SODIUM AND POTASSIUM ION EFFLUXES FROM SQUID AXONS UNDER VOLTAGE CLAMP CONDITIONS

L. J. MULLINS, W. J. ADELMAN, JR., *and* R. A. SJODIN
From the Department of Biophysics, University of Maryland School of Medicine, Baltimore, and the National Institutes of Health, Bethesda

ABSTRACT Squid giant axons loaded with Na^{24} were subjected to short duration (0.5 msec.) clamped depolarizations of about 100 mv at frequencies of 20/sec. and 60/sec. while in choline sea water. Under such conditions the early outward current was just about maximal at the time of termination of the clamping pulse. An integration of the early current versus time record gave 1.2 μcoulomb/cm^2 pulse, while a measurement of the extra Na^{24} efflux resulting from repetitive pulsing gave a charge transfer of 1.4 μcoulomb/cm^2 pulse. In sodium-containing sea water and with pulses 50–75 mv more positive than E_{Na} the Na^{24} efflux is about 3 times the measured charge transfer. The efflux of K^{42} from a previously loaded axon into normal sea water is only 50 per cent of the measured charge transfer when the membrane is held for about 5 msec. at a potential such that there is no early current, and such pulses are at 10–20/sec. The experiments appear to confirm the suggestion that the early current during bioelectric activity is sodium but provide unsatisfactory support for the identification of the delayed but sustained current solely with potassium ions. Resting Na^+ efflux is 0.6 pmole/cm^2 sec. mmole $[Na]_i$, while the apparent K^+ efflux is about 250 pmole/cm^2 sec. and is little affected by hyperpolarization.

INTRODUCTION

The ionic currents that flow during a voltage clamp of the membrane of the squid axon have been analyzed by Hodgkin and Huxley (1952) into an early, transient current of Na^+, and a later, sustained current of K^+. Their analysis predicts the exchange of these ions found during a propagated impulse at 18.5°C. by Keynes and Lewis (1951) and may be extended (FitzHugh) to account for the increased K^+ loss at low temperature found by Shanes and Berman (1956). But although the steady current flow in *Sepia* has been accounted for quantitatively by the flux of K^+ (Hodgkin and Huxley, 1953), it has not been so identified in the squid axon nor has the early transient voltage clamp current been correlated with a Na^+ movement in any preparation. In the present study, the effluxes of Na and K from squid

axons have been measured during voltage clamp pulses appropriate to provide tests of the Hodgkin-Huxley analysis.

In designing these experiments, the central idea has been to measure current flow during an interval of time and to measure the rate of movement of a specific ion. Ideally the measured ion movement should be over the same interval of time as that during which currents flow but practical considerations of specific radioactivity of substances, diffusion delays in experimental chambers, and the short durations of voltage clamp currents make it necessary to measure changes in the rate of movement of specific ions over times of the order of minutes while repetitively voltage clamping an axon at frequencies of the order of 10/sec. In order to relate movement of a specific ion to current flow during voltage clamp pulses, it is necessary to assume that a measured increment in ion movement occurs as a result of current flow and corrections are necessary for delays in collecting radioactive samples, for uncertainties in the relationship between influx and efflux, and for transients related to the onset and termination of the imposed clamp potential. The experimental conditions have been chosen to minimize these uncertainties.

METHODS

Axons were dissected from the hindmost stellar nerve of the squid *Loligo pealii*, and these were carefully cleaned prior to use.

Solutions. The following sea water formulations were used, as indicated in the text.

Solution	pH	Na	K	Ca	Mg	Tris	Choline	Cl	SO_4	HCO_3
Sea Water (s. w.)	7.9	432.19	9.18	9.46	49.40	0	0	504.90	26.0	2.19
Tris s. w.	7.1	0	20	20	0	455	0	515	0	0
Potassium s. w.	7.0	0	475	20	0	0	0	515	0	0
Choline s. w.	7.5	2.19	9.18	9.46	49.40	0	430	504.90	26.0	2.19

All concentrations are mmoles/liter. Tris sea water was made pH 7.1 in order that the ionization of trishydroxymethylaminomethane should be substantially complete. References to 90 per cent choline sea water in the text mean that 9 parts of choline sea water were added to 1 part of sea water.

Radioactive Ion Loading. As the surface to volume ratio in squid axons is small when compared with other fibers, special problems arise in connection with the loading of isotopes. For Na^{24}, it was found convenient to stimulate the fiber at a frequency of 40 to 60/sec. at 10°C. for 10 to 15 minutes during which Na^{24} containing sea water was circulated past the fiber as it lay in a groove in a block of teflon. Platinum electrodes were used both for stimulation and recording. The usual concentration of Na^{24} was about 1 mc/ml. and from 30 to 50 per cent of the $[Na]_i$ exchanged with Na^{24}. Some experiments were done with Na^{22}, and this was loaded in the same manner as for Na^{24}. For K^{42} such a technique for loading would involve a prohibitively long period of stimulation, and instead axons were immersed in about 10 ml of tris sea water with K^{42} for about twelve hours at 4°C. Control experiments showed that cleaned axons survive very

well an overnight immersion in a Na^+-free sea water as judged by normal resting and action potentials, although they may deteriorate somewhat more rapidly than normal when brought into Na^+-containing sea water. An alternate procedure for K^{42} loading was to immerse axons in a medium of high K^+ concentration (K–sea water) for short times (10 to 30 minutes). As loading in this medium was inferior to that obtained in tris sea water, only a few such experiments were done. In a double loading experiment, an axon was first loaded overnight in tris with K^{42} and then stimulated for twelve minutes in sea water containing Na^{22}.

Voltage Clamp. The apparatus used was that described by Cole and Moore (1960*a*). The axon was placed across the top of the center and guard electrode chambers as shown in Fig. 1. The membrane potential was measured between micropipette,

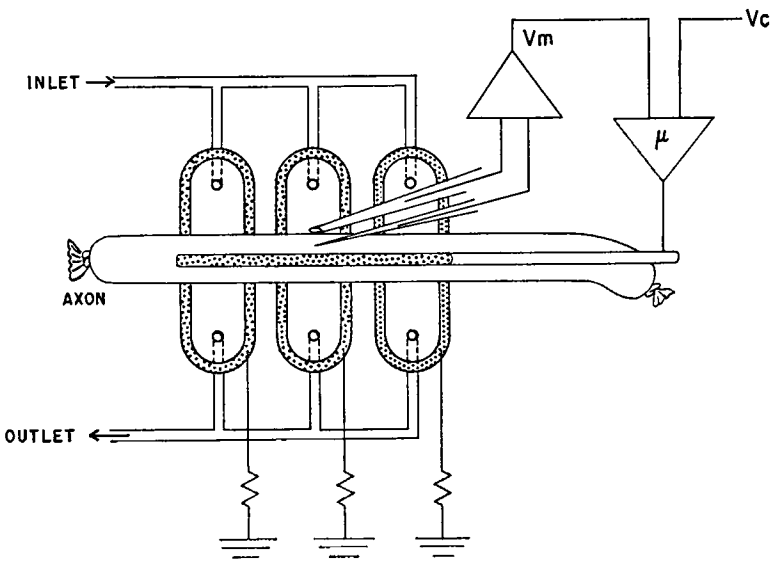

FIGURE 1 This shows the experimental chamber with the axon positioned across the central and two guard chambers. The stippled regions are electrically conducting and are the internal axial electrode and the ground electrodes in the chambers. Membrane potential is measured between an internal micropipette and external capillary electrode. These are connected to an electrometer amplifier and the measured V_m is compared with V_c, the command potential at the input of the feedback amplifier μ. Sea water circulation through the chamber is as indicated by the arrows.

internal, and capillary, external electrodes. Membrane potential was controlled by the electronic system which applied such a potential to the internal axial electrode as to force the membrane potential to follow the command potential E, within tens of microseconds and a few millivolts. The membrane currents were usually measured and recorded from the potential drop across the chamber electrode resistors.

Membrane Current Measurement. A record such as that shown in Fig. 2 can only be properly interpreted when the current that is measured can be divided between I_C, I_{Na}, I_K, and I_L, where these are, respectively, the capacitative, sodium, potassium and leakage currents.

To estimate I_{Na} with accuracy it was essential that I_L and I_K either remained constant during the pulse or varied in a way that could be measured independently. For large depolarizations (such as to +30 mv) I_K rose rather rapidly if the depolarization was started from the normal resting potential of −60 mv. To prevent any confusion from a varying I_K, the membrane was held hyperpolarized 55 mv beyond the resting potential for 7 msec. prior to the depolarizing step; this delayed changes in I_K for as much as 1 msec. (Cole and Moore, 1960*b*).

While initially we had assumed that I_L could be obtained on the basis of a constant leakage conductance g_L, simultaneous work by Adelman and Taylor (1961) indicated that this current was not linear and was indeed far larger at depolarizations to beyond E_{Na} than we had expected. However, at E_{Na} in normal sea water we had only the usual constant leakage, along with some small potassium current and it may be assumed that at this same potential these both remained unchanged as external Na^+ was replaced with $choline^+$. The increase of outward current in $choline^+$ is then to be ascribed to Na^+ and the area between the two current records of Fig. 2 was integrated graphically to measure Na^+ removed from the axon during the pulse.

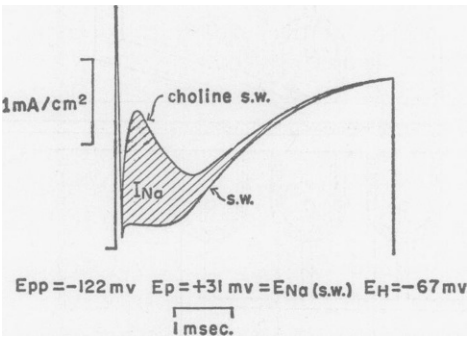

FIGURE 2 Squid axon 907. 20 pulses/sec. The current trace to a clamp to E_{Na} in sea water (lower line) is compared with a clamp to the same potential in choline sea water (upper line). The hatched area (I_{Na}) is the area to be integrated for the calculation of charge transfer due to Na^+. E_P is the potential of the pulse, E_H is the potential held between pulses, and E_{PP} is the pre-pulse potential applied 7 msec. before the pulse.

The measurement of I_K was made by either a long pulse to E_{Na} or to another potential where I_{Na} could be easily estimated. In both cases it was necessary to have other methods for the estimation of I_L.

Efflux. Flow from a sea water reservoir passed through the central and the two guard electrode chambers at a rate of about 2 ml/min. and the pooled efflux from all three chambers was collected in plastic test tubes connected in the suction line from the chamber. The temperature of the chamber was usually maintained at 10± 1°C., and samples of the effluent sea water were generally taken every two minutes. Five such sampling periods followed a single period of voltage clamping and the time constant for the loss of radio-activity from the chamber was about 1 to 1.5 minutes at the above flow rate. Axons were generally washed in inactive solutions for 1 hour before efflux measurements were begun. The geometry of the chamber was such that with an axon of 500 μ diameter, 0.25 cm^2 of its surface was exposed to the flowing sea water.

Counting. Because of the large volume (4 ml) and high salt content of efflux

samples to be counted, it was somewhat inconvenient to evaporate these in planchets. For K^{42} it was possible to make counts of the solutions in a β-scintillation well counter connected to a pulse height analyzer and scaler. This arrangement, because of its virtual 4π geometry, gave a higher efficiency of counting than that for dry samples with a GM tube. For Na^{24}, with its less energetic β-rays, the above method was less efficient and for weak samples it was necessary to resort to counting the samples dry in planchets. Samples of Na^{22} were counted in a γ-scintillation well with the analyzer set to 0.51 Mev. Differential counting of Na^{22} and K^{42} mixtures was easily made since there was virtually no K^{42} count in the γ-scintillation well.

Axoplasm Analysis. At the end of an experiment the chamber was drained of sea water and the axon carefully blotted with small bits of filter paper. The axial wire was then removed, and the axon tied off at the end where the wire had been inserted. The axon was then lifted out of the chamber, one end was cut off about 4 mm. from the tie and a sample of axoplasm from the central part of the axon was extruded onto a previously tared piece of plastic film. The film was weighed again and then placed in a platinum crucible. Crucibles were dried in an oven at 110°C. and then ashed at 550°C. for about 10 hours. The ash was dissolved in a few drops of N HCl and the volume of the solution brought up to 2 ml. Such samples, representing 3 to 5 mg of axoplasm, were compared in a flame photometer with 1 mM K and 0.2 mM Na solutions. Concentrations of cations in axoplasm are expressed as millimolal (mM). If concentrations per liter of water are desired, values in this paper must be divided by 0.865 which is the fraction of axoplasm that is water, as found by Kochelin (1955). An aliquot of the solution was counted in order to have an estimate of the final radioactivity of the axon.

RESULTS

If an axon, under voltage clamp conditions, is surrounded with a sea water not containing Na^+, the early current, attributed by Hodgkin and Huxley to Na^+, is outward, or in the same direction as the sustained K^+ current. A record of the membrane current taken at the sodium equilibrium potential in sea water (where there is no Na current) may be superposed on one taken at the same membrane potential in choline sea water and a subtraction at each point on the current records then should give the outward current[1] carried by Na^+. Such a record is shown in Fig. 2 where the membrane potential was held at a value of +30 mv for both current traces. If the duration of the control pulse of the voltage clamp is made about 0.5 msec. (see Fig. 3) there is essentially no K^+ current, and an integration of the area between the two curves gives the charge transferred. In the record (Fig. 3) such an integration gave 1.14 μcoulomb/cm^2 pulse. Such pulsing was continued at a frequency of 20/sec. for 120 sec. and the rate constant for the efflux of Na^{24} which at rest was about 3×10^{-3}/min. rose to about 10×10^{-3}/min. When the extra counts

[1] Data will be presented later in this paper to show that there is a considerable, but variable amount of K^{42} efflux under these experimental conditions. This efflux, however, appears in both choline and Na sea water (at the same potential) while current is only measured over the pulse interval if the axon is in choline sea water. It seems clear, therefore, that Na^+ movement should be correlated with current since this current appears when we remove Na influx.

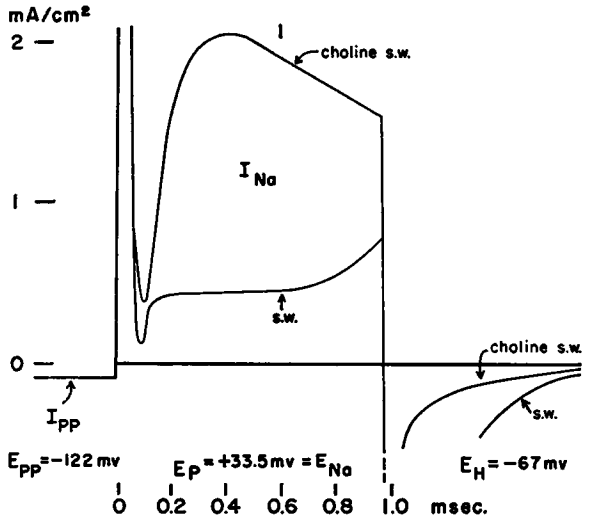

FIGURE 3 Axon 907. This tracing is similar to Fig. 2 but is for a 1 msec. pulse. Pulses were usually terminated after 0.6 msec. and this time is indicated by a vertical line. E_H is the holding potential, E_{PP} is the pre-pulse potential.

during pulsing were summed, multiplied by the reciprocal specific activity of the axoplasm (moles/CPM), and divided by the Faraday times the number of pulses, the Na^{24} efflux was equivalent to 1.34 μcoulomb/cm^2 pulse. As the axon was in choline sea water, Na^+ efflux and net efflux (or current) must be identical. The data for all such experiments are given in Table I where the transference number, T_{Na}, represents the fraction of the charge carried by Na^+ during a pulse.[2]

[2] The pulse transference number for Na is defined as:

$$T_{Na} = \frac{F(\bar{m}^* - \bar{m})t}{\sum_0^n \int_0^\tau I_{Na}\, d\tau}$$

where T_{Na} is the pulse transference number of Na, F is the Faraday, $\bar{m}^*$ is the measured net ion flux during activity, $\bar{m}$ is the net ion flux at rest, t is the time over which the extra flux of activity is measured, n is the total number of voltage clamp pulses, τ is the duration of a voltage clamp pulse, and I_{Na} is the current attributed to Na^+. In practice, as efflux m_o is measured by tracers, it is necessary to have some relationship between m_o and $\bar{m}$ in order to use the above equation. The use of choline sea water outside the axon makes $m_i = 0$ and m_o therefore equal to $\bar{m}$. Ideally we should define

$$T_{Na} = \sum_0^n \left[\frac{F \int_0^\tau (\bar{m}^* - \bar{m})\, d\tau}{\int_0^\tau I_{Na}\, d\tau} \right] \Big/ n$$

but the quantity in the numerator is not measured in these experiments because of the limitations inherent in tracer techniques.

TABLE I

CHARGE TRANSFER AND SODIUM EFFLUX INTO CHOLINE SEA WATER

Axon	E_m mv	E_{pulse} [$=E_{Na}$ s. w.]	Charge transfer $\mu coulomb/cm^2$ pulse: Measured from current	Measured by Na efflux	Pulse frequency per sec.	Pulse transference number for Na^+ T_{Na}
907	−63	+33	1.14	1.34	20	1.2
	−63	+34	1.14	1.37	20	1.2
	−64	+36	1.14	1.34	60	1.2
	−63	+36	1.14	1.60	60	1.4
	−64	+33	1.44	1.79	20*	1.2
906	−34	+36	0.39	0.36	20	0.95
	−24	+42	0.46	0.56	60	1.2
					Mean	1.2

* 5 msec. pulse; all others 0.5 msec. pulse

Axon 907 gave a constant integrated charge transfer of 1.14 for four successive periods where short duration pulses were delivered at 20 and 60/sec. A single period where 5 msec. pulses were used gave an appropriate increase in charge transfer and an unchanged pulse transference number of 1.2. That there is no appreciable error in these measurements because of the Na^+ that emerge during a depolarizing pulse being drawn back into the axon during the shut-off associated with repolarization is indicated by the following calculations. If we take the Frankenhaeuser-Hodgkin (1956) space as 300 A in thickness, then its volume per cm^2 of membrane surface is 3×10^{-9} liter. In a single pulse in choline sea water about 13 pmole of Na^+ emerge and the concentration of Na^+ immediately after a pulse is therefore 13×10^{-12} mole/3×10^{-9} liter = 4.3 mM. When pulses are at a frequency of 20/sec. there are almost two time constants for the F-H space between pulses. From this it can be calculated that the steady-state $[Na]_0$ will be around 10mM or 1/50th of its normal value. If influx *versus* external Na concentration were linear, this would introduce an error of 2 per cent (at pulses to E_{Na} in sea water).

The Relationship between Current and Efflux. If the medium surrounding a squid axon is sea water, the early current should represent the difference (Na influx − Na outflux) F. As our measurements were of Na outflux, and of current, and our aim was to decide whether early current and Na^+ efflux were connected, it is clearly important to know what sort of relationship between these quantities is to be expected. Unidirectional fluxes as a function of membrane potential and ion concentration can only be developed from some model of the membrane and the constant field assumption is that most generally used, but the data of Hodgkin and Huxley for the squid axon were fitted not to constant field equations but to a more simple linear relationship between ion current and driving potential (at large de-

polarizations). Such data can be fitted to constant field equations only by the introduction of further arbitrary assumptions; these have been discussed by Frankenhaeuser (1960).

The most general relationship between current and unidirectional ion flux as a function of membrane potential can be developed from the Behn (1897) relationship (*cf.* Teorell, 1949; Ussing, 1949). This equation can be written as in (1)

$$m_i/m_0 = \exp\left[-z(E_m - E_e)F/RT\right] \tag{1}$$

where m_i, m_o, are the influx and efflux of the ion, E_m is the membrane potential, and E_e the equilibrium potential of the ion, which is defined as $E_e = RT(zF)^{-1} \ln C_o/C_i$. This equation can be transformed into an expression for the net flux by multiplying and subtracting m_o from both sides and rearranging to give equation 2. With the convention that outward currents are positive equation 2 requires that net flux (or current) become identical with efflux when the exponential

$$m_0 - m_i = \bar{m} = -m_0\{[\exp\{-z(E_m - E_e)F/RT\}] - 1\} \tag{2}$$

expression becomes zero yielding $\bar{m} = m_o$ or $I = m_o F$. When $(E_m - E_e)$ is 75 mv., the value of the exponential is 0.05, or the current is 95 per cent of the efflux. In our experiments in Na sea water $(E_m - E_e)$ was 50 to 75 mv for Na efflux and about 100 mv for K efflux experiments.

Na^+ Efflux during Pulsing into Sea Water. The results obtained for axons in normal sea water subjected to pulses 50–75 mv more positive than E_{Na} are shown in Table II. The most prominent difference between these values and those in Table I is that here the mean T_{Na} is close to 3. This result[3] suggests that the larger fraction of the Na efflux is not contributing to the measured current, *i.e.* that its movement may be paired with the simultaneous movement of other ions. As there are far too many possibilities to make speculation about the nature of this pairing profitable, it seems best at the moment to show that very large transference numbers for Na have been observed (greater than 10) when the charge transfer during a pulse is small, while T_{Na} approaches 1 for pulse charge transfers that are large. All our results from Tables I and II and a considerable number of measurements on axons in from 0.1 to 0.9 choline sea water are shown in Fig. 4. The solid line, relating the apparent T_{Na} to charge transfer during a pulse (measured from current) has been calculated using the following equation: $T_{Na} = (a + x)/x$, where a is a constant (0.2 μcoulomb/cm^2 pulse), and x is charge transfer measured from current integration. The assumption involved in the derivation of the equation is that T_{Na} in a pulse is 1.0, but that a constant amount of Na efflux during a pulse

[3] Exchange diffusion of Na^+ cannot be involved here both because the resting Na efflux in squid axon is not decreased in going to choline sea water (see Resting Na Efflux, below) and because these measurements involve increments in Na^{24} efflux above resting values. The measurements could be reconciled with currents if influxes were much larger than we suppose but such an assumption would require that the flux ratio equation be not valid.

TABLE II

CHARGE TRANSFER AND SODIUM EFFLUX INTO SODIUM SEA WATER

Axon	E_m mv	E_{ap} mv	E_{pulse} mv	Charge transfer $\mu coulomb/cm^2$ pulse: Measured from current*	Measured from Na^+ efflux	T_{Na}
718	−57	75	+75	1.25	1.14	0.90
719	−59	82	+90	1.08	3.78	3.50
	−59	82	+98	0.84	2.28	2.72
802	−60	92	+90	0.92	3.00	3.26
809	−63	100	+96	1.16	1.67	1.46
					Mean	2.91

Pulse frequency 60/sec.

T_{Na} (theoretical) = 1.14 (based on a pulse 50 mv more positive than E_{Na})

* In these experiments the value of the leakage current at the pulse potential could not be known with certainty, and it was estimated by linear extrapolation from the value obtained at the sodium potential. Inasmuch as the leakage current rectifies and might indeed be larger than our estimates the values for charge transfer estimated from the sodium current are probably overestimates. E_{ap} is the magnitude of the action potential.

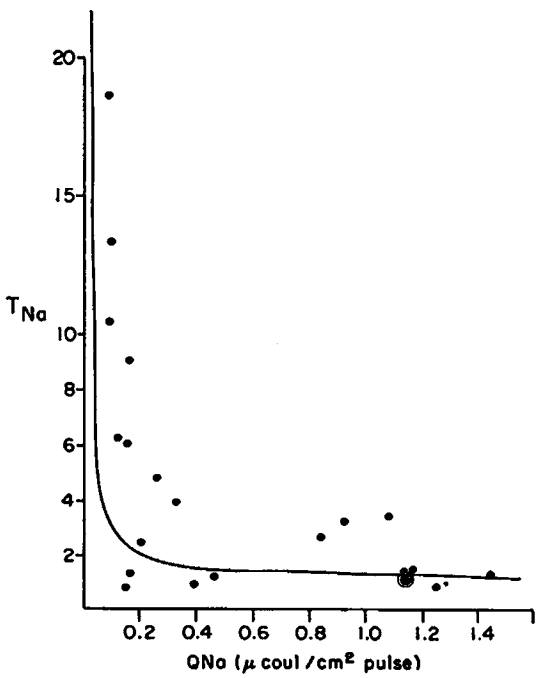

FIGURE 4 The apparent pulse transference number for sodium, T_{Na}, for all axons studied is plotted against the measured charge transfer, Q_{Na}, obtained from an integration of I_{Na} *versus* time. Points are experimental values, and the solid line is a solution of the equation $T_{Na} = (a + x)/x$, where a = 0.2 μcoulomb/cm² pulse and x is the measured charge transfer of Na.

takes place in addition to that represented by charge transfer. The value of the constant was chosen such as to make $T_{Na} = 1.0$ for measurements in choline sea water;[4] it is clear that a ought to be somewhat larger to fit closely with the very high values of T_{Na} that have been observed, but in the absence of any certainty that a does not vary considerably from axon to axon it is not considered worthwhile making an effort for closer fit.

Potassium Efflux during Long Pulses. When the squid axon membrane is subjected to a 5 to 6 msec. pulse to the Na^+ equilibrium potential (E_{Na}), a record of current versus time is obtained that is similar to that shown in Fig. 5. As the

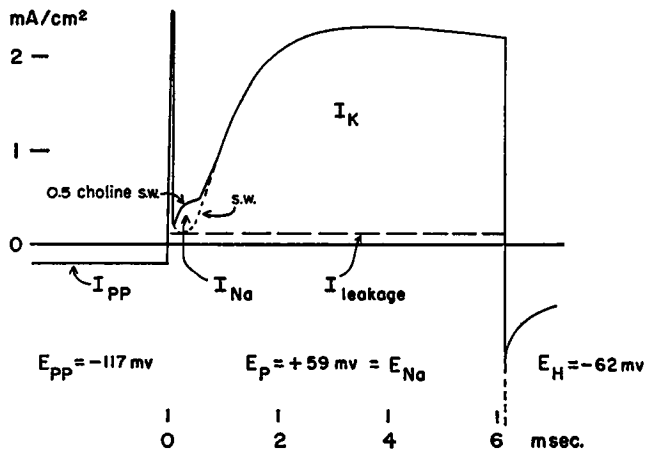

FIGURE 5 Axon 823. 20 pulses/sec. This current trace shows the method of estimating charge transfer effected by K^+. The axon is in 0.5 choline sea water and the clamp as close to E_{Na} as possible. A small subtraction of I_{Na} was necessary, as shown, and I_L was measured in sea water.

difference ($E_m - E_K$) is > 100 mv., one should expect substantial agreement between current and measured K^+ efflux. The results obtained are shown in Table III, where the mean transference number T_K is 0.53. This is apparently poor agreement with the hypothesis that the delayed, but sustained current in squid axons is K. From equation (2) it can be calculated that K efflux and current should be the same to within 1 per cent; in any event, an error from this source should make the isotope efflux larger than the measured current.

It might be supposed that the effect, described by Caldwell and Keynes (1960) where squid axons loaded with K^{42} by soaking gave anomalously high resting effluxes, would lead to an error in the estimation of T_K. This seems unlikely for the following

[4] It is of interest to note that from Table II the mean potential change during a pulse (ΔV) was 0.15 volt (from −60 to +90 mv); with a membrane capacitance of 1 μcoulomb/volt cm² the charge transfer (ΔVC) effected by the capacitative current is 0.15 μcoulomb/cm². If this current were carried solely by Na^+ derived from the axoplasm, it would correspond rather closely to the value (0.2) that we have chosen for a for other reasons.

TABLE III

CHARGE TRANSFER BY POTASSIUM DURING LONG PULSES TO E_{Na}

Axon	E_m mv	E_{ap} mv	E_{pulse} mv	External solution	Pulse frequency sec.$^{-1}$	Pulse duration msec.	Charge transfer μcoulomb/cm² pulse: From current	Charge transfer μcoulomb/cm² pulse: From K^+ efflux	T_K
720	−62	95	+72	Sea water	60	4.0	12.7	2.77	0.22
817	−63	102	+44	"	20	4.0	4.81	3.98*	—
823	−58	68	+59	0.5 choline s. w.	20	6.0	9.57	6.09	0.64
	−58	68	+59	"	31	2.2	2.48	1.03	0.42
829	−53	0	+43	0.9 choline s. w.	10	5.3	7.64	4.85	0.64
	−47	0	+43	"	10	5.3	6.96	5.31	0.76
Mean									0.53

* This axon was pulsed three times. The above long duration pulse sequence was preceded and followed by a short duration pulse sequence. Potassium effluxes during these short pulse sequences were 3.08 (1st) and nil (2nd). The early potassium efflux during the long pulse sequence might then be any value between 3.08 and zero. Under these conditions the efflux pertaining to the integrated potassium current should be less than 3.98 and might be as low as 0.90. T_K then might be any value between 0.81 and 0.19.

reason. If the response of the membrane to depolarization is a release of K^{42} of lower specific activity (*i.e.*, axoplasmic specific activity) than that in the Keynes-Caldwell space, we should measure an increment in K efflux above an anomalously high base line efflux. For axon 829 which had the highest apparent resting efflux (491 pmole/cm² sec.) we measured 1255 pmole/cm² sec. during pulsing at 10/sec or an extra efflux of activity of 764 pmole/cm² sec. For most axons, the apparent resting efflux was only 200 pmole/cm² sec.

A curious relationship between T_K and the level of resting membrane potential from which depolarizing pulses start can be developed from the data in that T_K rises toward 1.0 as the membrane potential falls, as shown in Fig. 6. Such a finding

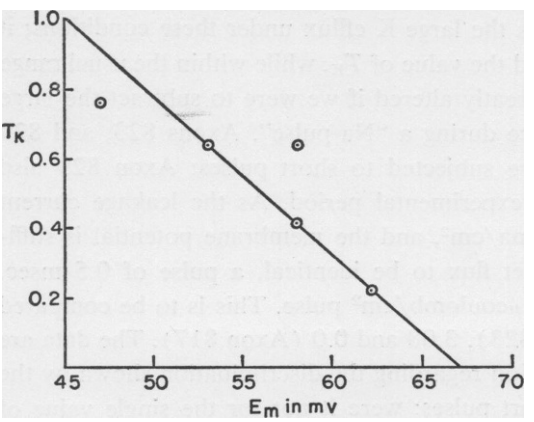

FIGURE 6 The pulse transference number for K^+ is plotted against the resting membrane potential of the axon.

suggests that if g_K is sufficiently high for the membrane at rest, then the increment in efflux and the current agree. The potential at which T_K would appear to reach unity is about −45 mv and it may be noted that at such a potential the Na conductance variable h is very small. There is no obvious connection between h and K flux, but it may be that processes that are active when h is close to 1 are involved in the low values for T_K when the membrane potential at rest is around −60 mv.

The Discrimination between Na^+ and K^+. One axon was successfully loaded with both Na^{22} and K^{42} and the efflux of both of these ions was measured under conditions that should give (*a*) only a Na current (a short pulse to 50 mv beyond E_{Na}) or (*b*) a Na flux but no current plus a large K current (a long pulse to E_{Na}). The results for axon 817 are shown in Table IV. The measurements on this

TABLE IV

SODIUM AND POTASSIUM ION EFFLUXES DURING A VOLTAGE CLAMP IN VARIOUS SODIUM-DEPLETED SOLUTIONS

Axon	Solution	Isotope	Charge transfer during pulse *μcoulomb/cm² pulse*				Pulse duration *msec.*	Pulse frequency *sec.*$^{-1}$	T_{Na}	T_K
			From I_K	From I_{Na}	Efflux Na	Efflux K				
817	0.5 choline	Na and K	0.02	0.12	0.69	3.08	0.5	60	5.75	
			4.81	—	0.68	3.98	4.0	20		0.81
			0.02	0.20	0.49	0	0.5	60	2.5	
823	0.5 choline	K	0.01	0.19		0.04	0.6	60		
829	0.9 choline	K	0	0.27		0.42	0.5	60		

axon gave a large value for T_{Na} but a very much larger efflux of K^+ that is not accounted for by current. This axon was in 0.5 choline sea water and it is a common finding that while axons in 1.0 choline sea water, or in normal sea water give reasonable values of T_{Na}, axons in mixtures of Na and choline give values much greater than 1. The most surprising finding is the large K efflux under these conditions; it is both variable and not explained, and the value of T_K, while within the usual range of values we have found, would be greatly altered if we were to subtract the large efflux of K that apparently takes place during a "Na pulse". Axons 823, and 829 were loaded with K^{42} only, and were subjected to short pulses; Axon 829 also showed a large K efflux during the experimental period. As the leakage current under these conditions is about 0.5 ma/cm², and the membrane potential is sufficiently far from E_K for efflux and net flux to be identical, a pulse of 0.5 msec. should give a charge transfer of 0.25 μcoulomb/cm² pulse. This is to be compared with 0.42 (Axon 829), 0.04 (Axon 823), 3.08 and 0.0 (Axon 817). The data are much too scanty for any firm conclusion regarding the discrimination shown by the membrane between Na and K in short pulses; were it not for the single value of

Axon 823, we could conclude that the K efflux during short pulses could almost be accounted for by leakage current (assuming this to be all K^+).

The Change of Specific Activity of Axoplasm With Time. We have no extensive analytical data on the Na^+ concentration of fresh axoplasm; two analyses of fresh axons gave 68 and 71 mM/kg axoplasm. These agree with the mean values of Kochelin (1955), based on much more extensive analyses, of 65 mM. The axons used in our experiments were usually analyzed for Na within 150 minutes after isolation. With a net influx of 50 pmole/cm² sec. (Shanes and Berman, 1956) we have

Initial Na in axon:

12.5×10^{-6} (kg axoplasm/cm²) × 65 mM/kg = 0.81 μmole/cm²

Resting Na net gain:

150 min × 60 (sec./min.) × 50 pmole/cm² sec. = 0.45 μmole/cm²

Net gain of Na by stimulation at 50/sec. for 600 sec:

50/sec. × 600 sec. × 4 pmole/cm² impulse = 0.12 μmole/cm²

Final $[Na]_i$ 1.38 μmole/cm² or 110 mM/kg axoplasm

This computed value is very close to the mean internal Na for axons subjected to 10 minutes of stimulation, and analyzed after 150 minutes. In a particular axon (No. 817) analysis was performed 70 minutes after isolation and the time course of this specific activity change can be expected to be shown below.

Time after isolation *min.*	Treatment	Computed $[Na]_i$	Axon radioactivity CPM	Specific activity × 10^{-5} CPM/mM
0	—	65	—	—
30	Cleaned	72	—	—
40	Stimulation	84	22,302	265
70	Experiment	93	17,870	191
	$[Na]_i$ by analysis	94	Per cent decrease in specific activity	28

The change in the specific activity for Na is thus a modest one for an experiment with a duration of the order of 30 minutes and this change has been allowed for in the calculations that have been made. For K^+, the changes in specific activity are much less because of the large $[K]_i$ and it has not been necessary to correct for changes in the specific activity of this isotope during an experiment. As the Na in sea water is replaced by choline, the change in specific activity with time will be diminished, and in 100 per cent choline sea water the specific activity of the axoplasm Na remains constant with time.

The Resting Na Efflux. Values for the Na efflux at rest for all the axons used in this study are shown in Table V. The mean for these is 0.57 pmole/cm²

TABLE V

RESTING SODIUM EFFLUXES

Axon	Initial efflux *pmole/cm² sec.*	Final efflux *pmole/cm² sec.*	$[Na]_i$ *mM*	Final efflux / $[Na]_i$ *pmole/cm² sec. mM*	Per cent exchange	Per cent exchange / 10^4 spikes
718	240	—	160*	—	36	12
719	85	119	178*	0.67	31	10
802	(180)	(360)	—	—	(30)	—
817	27	34	94	0.36	22	11
809	72	—	102	—	43	14
823b	141‡	113‡	106	1.0‡	19	9
830	270‡	265‡	260	1.0‡	19	9
		196		0.76		
907	192‡	176‡	158	1.1‡	34	10
	112					
906	(70)	—	—		(30)	—
824	165‡	174‡	256	0.68‡	10	5
831	134	88	180	0.49	63	10
	78‡	73‡		0.40‡		
						—
Mean				0.57		10
				0.83‡		

* Based on analysis of whole axon instead of axoplasm

‡ Efflux in choline sea water

Parentheses indicate that it was not possible to analyze axoplasm at end of experiment and flux has been computed on the basis of 30 per cent equilibration and with $[Na]_i$ = 100 *mM*.

sec. mM $[Na]_i$. This is in agreement with the values of Hodgkin and Keynes (1956) although their measurements were made at 20°C. Because the Na^+ efflux depends so much on the energy supply available to the axon, values for efflux must be expected to vary considerably. The Na^{24} efflux is, however, a good guide to the condition of the axon, and a sudden increase in the resting efflux signals the collapse of the membrane. The other point regarding these data is that the efflux of Na from an axon in choline sea water is somewhat greater than from an axon in sea water. This finding is in agreement with Keynes and Lewis (1951) that exchange diffusion is not demonstrable or is, in fact, negative as compared with frog skeletal muscle (*cf.* Keynes and Swan, 1959).

The Resting K^+ Efflux. Measured values for K^+ efflux in squid axons bathed in normal sea water are anomalously high, as has already been noted by Shanes and Berman (1956). This observation together with the fact that the influx of K^+ is seemingly too low suggest that a 'long pore effect' (Hodgkin and Keynes, 1955) may be operating. Caldwell and Keynes (1960) have compared axons injected with K^{42} and axons soaked in K^{42}. Their data suggest reasons for the anomalous K efflux at rest. The measured K^+ effluxes from 4 axons are shown in

TABLE VI

RESTING K^+ EFFLUX

Axon	Efflux *pmole/cm² sec.* Initial	Efflux *pmole/cm² sec.* Final	$[Na]_i$ *mM*	$[K]_i$ *mM*	E_m *mv*	Per cent exchange
720	160	—	—	210*	−69	1.5
817	156	160	94	337	−67	5.2
823*a*	246	—	104	266	−62	13.7
829	491	456	147	242	−58	16.0

* Based on analysis of whole axon rather than axoplasm.

Table VI. As the K^+ influx in normal sea water is about 50 pmole/cm² sec. if the efflux is taken as 250 pmole/cm² sec., there is a net loss of 200 pmole/cm² sec. The net Na^+ gain can hardly be greater than 50 pmole/cm² sec. so that the apparent unbalanced loss of K^+ is 150 pmole/cm² sec. In one respect the K^+ efflux at rest does not agree with the Hodgkin-Huxley equations. When an axon is placed in choline sea water, the membrane potential rises a few mv., and it is possible to apply current to raise E_m even further. For one axon the results of changes in E_m upon K^+ efflux are shown in Table VII. If one applies the rule that the K^+ conductance g_K falls e-fold for a 5 mv change in membrane potential, a change from −58 to −74 or 16 mv should have decreased g_K by a factor of e^3 or 20. In fact, however, there has been scarcely two-fold decrease in K^+ efflux.

Influxes of Na^+ and K^+. Estimates of the influx of both Na and K could be obtained from the extent to which an axon became loaded with isotope during ion uptake. In the case of Na, stimulation at 50/sec. for 600 sec. left the axon with an internal specific activity of 0.33 that of the external solution. If we take the figures for *Sepia* (Keynes 1951) of 10 pmole/cm² impulse for entry of Na and 4 pmole/cm² impulse for net gain, it can be calculated that for 50 impulses/sec. for 600 sec. there is a net gain of 15 mmole/kg axoplasm. The entry of 10 pmole/cm²

TABLE VII

THE EFFECT OF POTENTIAL AND SODIUM CONCENTRATION ON POTASSIUM EFFLUX (AXON 829)

Fraction of normal Na in sea water	E_m	K efflux *pmole/cm² sec.*
1.0	−58	491
0.1	−64	351
0.1	−74	250
1.0	−52	456

impulse under the same conditions as above and with the axoplasmic Na at 65 mM gives a time constant for Na exchange of 1620 seconds. As the time of stimulation was about 600 seconds the fraction of the internal Na exchanging was about 0.31. We can estimate the expected specific activity of the axoplasm by combining the Na exchange (at constant $[Na]_i$) with the net gain that must have unit specific activity. We have therefore (65 mM × 0.31 + 15 mM × 1.0)/80 mM = 0.43. This value is only slightly greater than the specific activity generally found for axons subjected to 10 min. of stimulation (*cf.* Table V, column 6).

Potassium loading was accomplished by allowing axons to remain in sea water containing various concentrations of K^+ for different times. The axoplasmic specific activity resulting from these treatments allow one to deduce values for the K^+ influx. If the equations obtained by Hodgkin and Huxley for potassium conductance in the steady-state were valid for times of the order of minutes, it should be possible to calculate the loading that would be brought about. In order to simplify the discussion, we will consider an axon with zero membrane potential. In the absence of an electric field, the net flux $\bar{m}_K = P_K ([K]_i - [K]_o)$. Under voltage clamp conditions, and in normal sea water, such an axon might give a current of 3 ma/cm² which corresponds to a flux of 3×10^4 pmole/cm² sec. With $[K]_i = 350$ μmole/cm³ and $[K]_o = 10$ μmole/cm³, $P_K = 1.1 \times 10^{-5}$ cm/sec. The undirectional fluxes $m_o (K) = P_K [K]_i$ and $m_i (K) = P_K [K]_o$ should be obtainable as indicated, with the only restriction on the applicability of the equations that there be zero field across the membrane. As the potassium conductance of the squid axon membrane in the steady state is supposed to be a function only of membrane potential, it should make no difference whether we hold E_m at zero by a clamp or by increasing $[K]_o$ in sea water to such an extent that E_m is zero. With an axon in 350 mM K^+ sea water, influx and efflux should both be 3×10^4 pmole/cm² sec. as the membrane potential should be zero and P_K should have a value appropriate to this potential. As 1 cm² of axon (500 μ diameter) has within its volume 5×10^6 pmole K^+, the time constant for K exchange should be $5 \times 10^6/3 \times 10^4 = 166$ sec. In an experiment where an axon was immersed in K-sea water (450 mM K), the membrane potential should have been close to zero, yet the specific activity of the axoplasm after 10 minutes immersion was 0.07 that of sea water. As 10 minutes is 3.6 time constants, the relative specific activity should have been close to 0.97 and it is evident that there is a large discrepancy. The experimentally measured influx was about 500 pmole/cm² sec. and P_K must have been 500/30,000 of 1.1×10^{-5} cm/sec. or 18 A/sec. instead of 1100 A/sec. Thus P_K over times of the order of minutes appears to have a value of about 1.5 per cent that measured during short-time voltage clamps.

In another experiment, an axon was immersed in a sea water containing 122 mM K^+. The specific activity of the axoplasm was 0.052 that of sea water after 10 minutes which corresponds to an influx of 362 pmole/cm² sec. while the normal

influx of K into an axon from a sea water containing 17 mM K is about 50 pmole/cm² sec. Thus both the ratio of influxes and the ratio of concentrations is about 7.2 although the membrane potential is calculated to be −30 mv and there should have been a large change in g_K.

APPENDIX A

The results presented in this paper have been analyzed without reference to the parameter ion conductance. This has been done in order that such data be independent of any particular model of the membrane. In connection with the relationship between K^+ efflux and current, however, a discussion of possible sources of error in the treatment of the data requires a consideration of ion conductance.

During a long pulse to E_{Na} about 50 pmole/cm² of K^+ emerge into the F-H space of 3×10^{-4} liter/cm² so that the [K] in the space rises from an initial value of 10 mM to 27 mM; the [K] of the space declines with a time constant of perhaps 30 msec., so that as K^+ are being released over a period of 5 msec. the final [K] of the space just at the end of a pulse is about 20 mM. Shutting off of the increment of the potassium conductance g_K by repolarization to the resting potential gives an inward current which when integrated with respect to time gives a charge transfer very close to 30 per cent of the charge transfer during a 5 msec. pulse so that 0.3 × 17 mM = 5.1 mM is the decrease in [K] brought about by shut-off and the final [K] of the space is 14.9 mM. At a frequency of 10/sec. there are more than 3 time constants of the F-H space between pulses and 14.9mM can be taken as the mean [K].

If we take E_K as −75 mv, $E_{Na} = E_{pulse}$ as +35 mv, and the steady-state I_K as 5 ma/cm², then from the relation $g_K = I_K/E - E_K$, $g_K = 45$ mmho/cm². When the membrane potential is switched from E_{Na} to the resting potential (−60 mv), there is an inward current the magnitude of which suggests that E_K during the pulse has been shifted from −75 to −40 mv. Because the membrane potential does not closely follow [K] in this range of concentration, we cannot calculate the shift expected from the efflux directly. With g_K at 45 mmho immediately following the potential change we have an inward current of about 1 ma/cm². If the flux ratio equation (equation 1) is applied to both cases then during the K pulse, efflux to influx is about 60 to 1 and we can ignore any contribution of the influx to current. After shut-off, with a 20 mv difference between E_m and the apparent E_K, efflux to influx has a ratio of 0.45 (equation 1). We have therefore the following relations for fluxes when the membrane is repolarized to −60 mv: $I_K = (m_o - m_i)\ F = 1$ ma/cm², and $m_o/m_i = 0.45$. Solving for the fluxes gives $m_i = -1.83$ ma and $m_o = +0.83$ ma. If we now assemble the information that is available:

[K] in F-H space at end of 5 msec. pulse	20 mM
Contribution of inactive K of s. w. to above	10 mM
Contribution of axoplasmic K to line 1	10 mM
Specific activity of F-H space K relative to internal specific activity	0.5
Initial influx of K at shut-off	1.83 ma.
Initial influx of radioactive K at shut-off	0.92 ma.
Initial efflux of K at shut off	0.83 ma.
Initial efflux of radioactive K at shut-off	0.83 ma.
Net return of K^{42} during shut-off	0.09 ma.

Thus the above calculation indicates that something of the order of 10 per cent of the current during shut off will cause a return of K^{42} to the axon. This is so because although efflux is less than influx, the specific activity of the efflux K is twice that for influx. The above solution has been worked out only for the initial value of g_K immediately after shut-off. As the flux ratio equation depends only on potential, and this is constant with time, the relationship between influx and efflux will be constant as g_K declines to resting values. As the total charge transfer during shut-off is 30 per cent that of a pulse, the calculated error is 10 per cent of this or a return to the axon of 3 per cent of the K released during a pulse.

The error of 3 per cent just calculated would be substantially reduced if consideration is given to the shift in E_K that takes place following shut-off; this is in a direction to make the flux ratio approach unity and therefore to cancel the calculated error. The fact that the error is so small makes it unprofitable to correct further. The ordinary analytical errors for Na and K, and for radioactive measurement are estimated to be $\pm$ 3 per cent while errors in weighing and volumetric manipulation are $\pm$ 1 per cent. It seems likely, therefore, that a T_K of 0.53 represents a good estimate of the contribution of K to the steady-state current.

The authors wish to express their appreciation to the Marine Biological Laboratory for making their excellent facilities available to us, to Mr. L. Binstock for his extremely valuable technical and electronic assistance and to Dr. K. S. Cole for his close cooperation and continued assistance in the experimental work, in the preparation of the manuscript, and for his initial suggestion of the problem to us.

The isotope work in this study was aided by a grant (B-2075) from the National Institute of Neurological Diseases and Blindness, Bethesda.

Received for publication, December 12, 1961.

BIBLIOGRAPHY

ADELMAN, W. J., and TAYLOR, R. E., *Nature*, 1961, **190**, 883.
BEHN, U., *Ann. Physik.*, Leipsic, 1897, **62**, 54.
CALDWELL, P. C., and KEYNES, R. D., J. Physiol., 1960, **154**, 177.
COLE, K. S., and MOORE, J. W., *Biophysic. J.*, 1960*a*, **1**, 1.
COLE, K. S., and MOORE, J. W., *J. Gen. Physiol*, 1960*b*, **44**, 123.
FITZHUGH, R., unpublished data.
FRANKENHAEUSER, B., *J. Physiol.*, 1960, **152**, 159.
FRANKENHAEUSER, B., and HODGKIN, A. L., *J. Physiol.*, 1956, **131**, 341.
HODGKIN, A. L., and HUXLEY, A. F., *J. Physiol.*, 1952, **115**, 500.
HODGKIN, A. L., and HUXLEY, A. F., *J. Physiol.*, 1953, **121**, 403.
HODGKIN, A. L., and KEYNES, R. D., *J. Physiol.*, 1955, **128**, 61.
HODGKIN, A. L., and KEYNES, R. D., *J. Physiol.*, 1956, **131**, 592.
KEYNES, R. D., *J. Physiol.*, 1951, **109**, 240.
KEYNES, R. D., and LEWIS, P. R., *J. Physiol.*, 1951, **114**, 151.
KEYNES, R. D., and SWAN, R., *J. Physiol.*, 1959, **147**, 591.
KOCHELIN, B. A., *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 511.
SHANES, A. M., and BERMAN, M., *J. Gen. Physiol.*, 1956, **39**, 279.
TEORELL, T., *Arch. Sc. Physiol.*, 1949, **3**, 205.
USSING, H. H., *Acta Physiol. Scand.*, 1949, **19**, 43.