-flux method; open circles). Internal and (nominal) external potassium concentrations as indicated. The numbers near the symbols with standard error bars indicate number of experiments performed. The weighted least squares regression line was fitted to the circles only (\( K_i = 350 \) mM; nominal \( K_o = 20 \) mM); it obeys the equation: 
\[
\eta' = 2.13 - 0.03 V_m \quad \text{(C)} \quad [\text{K}^+]_i = 350; \quad [\text{K}^+]_o = 20. \quad (\square) \quad [\text{K}^+]_i = 350; \quad [\text{K}^+]_o = 40. \quad (\Delta) \quad [\text{K}^+]_i = 200; \quad [\text{K}^+]_o = 40.
\]

FIGURE 4. Efflux and influx of \(^{42}\)K in an axon perfused with 350 mM K\(^+\) and bathed in ASW containing 20mM K\(^+\) and 300 nM TTX. During the first part of the experiment, the internal perfusion solution was labeled with \(^{42}\)K, and tracer was collected in the ASW. During the interruption indicated, internal isotope was flushed out as much as possible, and \(^{42}\)K was added to the bathing ASW to a specific activity about 10-fold higher than for the efflux experiment. Most of the "influx" of the second part of the experiment represents contamination by residual \(^{42}\)K from the axon and associated internal perfusion apparatus. Membrane potential was held at \(-78\) mV, except during the interruption indicated, when it was not controlled, and during the pulse periods, when it was clamped to \(-28\) mV for 40 ms every 400 ms. Axon No. 7123; diameter, 475 \(\mu\)m; temperature, 12.5°C.
cient was 0.80. Both the influx and two-flux techniques were employed on several axons with very good agreement in $n'$ values, (correlation coefficient, 0.96). In view of the satisfactory agreement between these two methods, the regression line in Fig. 3 was fitted by least squares to all data points obtained on (nominal) 20-mM $K_o$, 350-mM $K_i$ axons by either influx or the two-flux methods.

**Efflux Method**

As described under Methods, this technique employed pulses to two different voltages, one for area normalization and one for determining influx. The example in Fig. 5 shows the measured efflux for pulses to $-18$ and $-38$ mV. (The small progressive decrease in efflux discussed in Methods is observed here for the pulses to $-18$ mV). The average $I_K$ during the pulse to $-18$ mV was $4,970$ pmol/cm$^2$s, or 1.10 times the simultaneously measured $^{42}K$ efflux, which was $4,500$ pmol/cm$^2$s. The average $I_K$ during the pulse to $-38$ mV was $1,740$ pmol/cm$^2$s, and the efflux (scaled by a factor of 1.10) was $1,925$ pmol/cm$^2$s. The calculated influx is then $185$ pmol/cm$^2$s by difference. The inaccuracy of this technique is obvious: a 10% error in efflux measurement produces a 100% error in computed influx. At any rate, with the above influx and efflux values and $E_K = -63$ mV, a flux ratio exponent of 2.24 is obtained.

**Table II**

| Axon No. | $V_m$ (bath) | $|K_o|$ (periaxonal) | $|K_i|$ (periaxonal) | $I_K$ | $M_s$ | $M_i$ | $M_s/M_i$ | $n'$ |
|----------|--------------|---------------------|---------------------|-------|-------|-------|-----------|------|
|          | mV | mM | mM | mV | pmol/cm$^2$s | pmol/cm$^2$s | pmol/cm$^2$s |
| 7121     | $-18$ | 20 | 350 | 37 | -55 | 4,150 | 86 | 48.3 | 2.57 |
|          | $-38$ | 25 | -65 | 1,800 | 43 | 41.9 | 3.39 |
| 7122     | $-18$ | 20 | 350 | 39 | -54 | 7,250 | 86 | 84.3 | 3.20 |
|          | $-38$ | 28 | -62 | 2,300 | 33 | 69.7 | 4.33 |
| 7123     | $-28$ | 20 | 350 | 34 | -57 | 3,750 | 59 | 63.6 | 3.50 |
|          | $-38$ | 26 | -63 | 1,000 | 50 | 62.5 | 1.80 |
| 7132     | $-38$ | 20 | 350 | 36 | -55 | 4,780 | 240 | 19.9 | 1.98 |
|          | $-18$ | 36 | -55 | 4,780 | 240 | 19.9 | 1.98 |

**Membrane potential**

| $n'$, mean ± SEM |
|------------------|
| $-38$ mV | $-28$ mV | $-18$ mV |
| 3.17 ± 0.74 (3) | 3.50 | 2.58 ± 0.35 (3) |

* The average of $I_K$ during the efflux period and $V_m$ during the influx period.

Number of experiments is given in parentheses.

Table III summarizes the results of this and two additional experiments. In the experiments of Table III, pulses to $-38$ mV were used, and the average $n'$ value was $2.35 ± 0.19$, rather lower than the value of $3.26 ± 0.32$ obtained by the influx method (Table I). A direct comparison between the influx and efflux methods can be made because both were used on the axons listed in Table III. Systematic underestimation of $n'$ by the efflux technique could
occur if efflux were consistently underestimated. As discussed in Methods, K efflux may be slightly underestimated by the amount of $^{42}$K that reenters the fiber during the "tail" current. Since, in this method, $n'$ is very sensitive to errors in efflux measurement, the poor agreement with the influx technique is not altogether surprising.

**Potassium Conductance Method**

This last technique was applied to only two axons. Both $^{42}$K influx and $g_K$ were measured at the potassium equilibrium potential, $V_K$. To make $V_K$ sufficiently positive so as to render $g_K$ measurable, we lowered internal [K$^+$] to 100 or 200 mM and raised external [K$^+$] to 40 mM.

The results of one of these experiments are shown in Fig. 6. Calculated $V_K$ was $-22$ mV, close to the observed reversal potential for potassium current ($-26$ mV). The influx of $^{42}$K was measured during repetitive pulses from $-72$ mV to $V_K$ as well as during continuous holding at $-26$ mV; the calculated values for $n'$ were 4.0 and 2.3, respectively. These and the $n'$ value from a second experiment are listed in Table IV. The values cannot be compared
directly with those obtained with the other methods because rather different voltages and K concentrations were used. Also, the large difference between $n'$ as obtained with pulses to $V_K$ and that found with a constant potential is unexplained. All that can be confidently said of the $n'$ values obtained with this technique is that they exceed unity.

**Figure 6.** Influx of $^{42}$K into an axon internally perfused with 100 mM K$^+$ and bathed in ASW containing 40 mM K$^+$ and 300 nM TTX. For the first 21 min, membrane potential was held at -72 mV, except during the period indicated, when it was clamped every 400 ms to the reversal potential for potassium current (-26 mV). Membrane potential was then clamped for several minutes at -26 mV. Axon No. 7071; diameter, 500 μm; temperature, 10ºC.

| Axon No. | $V_m$ | $\left[K_o\right]$ | $\left[K_i\right]$ | $g_K$ | $M_i$ | $n'$ |
|----------|-------|-------------------|-------------------|-------|-------|------|
| 7063     | -40 (pulses) | 40 | 200 | 3.7 | 500 | 1.7 |
| 7071     | -26 (pulses) | 40 | 100 | 8.0 | 480 | 4.0 |
| 7071     | -26 (hold)   | 40 | 100 | 2.7 | 280 | 2.3 |

**Discussion**

Our findings show that, for potassium ion movements in squid giant axons subjected to short voltage-clamp pulses, the flux ratio exponent, $n'$ (Eq. 2), is $>1$, and generally between 2 and 3. This value is very similar to that obtained by Hodgkin and Keynes (1955) on cuttlefish axons subjected to prolonged depolarizations. Our data of Tables I and II and Fig. 3 suggest, in addition, that $n'$ varies with voltage, decreasing as $V_m$ is made more positive. They also suggest that $n'$ may increase with internal [K].
Hodgkin and Keynes (1955), and others after them, have interpreted \( n' > 1 \) as evidence for the existence of “single-file” or “long-pore” diffusion, where ions cannot pass one another as they travel through the channel. In simple models of ion permeation that assume ions to move from site to site across the membrane, \( n' \) appears either as the number of sites or one plus the number of sites, depending on whether all sites are filled and on whether ions undergo “knock-on” collisions (Hodgkin and Keynes, 1955; Hladky and Harris, 1967). Calculations by Heckmann (1965 and 1972) for uncharged particles and by Hille and Schwarz (1978) for ions, have shown that for non-“knock-on” models the maximum number of particles allowed in the channel sets an upper limit to \( n' \). Maximum \( n' \) values are expected when no more than a single vacancy exists in the channel and when it is mobile enough to move across the membrane before being filled from the bathing medium. Therefore, any factor (such as ion concentration or voltage) that changes the number and/or mobility of vacancies may affect \( n' \). A two-site model by Hille and Schwarz (1978) predicts that, at relatively low ion concentrations, \( n' \) values will increase with concentration. Our data (Table I and Fig. 3) are consistent with that prediction. Calculations in this laboratory on a rather similar model (see Begenisich and Cahalan [1980] for details), with conditions close to the experimental situations described in this paper, show that, as also observed here, \( n' \) should decrease as \( V_m \) is depolarized in the range of \(-40 \) to \( 0 \) mV and should increase with \([K]\). Data over much wider concentration and voltage ranges will be required to discriminate between models.

Because we have obtained average \( n' \) values as large as \( 3 \), the conclusion appears inescapable that the potassium channel of squid giant axon is a single-file pore with at least two sites. This minimum number, however, requires the assumption of “strict knock-on” movement of ions in the channel, which in turn predicts a constant \( n' \), a condition not met by our data. We are left with the likelihood that the single-file potassium channel of squid giant axon harbors three or more sites, and that the mechanism of ion passage across the membrane includes motions other than of the “strict knock-on” type.

We thank the Director of the Marine Biological Laboratory, Woods Hole, Mass., for the facilities placed at our disposal, Drs. L. J. Mullins and C. M. Rovainen for their comments, and J. Jones for typing the manuscript.

This study was supported by National Institutes of Health (NIH) grants NS 06084 and NS 11223. T. Begenisich was holder of NIH Research Career Development Award NS 14138.

Received for publication 14 December 1979.

REFERENCES

Abercrombie, R. F. 1977. Single-file diffusion: a relationship between membrane conductance and unidirectional flux. Am. J. Physiol. 232:C163.

Adam, G. 1973. The effect of potassium diffusion through the Schwann cell layer on potassium conductance of the squid giant axon. J. Membr. Biol. 13:353-386.

Adelman, W. J., Jr., Y. Palti, and J. P. Senft. 1973. Potassium ion accumulation in a periaxonal space and its effect on the measurement of membrane potassium ion conductance. J. Membr. Biol. 13:387-410.
Begenisich, T., and M. Cahalan. 1980. Sodium channel permeation in squid axons. I. Reversal potential experiments. J. Physiol. (Lond.). In press.

Begenisich, T., and P. De Weer. 1977. Ionic interactions in the potassium channel of squid giant axons. Nature (Lond.). 269:710–711.

Begenisich, T., and P. De Weer. 1979. Evidence that the K channel of squid axons is a multiple ion pore. Biophys. J. 25:194a. (Abstr.).

Begenisich, T., and C. Lynch. 1974. Effects of internal divalent cations on voltage-clamped squid axons. J. Gen. Physiol. 63:675–689.

Connor, J. A., and C. F. Stevens. 1971. Inward and delayed outward membrane currents in isolated neural somata under voltage clamp. J. Physiol. (Lond.). 213:1–119.

Ehrenstein, G., and D. L. Gilbert. 1966. Slow changes of potassium permeability of the squid giant axon. Biophys. J. 6:553–566.

Frankenhaeuser, B., and A. L. Hodgkin. 1956. The after-effects of impulses in the giant nerve fibres of Loligo. J. Physiol. (Lond.). 131:341–376.

Heckmann, K. 1965. Zur Theorie der "single file"—Diffusion. II. Z. Phys. Chem. 46:1–25.

Heckmann, K. 1972. Single file diffusion. In Biomembranes. F. Kreuzer and J. F. G. Slegers, Editors. Plenum Press, New York. 3:125–153.

Hille, B., and W. Schwarzb. 1978. Potassium channels as multi-ion single file pores. J. Gen. Physiol. 72:409–442.

Hladky, S. B., and J. D. Harris. 1967. An ion displacement membrane model. Biophys. J. 7:535–542.

Hodgkin, A. L., and A. F. Huxley. 1952. The components of membrane conductance in the giant axon of Loligo. J. Physiol. (Lond.). 116:473–496.

Hodgkin, A. L., and R. D. Keynes. 1955. The potassium permeability of a giant nerve fibre. J. Physiol. (Lond.). 120:61–88.

Kirsch, G. E., and T. Narahashi. 1978. 3,4-Diaminopyridine: a potent new potassium channel blocker. Biophys. J. 22:507–512.

Meves, H., and Y. Pichon. 1977. The effect of internal and external 4-aminopyridine on the potassium currents in intracellularly perfused squid giant axons. J. Physiol. (Lond.). 268:511–532.

Mullins, L. J., W. J. Adelman, Jr., and R. A. Sjodin. 1962. Sodium and potassium ion effluxes from squid axons under voltage clamp conditions. Biophys. J. 2:257–274.

Shagina, L. V., A. E. Grinfeld, and A. A. Lev. 1978. Interactions of cation fluxes in gramicidin A channels in lipid bilayer membranes. Nature (Lond.). 273:243–245.

Ussing, H. H. 1949. The distinction by means of tracers between active transport and diffusion. Acta Physiol. Scand. 19:43–56.

Wanke, E., E. Carbone, and P. L. Testa. 1979. $K^+$ conductance modified by a titratable group accessible to protons from the intracellular side of the squid axon membrane. Biophys. J. 26:319–324.

Yeh, J. Z., G. S. Oxford, Ch. H. Wu, and T. Narahashi. 1976. Interactions of aminopyridines with potassium channels of squid axon membranes. Biophys. J. 16:77–81.