Tracer and Nontracer Potassium Fluxes in Squid Giant Axons and the Effects of Changes in External Potassium Concentration and Membrane Potential

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ABSTRACT The efflux of labeled and unlabeled potassium ions from the squid giant axon has been measured under a variety of experimental conditions. Axons soaked in sea water containing ^{42}K ions lost radioactivity when placed in inactive sea water according to kinetics which indicate the presence of at least two cellular compartments. A rapidly equilibrating superficial compartment, probably the Schwann cell, was observed to elevate the specific activity of ^{42}K lost from such axons to K-free sea water for a period of hours. The extra radioactive potassium loss from such axons during stimulation, however, was shown to have a specific activity identical within error to that measured in the axoplasm at the end of the experiment. The same was shown for the extra potassium loss occurring during passage of a steady depolarizing current. Axons placed in sea water with an elevated potassium ion concentration (50 mM) showed an increased potassium efflux that was in general agreement with the accompanying increase in membrane conductance. The efflux of potassium ions observed in 50 mM K sea water at different membrane potentials did not support the theory that the potassium fluxes obey the independence principle.

INTRODUCTION

The movements of various radioactive ionic species across the membranes of invertebrate giant axons have been studied by many investigators. The results obtained with radioisotopes of potassium and sodium ions have generally confirmed the ionic hypothesis of Hodgkin and Huxley (1952). Keynes (1951) measured the increased fluxes of potassium and sodium ions occurring during the passage of nerve impulses in giant axons from *Sepia*. Similar measurements have been made on squid giant axons by Shanes (1954) and by Caldwell and Keynes (1960) using ^{42}K to trace potassium movement. The

results obtained by these investigators are in general agreement with the ionic hypothesis. Furthermore, Mullins, Adelman, and Sjodin (1962) tentatively identified the early current in a voltage clamp of the squid giant axon as a current carried by sodium ions and the late current as mainly a potassium current using radioactive isotopes of these cations as tracers. Also, Brinley and Mullins (1965) have shown that the resting membrane of squid giant axons from *Loligo pealii* has a high selectivity for potassium ions compared to the selectivity shown by the membrane for sodium or chloride ions. Meves and Chandler (1965) and Chandler and Meves (1965) using internally perfused squid giant axons, have worked out the selectivities of the early and late current channels to a series of cations. The relative selectivity of the early current channels for sodium ions and the late current channels for potassium ions was high.

In spite of the large amount of information available relative to ion transfers across giant axonal membrane, some questions remain unanswered. It is well known that potassium efflux across squid giant axon membranes reckoned from ^{42}K loss by axons previously soaked in ^{42}K-containing sea water is much higher than the rate of loss suggested by net movement of total K (Shanes and Berman, 1955; and Caldwell and Keynes, 1960). Shanes and Berman referred the anomaly to an "X-phase" containing ^{42}K ions at a higher specific activity than that found in the axoplasm. Caldwell and Keynes tentatively identified the X-phase with the Schwann cells surrounding the axon. Labeled potassium collected from the Schwann cells would be expected to influence the resting potassium efflux deduced from the loss of radioactivity by axons previously soaked in ^{42}K-containing sea water, but not the extra potassium lost per nerve impulse as the latter potassium is believed to originate solely within the axoplasm. The purpose of this investigation was, in part, to test this hypothesis by measuring the specific activity of ^{42}K lost by ^{42}K-soaked axons to a potassium-free sea water at rest and during excitatory activity and comparing the results with the specific activity measured in the axoplasm. Similar experiments are also reported under conditions of a potassium loss promoted by passage of a steady depolarizing current.

Another part of the investigation was concerned with the membrane potential and concentration dependence of potassium efflux in squid giant axons. Hodgkin and Keynes (1955) varied the external potassium concentration and noted the effect on potassium efflux under conditions of approximately constant membrane potential. The results suggested that potassium influx and efflux are not independent processes in giant axons from *Sepia*. The question arose as to the existence of a similar situation in giant axons from the squid. Some of the data provided by this investigation should help to answer this question.

METHODS

Axons from the squid, *Loligo pealii*, were carefully dissected from the hindmost stellar nerve. The giant axon was separated from the nerve trunk and a variable length of axon was carefully cleaned of adhering small fibers. The length cleaned depended on the experimental arrangement. In the experiments designed to measure the specific activity of ^{42}K lost during rest and stimulation periods, a 6 cm portion of axon was cleaned. The axon was then mounted in a narrow well-type chamber equipped with platinum electrodes for stimulating and recording the action potential. The tied ends of the axon were covered with a vaseline–mineral oil mixture and the remainder of the cleaned axon in the well was immersed in 1 cc of K-free sea water, the composition of which is given in a section to follow. Axons for this part of the investigation were previously soaked for periods of a few hours in a ^{42}K-containing sea water. At 10 min intervals, the solution surrounding the axon was replaced with fresh solution and the solution withdrawn was analyzed for potassium content and was assayed for radioactivity. In a given experiment, a 10 min interval in which the axon was not stimulated was employed to obtain the resting K and ^{42}K efflux. The axon was then stimulated for 10 min intervals at a frequency of 50 impulses per sec. Both K and ^{42}K effluxes were again determined. The extra efflux during stimulation was obtained by subtraction of the resting values. In an experiment to determine the K vs. ^{42}K efflux characteristics at rest over a long period, a similar technique was used except that efflux intervals were 30 min and the axons were not stimulated. The axon diameter varied considerably over a 5 to 6 cm length of axon and this produced some uncertainty in the estimate of axonal surface area. The technique employed was to measure the diameter in three positions, approximately 1.5 cm apart. An average diameter was used to obtain the surface area. At the end of each experiment, the axon was cut 1 cm from a vaselined end and an approximately 3 cm length of axoplasm was extruded on to a small clean piece of Saran by using a small roller. The axoplasm sample was weighed and ashed in a platinum crucible for a period of 10 hr. The ash was then dissolved and potassium and sodium analyses were made by flame photometry. An aliquot of the axoplasm solution was used to determine the ^{42}K radioactivity.

In the experiments in which it was desired to pass current through a portion of membrane, a 4 cm length of cleaned axon was mounted in the chamber diagramed in Fig. 1. The arrangement is essentially a "sucrose-gap" for maintaining electrical insulation in two regions of membrane. The two end-pools contained KCl isosmotic with sea water. The two intermediate pools contained a flowing isosmotic sucrose solution. The center pool contained the experimental portion of axon membrane and this region was externally perfused with the desired artificial sea water which was collected in tubes by means of a suction device. The perfusate collected was analyzed for K and ^{42}K only, depending upon the type of experiment. Collection intervals were 2 min each in these experiments. The membrane potential in the center region was measured by means of a 3 M KCl-filled microelectrode and a low grid current electrometer type bioelectric amplifier. Resting and action potentials were displayed

on a Tektronix oscilloscope. The membrane potential in the center region could be altered by passing current between the end and center pools by means of the voltage divider circuit diagramed. Current densities were measured by introducing a microammeter into the current-passing circuit. The arrangement allows the determination of a steady-state current-voltage characteristic and the determination of the effects of membrane potential and external potassium concentration on potassium efflux. As before, an axoplasm sample was taken for analysis at the end of each experiment.

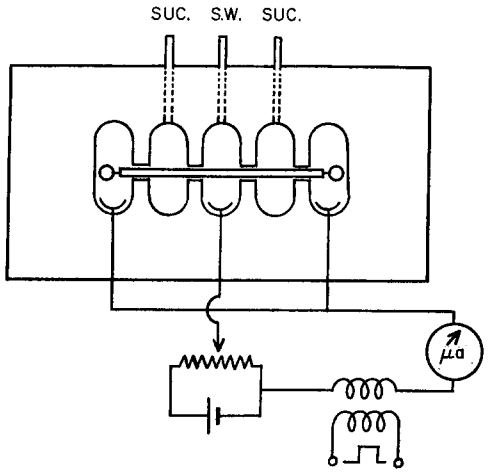

FIGURE 1. The chamber used to measure efflux from axons under conditions where the external potassium concentration was changed and hyperpolarizing currents were passed is shown diagramatically. Cooled sea water (concentrations of ions stated in text) was passed into the center compartment and subsequently collected for the assay of potassium ions. Current was passed through the membrane using the electrodes in the center and end compartments and the voltage divider illustrated. Current was measured by means of a microammeter placed in the circuit. An isosmotic sucrose solution was passed into the pools labeled "suc." to provide electrical insulation of the portions of axon situated in these pools.

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

Solution	pH	$[Na^+]$	$[K^+]$	$[Ca^{++}]$	$[Mg^{++}]$	$[Cl^-]$	$[SO_4^=]$	$[HCO_3^-]$	[Choline]
			mmoles/liter						
Normal sea water	7.9	423	10.0	9.3	48.5	497	25.5	2.2	
K-free sea water	7.9	433	0	9.3	48.5	497	25.5	2.2	
50 mM K sea water	7.9	383	50.0	9.3	48.5	497	25.5	2.2	
Choline sea water	7.7	0	0	9.3	48.5	494	25.5	2.0	430

Radioactive Solutions and Axon Labeling ^{42}Potassium was procured as the chloride salt in an acid solution. After neutralization it was added to K-free sea water to

produce the ^{42}K soak solution. The potassium concentration in the soak solution varied between 10 and 30 mM depending upon the amount of activity added. The specific activity of potassium in the soak solution was always determined.

In the experiments performed to determine the effects of membrane potential and potassium concentration on potassium efflux, axons were internally injected with isosmotic K_2SO_4 labeled with ^{42}K using a microinjection apparatus similar to the one described by Hodgkin and Keynes (1956) and identical, except for modifying details, to the one described by Brinley and Mullins (1965). An approximately 1 cm length of axon was uniformly labeled with ^{42}K in this way. The use of a dye in the injection fluid assured that the injected portion would always be mounted in the center pool region of the chamber.

Analytical Procedures Flame photometry of potassium and sodium was performed with a specially constructed instrument designed by L. J. Mullins and previously described (Sjodin and Henderson, 1964). Potassium and sodium interference filters of 2 mμ band width allowed analyses to be made with no detectable interference. The potassium filter had a narrow enough spectral range to permit accurate analyses to be carried out in the presence of sea water sodium concentrations. In the latter case potassium standard solutions were made by adding small amounts of potassium to K-free sea water. Analyses were performed by zeroing the instrument using K-free sea water and comparing the unknowns with the sea water standard potassium solutions. Potassium concentrations in the 10 to 200 μM range could be determined with the usual flame photometer accuracy in this way. In some experiments, the extra K and ^{42}K flowing out of the axon during passage of a depolarizing current were determined. This necessitated the use of the arrangement diagramed in Fig. 1 and previously discussed. Due to the smaller length of axon exposed to sea water and the use of shorter collection times, the potassium collected into a K-free solution resulted in a concentration in the 1 to 4 μM range. With sea water sodium concentrations present, it was not possible to obtain accurate potassium analyses in this concentration range. To accomplish the experiment, use was made of a K-free perfusion solution in which choline was substituted for sodium. These potassium analyses were somewhat less accurate than those usually made by flame photometry, accuracy being $\pm 5\%$ instead of the usual $\pm 1\%$.

Radioactive counting was performed by drying all samples on planchets and counting by means of an automatic low-level counting system.

Efflux Measurements Potassium efflux was determined from ^{42}K loss by dividing the ^{42}K efflux by the specific activity of the ^{42}K measured in the axoplasm.

Temperature Control Experiments not performed in the chamber depicted in Fig. 1 were performed at room temperature which is stated for each experiment. Experiments performed in the chamber of Fig. 1 were at a temperature lower than room temperature. In these cases the external perfusion solution passed through a thermoelectric cooling device and the temperature was monitored with a thermistor and voltmeter thermometer. The temperatures attained were in the range of 10° to 16°C and are stated in each case.

RESULTS

K and ^{42}K Effluxes during Electrical Stimulation

The results of the stimulation experiments in a K-free solution are summarized in Table I. Axons were soaked in ^{42}K-containing sea water for periods of from 2.5 to 6 hr and subsequently treated in the manner indicated under Methods. The extra potassium loss per cm^2 of axon per impulse was computed by two methods: (*a*) the assumed uniform specific activity in axoplasm was used to calculate total axoplasmic K output from the extra ^{42}K output and

TABLE I

TRACER AND NONTRACER POTASSIUM LOSS FROM STIMULATED AXONS PREVIOUSLY SOAKED IN ^{42}K-CONTAINING SEA WATER

Axon No.	Temperature	Stimulation interval	Extra potassium loss per impulse		Specific activity of extra K lost / Specific activity of K in axoplasm	^{42}K equilibration of axoplasm
			Tracer	Nontracer		
	°C	min	pmoles/cm^2			%
0525 B	18.5	10	6.4	6.1	1.05	34
		10	6.5	6.1	1.07	
		10	5.9	5.4	1.09	
0525 C	19.5	10	5.5	5.6	0.98	56
		10	5.5	5.7	0.97	
		10	5.1	4.8	1.06	
0526 A	20.3	10	5.6	5.0	1.12	54
		10	4.9	4.9	1.00	
		10	5.9	5.6	1.05	
		10	3.8	3.9	0.98	
		10	3.6	4.1	0.88	

(*b*) the total extra output of analytical K was used directly to obtain the same quantity. The table shows that the two methods yield values that agree to within experimental error.

K and ^{42}K Effluxes at Rest

The next experiments performed were for the purpose of determining the influence of a more rapidly equilibrating superficial compartment on the resting ^{42}K efflux from axons previously soaked in ^{42}K-containing sea water. A typical experiment gave the data shown in Table II. The axon in this experiment was soaked in ^{42}K sea water for a period of 4.67 hr. Efflux of both K and ^{42}K was followed for six intervals or a total of 3 hr. The relative specific activity of ^{42}K in the efflux samples is presented in the table and is plotted against time in Fig. 2. By considering the efflux samples to contain a

mixture of ^{42}K from two sources of different specific activity, e.g. axoplasm and Schwann cell, the observed specific activity can be analyzed into two fractions. This was accomplished by means of the equations $6.5x + 30.5y =$ S.A. and $x + y = 1$ where 6.5 is the relative S.A. in the axoplasm, 30.5 is that for the soak solution and the Schwann cell, and x and y refer to fractions of slow and fast compartment counts respectively. The fast fraction, y, appears as a column in Table II and is plotted semilogarithmically in Fig. 2B.

TABLE II

TRACER AND NONTRACER POTASSIUM LOSS FROM ^{42}K-SOAKED AXONS DURING REST

Temperature = 19.5°C

Sample	Relative specific activity	Total cpm in sample	Fast fraction		Back-added cpm
			y	cpm	
1	16.0*	1929	0.40‡	772	2505
2	14.2	1370	0.32	438	1733
3	13.0	1218	0.27	329	1295
4	11.8	1097	0.22	241	966
5	10.6	1013	0.17	172	725
6	9.6	1080	0.13	140	553
Axoplasm	6.5		Residual	413	413
Soak-solution (Schwann cell)	30.5				

* Read from graph, Fig. 2A.

‡ Calculated as indicated in text.

K and ^{42}K Loss during Passage of Depolarizing Current

When a steady depolarizing current is passed across the membrane of squid giant axon an increased potassium efflux occurs (Brinley and Mullins, 1965). As in the case of the stimulation experiments, all the extra potassium moving as a consequence of current passage is believed to originate in the axoplasmic compartment. Though the specific activity of ^{42}K lost to a K-free solution during rest is expected to be higher than that found in the axoplasm, the specific activity of the extra potassium lost during a depolarization should be equal to the specific activity in axoplasm to within experimental error. The results of a typical experiment are shown in Fig. 3. This particular axon had an initial resting potential of −62 mv and an action potential of 100 mv. Plotted in Fig. 3 are the amounts of K and ^{42}K in each efflux sample. Though there is considerable scatter in the resting base line for total K output, an average value can be used to estimate the resting efflux of total potassium. For comparison, the resting potassium efflux can also be estimated from ^{42}K output and a knowledge of the specific activity in axoplasm. The resting efflux from total K output had a value of 71 pmoles/cm^2 sec while that from

^{42}K output was 125 pmoles/cm^2 sec. It is clear that the efflux samples during rest contain considerable ^{42}K derived from a compartment with a specific activity higher than that in the axoplasm. At the point represented by 16 min in Fig. 3, the membrane was depolarized to a potential of −45 mv for 2 min. It is evident that the correlation between extra K and extra ^{42}K output

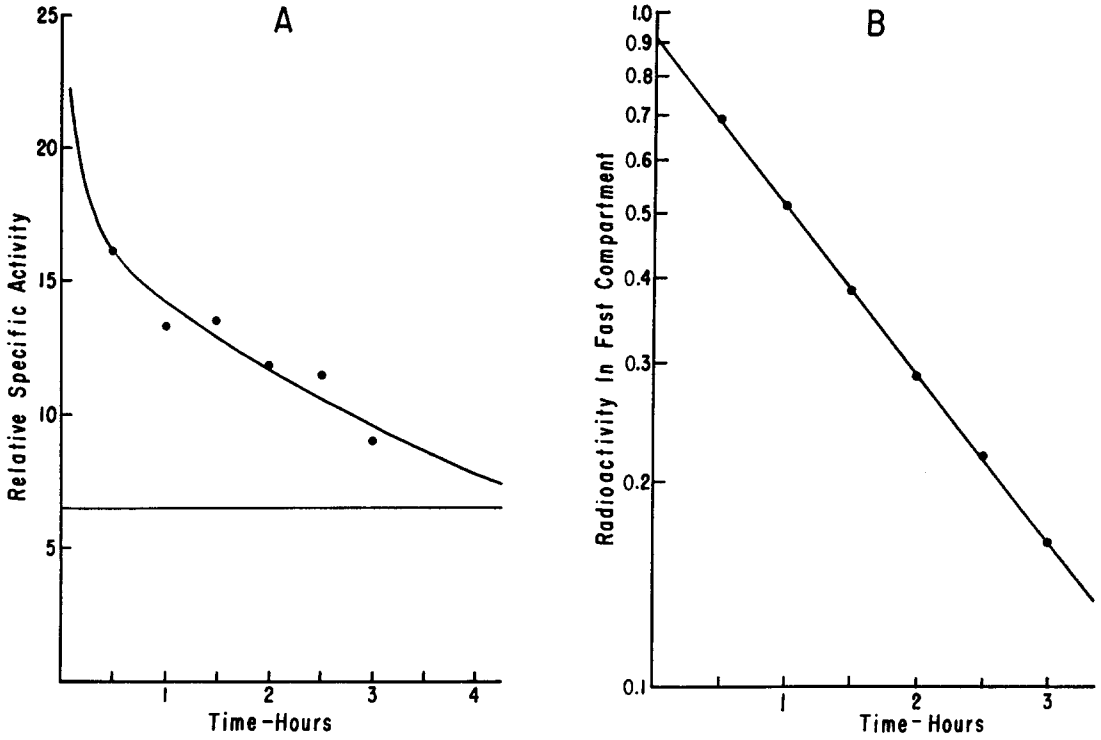

FIGURE 2. The graph in *A* is a plot of the measured specific activity of radioactive potassium lost from axons soaked in ^{42}K-sea water against the time in contact with K-free sea water. The radioactivity due to ^{42}K residing in a superficial rapidly equilibrating compartment is plotted semilogarithmically against time in graph *B* (see text). It was assumed that potassium ions are lost from the fast compartment with a single rate constant.

is excellent. By subtracting the resting levels of K and ^{42}K output from the total respective outputs during depolarization, the extra amounts of K and ^{42}K are obtained. The specific activity of the extra potassium output from this axon was 92% of the axoplasmic specific activity. This must be taken to indicate agreement within experimental error as the deviation can easily be accounted for by uncertainty in the base lines, especially for total potassium output. This experiment shows clearly the influence of ^{42}K in a superficial axon compartment on the resting efflux and also its lack of in-

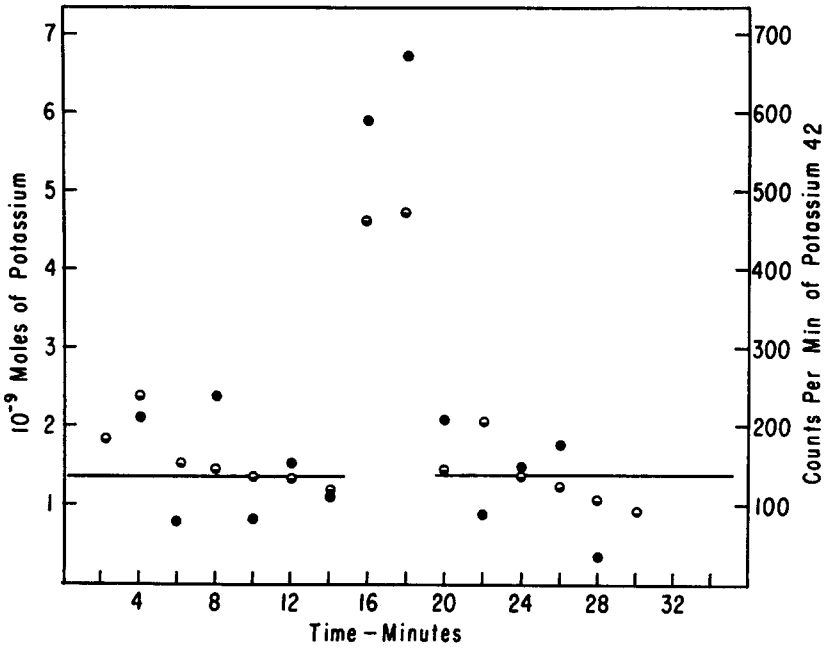

FIGURE 3. The extra loss of labeled and unlabeled potassium ions from an axon during passage of a steady depolarizing current is illustrated. The filled-in circles refer to the left-hand axis for the amount of unlabeled potassium. The half-filled circles refer to the right-hand axis for the amount of labeled potassium. In the time interval between 16 and 18 min, the membrane in the center compartment (Fig. 1) was depolarized to −45 mv by passing a steady outward current. The membrane was under resting conditions with a membrane potential of −62 mv for all the other time intervals. Potassium was collected in all cases into a K-free choline-substituted sea water (see text).

fluence on any extra efflux originating as a consequence of current flow through the membrane.

The Influence of Potassium Concentration and Membrane Potential on Potassium Efflux

^{42}Potassium was injected into each axon as an isosmotic potassium sulfate solution in all remaining experiments. Experiments were performed in the apparatus shown in Fig. 1. A series of resting efflux samples was taken after which the perfusion solution was changed to a sea water with $[K]_o$ = 0 or 50 mM. The new resting potential was recorded and a new series of resting efflux samples was taken. The membrane was next hyperpolarized by passing current across the membrane in the center pool of the chamber. The membrane potential during the hyperpolarization and the current necessary to sustain it were recorded and another set of efflux samples was taken. A final set of efflux samples was taken after hyperpolarization and also after restoring

TABLE III

THE EFFECTS OF OF EXTERNAL POTASSIUM CONCENTRATION AND MEMBRANE POTENTIAL VARIATION ON POTASSIUM EFFLUX

Axon No.	Temperature	Efflux* $[K]_o$ = 10 mM	E_{m_1}‡ Normal R.P.	New concentration $[K]_o$	Efflux at new concentration	E_{m_2} New R.P.	Efflux during hyperpolarization	E_{m_3} Hyperpolarized membrane potential	Specific K efflux at new concentration	Calculated conductance g_K
	°C			mM						mMho/cm²
725	13.0	80	60	0	82	63	38	70	44	0.82
730	12.5	44	61	0	46	68	17	88	29	0.54
801	11.2	54	60	0	46	65	20	82	26	0.49
802	12.5	102	60	0	74	64	46	80	28	0.52
813	12.6	240	60	0	210	65	160	90	50	0.93
712	12.0	99	61	50	334	44	274	80	235	0.90
713	15.0	89	60	50	267	45	131	67	168	0.64
716	12.5	79	60	50	204	46	126	82	105	0.40
717	12.5	252	59	50	671	45	417	85	572	2.18
720	13.5	200	60	50	950	47	732	90	851	3.24

* All fluxes are in units of pmoles/cm² sec.

‡ All membrane potentials are in units of millivolts, inside negative.

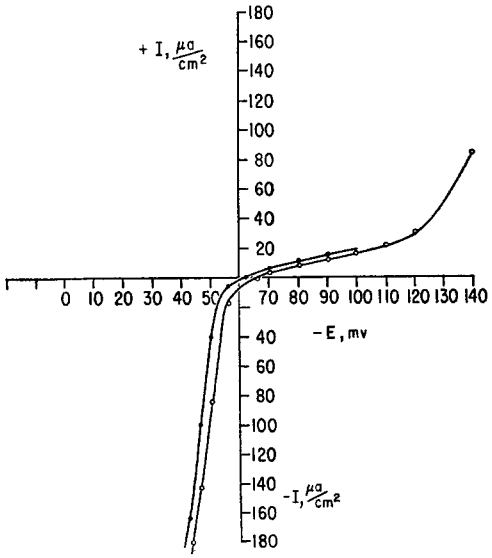

FIGURE 4. The current-voltage relation obtained on an axon in K-free sea water is compared with that obtained in normal 10 mM K sea water. The filled circles refer to values obtained in normal sea water. The origin of the graph is the resting potential in 10 mM K sea water. The sign convention used is that the positive numbers on the voltage axis denote negativity inside the axon. The positive currents are inward or hyperpolarizing currents whereas the negative currents are outward.

the initial conditions. The data obtained are summarized in Table III. To compare flux data with conductance data, current-voltage relations were measured in normal sea water, in K-free sea water, and in 50 mM K sea water.[1] The results appear in Figs. 4 and 5.

[1] The Appendix should be consulted for a discussion of possible current density nonuniformities.

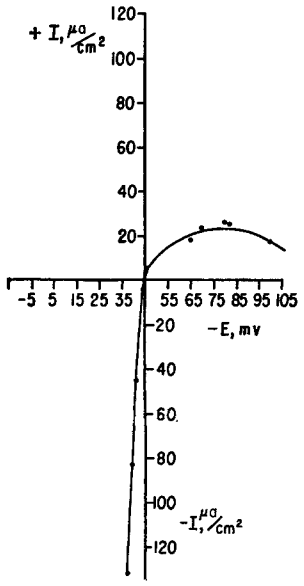

FIGURE 5. The current-voltage relation obtained when an axon was in 50 mM potassium sea water is presented. The origin of the graph is the resting potential of −45 mv obtained in 50 mM K sea water in the absence of current flow. Sign conventions for voltage and current are identical to those stated for Fig. 4. (See Appendix for discussion of possible current density nonuniformities.)

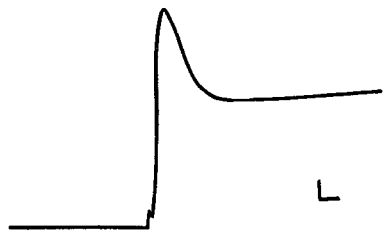

FIGURE 6. The action potential given by an axon hyperpolarized when in 50 mM K sea water is illustrated. The vertical calibration mark represents 15 mv and the horizontal calibration represents 1 msec. The resting potential in 50 mM K sea water for this axon was −41 mv. The hyperpolarized holding potential was −128 mv. The membrane potential was quite stable at this value and it was difficult to hold the potential at less hyperpolarized values due to instability. The temperature was 15°C. The action potential had an overshoot of 37 mv.

Action Potentials in Solutions with Elevated Potassium Concentration

A final point deals with the excitability of axons hyperpolarized in 50 mM K sea water. These axons gave action potentials when electrically stimulated in the usual manner. A typical action potential is shown in Fig. 6. The falling phase eventually returns the membrane potential to a value agreeing with the equilibrium potential for potassium ions under these conditions as expected. Restoration of action potentials by hyperpolarization in high K media has been previously observed by Narahashi (1964).

DISCUSSION

The resting potassium efflux magnitudes measured by the ^{42}K microinjection technique lie close to the range of values observed by Caldwell and Keynes (1960). Though some of the presently measured values are somewhat above this range, this would be expected if membrane regions just adjacent to the vaseline seals were more leaky to potassium. When ^{42}K was introduced into axons by soaking them in ^{42}K sea water, however, resting potassium effluxes determined by the tracer method using the specific activity in the axoplasm did not agree with the analytically determined potassium losses. The reason for this is the presence of a rapidly equilibrating superficial compartment. The rate constant of the fast compartment obtained from the slope in Fig. 2B has the value 0.57 hr^{-1}. This is within the range reported by Caldwell and Keynes and it appears likely that the Schwann cells are the source of the observed specific activity elevations in the efflux samples. The extra potassium effluxes observed during electrical stimulation, however, were within the normal range even though the axons had been soaked in ^{42}K sea water for considerable periods. The highly equilibrated superficial compartment does not interfere in any detectable way with ^{42}K efflux across the axolemma.

The effects of variation in external potassium concentration on potassium efflux are in general agreement with the effects on membrane conductance presented in Figs. 4 and 5. The results are also in general agreement with the Hodgkin-Huxley (1952) findings concerning the effects of membrane depolarization on potassium conductance. Some features of the data in Table III will be pointed out and any discrepancies noted. Upon reducing the external potassium concentration from 10 mM to zero little change in efflux was observed. The current-voltage relation change observed (Fig. 4) is consistent with this as a shift in the curve was observed with little change in resting membrane conductance. Further hyperpolarization reduced potassium efflux to about 50% of the initial value. Since Cole and Moore (1960) found a continuous decrease in potassium conductance with increasing hyperpolarization, it appears likely that the potassium efflux observed during hyperpolarization represents a nonspecific or leakage flux (Mullins, 1966). These values were thus subtracted from the effluxes observed in the K-free sea water to obtain the "specific K efflux" column in Table III. From the current-voltage plot in Fig. 4, the limiting slope at high potentials yields a conductance of 0.3 mmho/cm^2. At the resting potential (K-free sea water) the conductance is 0.8 mmho/cm^2 and subtraction of leakage as in the case of efflux gives a specific potassium conductance of 0.5 mmho/cm^2. The specific K effluxes reported can be used to calculate specific potassium conductances for comparison. The equation employed is a nonsteady-state relation ob-

tained by Sjodin (1959) applying the assumption of a constant electrical field. For zero potassium influx, the equation is:

$$G = \frac{F^2}{RT} m_o \left[\left(\frac{1}{1 - e^{-EF/RT}} \right) - \frac{RT}{EF} - \frac{RT}{F} \frac{\partial \ln P}{\partial E} \right]$$

where m_o refers to efflux and all other quantities have their usual significance. The coefficient $\frac{RT}{F} \frac{\partial \ln P}{\partial E}$ is obtained from the Hodgkin-Huxley assumption that the potassium conductance increases e-fold for every 5 mv of depolarization. By applying a small correction for the influence of a 5 mv change in driving force, the coefficient is easily obtained and is found to have the value of −4.4. Values of conductance calculated from specific K efflux are found as the first five entries in the last column of Table III. Three of the values are very close to the conductance estimated from the current-voltage diagram, i.e. 0.5 mMho/cm².

When the external potassium concentration was changed from 10 to 50 mM, the data show that potassium efflux becomes elevated by a factor of about 3. The membrane potential declines from −60 to −45 mv on the average. The resulting change in outward driving force as estimated by use of the constant-field flux equation is an increase by a factor of about 1.5. On this basis, one would conclude that the potassium resting permeability doubles upon making the change from 10 to 50 mM K. At the resting membrane potential in 50 mM K sea water, the membrane conductance is estimated at 4 mMho/cm². In view of the possible errors in the determination of the latter, as discussed in the Appendix, the conductance increase is in fair agreement with the flux increase. The results are also consistent if estimated potassium leakages are subtracted from total flux values. To accomplish this for the 50 mM K data, the average leakage in the K-free experiments (56 pmoles/cm² sec) is corrected for additional leakage due to 15 mv of depolarization by using the leakage conductance of 0.3 mMho/cm² previously determined. The total leakage estimated is 99 pmoles/cm² sec. This value is subtracted from the 50 mM K effluxes to continue the specific K efflux column in Table III. The increase in specific K efflux upon increasing the external K concentration to 50 mM is consistent with the increase in potassium conductance arrived at from current-voltage measurements. Though it appears that the efflux increase and conductance increase are consistent, the absolute values of calculated conductances in 50 mM K sea water do not agree well with measured values. In the 50 mM K case, values were calculated

from the steady-state relation $g_K = \frac{F^2}{RT} m_{o_K}$. The value determined from current-voltage measurement was 3.7 mMho/cm² and this value is approached by only two of the five axons studied.[2]

When axon membranes exposed to 50 mM K sea water were hyperpolarized, generally to values between −80 and −90 mv, a reduction in potassium efflux was observed. The observed reduction was insufficient, however, to support the principle of the independence of potassium influx and efflux. According to this principle, potassium efflux should depend upon the membrane potential and the internal potassium ion concentration. Since the inside potassium ion concentrations did not change much during these experiments, all axons may be regarded as having comparable values. This was verified by flame photometry. Since all hyperpolarized membrane potential values were similar (column E_{m_3}), the last five and the first five entries in the "Efflux during hyperpolarization" column should be comparable. Clearly, they are not. When the external potassium concentration was restored to the initial value (10 mM) during hyperpolarization, potassium efflux fell to values much closer to the initial fluxes. Any discrepancies between the latter values could easily be explained by a gradual deterioration of the preparation during the course of the experiment. It is evident that external potassium ions exert an influence on potassium efflux even at constant membrane potential. A failure of the potassium fluxes to obey the independence principle was also observed in giant axons from *Sepia* by Hodgkin and Keynes (1955). In this case, however, the deviation was in the opposite sense to that observed in this work.

Some possible mechanisms for the presently observed departures from the independence principle will be briefly examined. One possibility is that the hyperpolarizing current does not polarize the region of membrane in which conductance control is normally exerted. This possibility seems to be an extremely unlikely one. Another possibility is that a fraction of the K^+ movement engages in exchange diffusion. A difficulty with this explanation is that no decrease in potassium efflux occurs when the external potassium concentration is lowered from 10 mM to zero. This would normally be the region of most pronounced exchange diffusion effect. Another difficulty is that transport by exchange diffusion is incapable of carrying electrical current. If much potassium transport is by this mechanism, it becomes difficult to ac-

[2] It is possible that the potassium fluxes do not correspond to an exact steady state even in the solution with elevated potassium concentration. In this event, the previous equation would provide the necessary correction which would be in the direction of elevating the conductance calculated from the flux data. Since influx was not measured under these conditions, no attempt was made to correct for possible departures from the steady state. Possible errors in the conductances estimated from current-voltage data are discussed in the Appendix.

count for the flux-conductance correlations. A third possibility is that there is a critical region in the membrane where the potassium concentration itself exerts permeability or conductance control. This appears, at present, to be the most likely explanation. The sequence of events could be visualized in the following manner. Membrane depolarization in a solution with normal potassium concentration leads to some potassium accumulation in an outer region of membrane which in turn leads to conductance increase. In a high K solution, the external K feeds the region and also depolarizes the membrane, both factors leading to conductance increase. In a high K medium during membrane hyperpolarization, the elevated potassium concentration keeps the outer portion of membrane enriched with potassium, the hyperpolarizing current being insufficient to deplete the critical region of potassium ions. The actual mechanism is likely to be very complicated, involving an interaction of both electrical potential and potassium concentration profiles.

APPENDIX

In all the cases in which the membrane potential was altered by means of current flow using the experimental arrangement depicted in Fig. 1, the question of the uniformity of current density and membrane potential arises as well as the question of the possibility of current leaks in the vaseline seal regions. Regarding the latter, all attempts to detect such leakage by collecting current using an electrode placed into the vaseline seal regions failed to indicate the occurrence of current leaks. Perhaps the best evidence that significant current leaks did not occur in these experiments is that the current-voltage relations obtained in normal sea water were very similar to those obtained by Cole and Moore (1960) employing a central wire technique. The slope of the current-voltage curve in Fig. 4 at the resting potential yields a conductance of 0.8 mMho/cm^2 or a membrane resistance of 1200 ohm·cm^2. The mean value cited by Cole and Moore (1960) was 1400 ohm·cm^2.

The uniformity of the current density and membrane potential over the length of axon in the center pool of sea water depends on the length constant of the axon. This parameter, in general, varies with the membrane potential. For small displacements from the resting potential and for regions of approximate linearity in the current-voltage plot, the length constant remains essentially invariant. In the regions of hyperpolarization employed in this investigation, the length constant for a 500 μ diameter axon has a value around 1.1 cm. The length of axon in the center pool was 0.5 cm in the experiments reported and microelectrode penetrations were made in the center of the pool. The lengths over which current density nonuniformities can occur are thus 2.5 mm on either side of the microelectrode. For these lengths, the nonuniformity in the region of hyperpolarization has a maximum value of around 20%. With this degree of nonuniformity, the membrane potential recorded by the microelectrode during hyperpolarization to -80 mv will be within 5% of the average membrane potential determined on a weighted length basis. This uncertainty is within the limits of error in the flux determinations made by the radioactive tracer methods employed.

In the case of large depolarizations, the length constant decreases to values around 0.3 cm, and the current density nonuniformities become considerable. In these instances (the determination of the curve in Fig. 5, for example) microelectrode penetrations were made near the half-way point from the center to one of the vaseline seals in an attempt to record a membrane potential representative of the average value. The slopes determined from the data illustrated in Fig. 5 are consistent with those obtained by Cole and Moore (1960) in normal sea water in a comparable region of depolarization. Any errors occurring in the region of large depolarization as a consequence of these factors would be in the direction of overestimating the conductance. This may account for some of the apparent discrepancy between the conductance estimated from K efflux in 50 mM K sea water and that estimated from electrical data under the same conditions.

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