Cation Interdiffusion in Squid Giant Axons

ICHIJI TASAKI, IRWIN SINGER, and AKIRA WATANABE

From the Laboratory of Neurobiology, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, and The Marine Biological Laboratory, Woods Hole, Massachusetts. Dr. Watanabe's present address is the Department of Physiology, Tokyo Medical and Dental University, Tokyo, Japan

ABSTRACT Radiotracer techniques were used to study the influxes and effluxes of various univalent cations in internally perfused squid giant axons. Membrane currents and conductances were determined by the voltage-clamp technique under analogous internal and external conditions. Both sodium-containing and sodium-free internal and external media were studied. Membrane impedance was measured with an ac impedance bridge to determine the general magnitude and time course of the impedance loss which accompanied the excitation process in both varieties of external media. Maximum transmembrane currents were found to be of comparable magnitude to the charge transfer associated with the peak interdiffusion flux measured under the same conditions. The product of the membrane resistance and the interdiffusion flux remained constant over a wide range of resistance and flux values, both at rest and during activity, both in sodium-containing and sodium-free media. The implications of these findings for excitation theory are discussed.

Recent reports from this laboratory have shown that squid giant axons internally perfused with favorable solutions remain excitable in various sodium-free media (Tasaki, Singer, and Watanabe, 1965 and 1966; Tasaki and Singer, 1965 and 1966). Since the excitation process in sodium-free media did not seem qualitatively different from excitation in sodium-containing media, the present study was undertaken to compare these excitation processes more quantitatively. For this purpose cation fluxes were measured by radiotracer methods, and membrane currents were measured by voltage-clamp methods, under intracellular perfusion in both sodium-free and sodium-containing external media.

The fluxes of cations interdiffusing across the membrane of the internally perfused squid giant axon can be measured easily by radiotracer techniques (Tasaki, 1963). Provided that the internal and external media contain no cation species in common, the influxes and effluxes of cations can be traced

faithfully by using radioisotopes of the cations concerned. The difference in mobility between two different isotopes of one chemical element is small enough to be ignored in these tracer measurements. This radiotracer method is applicable both to resting and to stimulated axons. Since the fluxes during a single cycle of excitation are very small and rapid, repetitive stimulation is required to measure the fluxes during excitation. Although the precise time course of the fluxes cannot be measured with repetitive stimulation, the total flux which takes place during a series of excitation cycles can be measured without ambiguity. If membrane resistances and currents are measured under analogous conditions, the results may be compared with fluxes observed by the radiotracer method. This comparison should give a better understanding of the process of excitation in the membrane, since both the interdiffusion flux and the membrane resistance are determined by the ion concentrations and the ion mobilities within the major diffusion barrier of the membrane (Helfferich, 1962, p. 357).

In the present series of experiments, results obtained by the radiotracer technique are compared with results obtained by the voltage-clamp technique under various experimental conditions. Measurements of cation fluxes are compared to membrane currents both at rest and during excitation; the results confirm that excitation in sodium-free media is very similar to excitation under the usual experimental conditions. In particular, the product of the flux and the membrane resistance was found to remain constant under various experimental conditions, including both the resting and excited states, and both sodium-containing and sodium-free media. The constancy of the flux-resistance product has been given a theoretical basis and is generally applicable to many charged membranes, both biological and nonbiological. Preliminary reports of some of these findings may be found elsewhere (Singer, Tasaki, Watanabe, and Kobatake, 1966; Tasaki and Singer, 1966), and a theoretical discussion of these results will be found in a subsequent article from this laboratory.

METHODS

Giant axons obtained from *Loligo pealii*, available at The Marine Biological Laboratory, Woods Hole, Mass., were used for most experiments. Giant axons were partially cleaned of small nerve fibers and connective tissue for determinations of membrane current; extensive cleaning was necessary for radiotracer experiments to reduce any delay in isotope movement across the tissues surrounding the axon. Dissection was carried out in natural seawater, under a dissecting microscope with dark-field illumination. The axon diameters were 400–500 μ in most cases.

The method of intracellular perfusion was similar to that used previously (Oikawa, Spyropoulos, Tasaki, and Teorell, 1961; Tasaki, Singer and Takenaka, 1965). The Lucite perfusion chamber was 32 mm in width. The axon was supported by the chamber and two Lucite side supports, separated from the chamber by a 2 mm air

gap. The axon interior was exposed to artificial perfusion solutions in the central 11–12 mm portion of the chamber; the unperfused lateral segments of the axon were separated from the middle portion by narrow vaseline partitions. These lateral segments were treated with a solution of 0.4 M $MgCl_2$ to suppress excitability. The air gaps prevented direct mixing of internal and external solutions near the cut ends.

Double cannulation was performed in the Lucite chamber containing natural seawater. A large glass cannula, roughly 250 μ in diameter, was introduced into one end of the axon to serve as the outlet for the perfusion fluid; another glass cannula, approximately 150 μ in diameter, was introduced into the other end to serve as the inlet. With the two cannulae overlapping concentrically, the vaseline partition was formed, and the $MgCl_2$ solution was applied to the lateral portions. Internal perfusion was initiated by raising the perfusion fluid reservoir to provide a small hydrostatic pressure, and then separating the two cannulae to allow perfusion of the central zone between their tips. (Further details of the double cannulation technique may be found in the articles cited above.) For studies of cation fluxes during excitation, external biphasic stimulation was performed with platinum wire electrodes placed at one end of the perfusion zone.

Effluxes of internal cations were studied by adding a radioisotope of the cation to the internal perfusion solution. Perfusion fluid flow was maintained for at least 5–10 min. Radioactive samples of the external medium were collected after removal of the two perfusion cannulae. (Removal of the external solution while the sharp tips of the cannulae were still in place often injured the axon.) The entire external solution was collected at 5- or 6-min intervals as a single sample.

To determine the efflux J_k, of cation species k, the following formula was used:

$$J_k = \frac{n_k m_k}{t A n_k^0}$$

where n_k represents the radioactivity (expressed in counts per minute or CPM) in the sample of external medium collected in period t (expressed in seconds); A represents the area of the perfused axon membrane in the chamber; m_k represents the amount of nonradioactive cation of species k (expressed in pmoles) in a sample of the internal medium with radioactivity n_k^0 (expressed in CPM). The ratio n_k^0/m_k was generally 20–60 CPM/pmole. The axon area, A, was 0.12–0.14 cm^2 in most cases. For internal cations, the efflux per impulse was obtained by dividing the "extra efflux", i.e. the efflux above the resting level resulting from repetitive stimulation of the axon, by the number of nerve impulses elicited during the collection period concerned.

Cation influxes were measured by adding radioisotopes of the external cations to the external fluid medium. Samples of internal perfusion fluid were then collected from the outlet cannula at regular 5- to 6-min intervals. Since continuous flow of internal perfusion fluid was required, the two perfusion cannulae were not removed. Cation influx at rest and during excitation was calculated as above.

Radioactive material was obtained from Isoserve, Inc., Boston, Mass. Samples containing radiotracers were dried in planchets before counting. Radioactivity was determined with a low background counter (Nuclear-Chicago Corporation, Des Plaines, Ill., models C112 and C110B) in conjunction with a gas-flow detector (model

D-47, with window) and a printing timer (model C111B). The background radioactivity was 1–2 CPM.

Membrane currents were measured by the voltage-clamp method (Cole and Moore, 1960). The perfusion chamber and double-cannulation procedure were the same as described above. The perfusion zone was 8–10 mm in length. A pair of enameled platinum wire electrodes, each about 50 μ in diameter, was used. One electrode had an uninsulated platinized surface, about 8 mm in length, and was used to pass electric currents (for voltage clamping) through the membrane. The other electrode had an uninsulated, platinized surface, about 1 mm in length; the uninsulated (recording) portion was positioned adjacent to the uninsulated surface of the current electrode. This pair of electrodes was inserted concentrically within the outlet cannula, and the uninsulated portions were brought to the middle of the perfusion zone. A large coil of uninsulated platinum immersed in the external medium served as the ground electrode.

After the mounted axon was double-cannulated and the electrodes were positioned, the natural seawater in the chamber was replaced with an isotonic solution of tetramethylammonium chloride (TMA-Cl) and $CaCl_2$. This sodium-free external medium was replaced several times during the following 5–10 min to ensure complete exchange of the seawater. The test solution was then introduced into the chamber, and perfusion was instituted by separating the two cannulae. When favorable external media were used, axon excitability was restored. After the action potential reached a reproducible, almost constant amplitude, the voltage-clamp device was connected to the electrodes. Records of membrane currents were obtained at varying levels of membrane depolarization.

All experiments were performed at 18–22° C.

RESULTS

(1) *Efflux of Potassium Ions at Rest and during Excitation (in Sodium-Containing External Media)*

In the first series of experiments, the efflux of potassium ions into artificial (sodium-containing) seawater was determined. An example of these results is shown in Fig. 1, left. The K-free external medium contained 440 mM NaCl, 50 mM $MgCl_2$, and 10 mM $CaCl_2$. The internal medium contained 500 mM KF, and was both Na-free and divalent cation-free. (The external medium contained less than 5 mM tris (hydroxymethyl) aminomethane buffer, at a pH of 8.0–8.2; the internal medium contained less than 5 mM potassium phosphate buffer, at a pH of 7.2–7.4.) Samples of the external medium were collected at regular 5-min intervals (see Methods). Three resting periods preceded the first stimulation period and followed the last stimulation period. Two resting periods were interposed between stimulation periods.

The average efflux of potassium ion (^{42}K) was about 170 pmoles·cm^{-2}·sec^{-1} at rest. There was little variation in resting efflux in different periods; resting efflux in the last period was about the same as that in the first, or be-

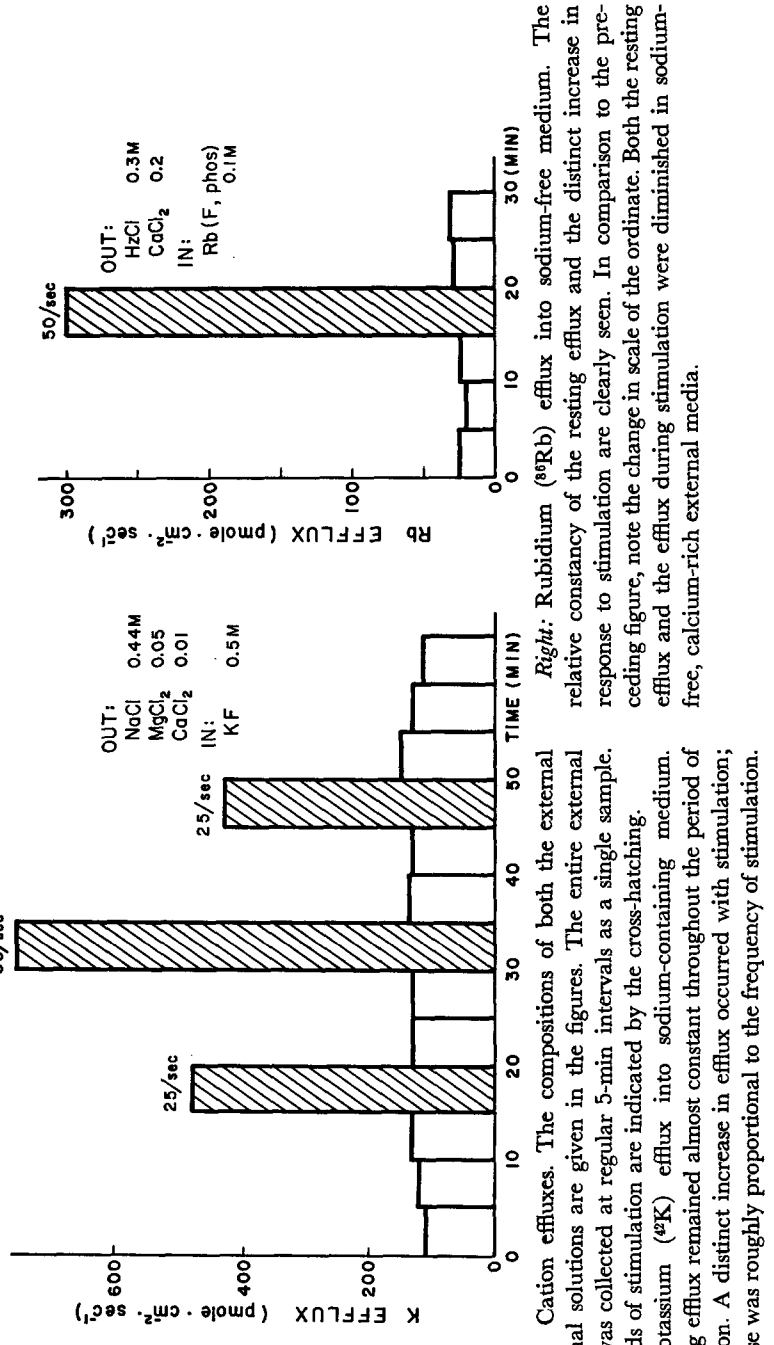

FIGURE 1. Cation effluxes. The compositions of both the external and internal solutions are given in the figures. The entire external medium was collected at regular 5-min intervals as a single sample. The periods of stimulation are indicated by the cross-hatching.

Left: Potassium (^{42}K) efflux into sodium-containing medium. The resting efflux remained almost constant throughout the period of investigation. A distinct increase in efflux occurred with stimulation; the increase was roughly proportional to the frequency of stimulation.

Right: Rubidium (^{86}Rb) efflux into sodium-free medium. The relative constancy of the resting efflux and the distinct increase in response to stimulation are clearly seen. In comparison to the preceding figure, note the change in scale of the ordinate. Both the resting efflux and the efflux during stimulation were diminished in sodium-free, calcium-rich external media.

TABLE I

CATION INTERDIFFUSION FLUXES

The results obtained in 19 experiments are summarized. The internal radiotracers were used in perfusion solutions of the corresponding nonradioactive species. The guanidine tracer (Gu*; ^{14}C labeled) was used in an external medium also containing sodium. All concentrations are expressed in millimoles per liter (mM) for the internal and external solutions. The fluxes given refer to the total univalent cation flux in cases 1*A* through 8*B*; in the remainder, the fluxes refer only to the radioactive species. The fluxes during excitation (Act) are given with their corresponding frequencies of stimulation (Frequency). In general, the average cation fluxes during a collection period increased in proportion to the frequency of stimulation, although the flux per impulse tended to diminish at high frequencies. Further details may be found in the text.

Axon	Tracers		External cations					Internal cations		Flux		Frequency
	In	Out	Na	Hz	Gu	Ca	Mg	K	Rb	Rest	Act	
			mM	*mM*	*mM*	*mM*	*mM*			*pmol·cm⁻²·sec⁻¹*	*pmol·cm⁻² per impulse*	*imp/sec*
1*A*	^{42}K	—	440	—	—	10	50	550	—	150	12.0	50
1*B*	^{42}K	—	440	—	—	10	50	550	—	170	10.4	50
2*A*	^{42}K	—	440	—	—	10	50	550	—	230	12.0	25
2*B*	^{42}K	—	440	—	—	10	50	550	—	225	11.0	50
3*A*	^{42}K	—	440	—	—	10	50	550	—	125	15.0	25
3*B*	^{42}K	—	440	—	—	10	50	550	—	130	12.5	50
4*A*	^{86}Rb	—	450	—	—	100	—	—	100	20	4.1	50
4*B*	^{86}Rb	—	450	—	—	100	—	—	100	35	3.0	100
5	^{86}Rb	—	300	—	—	200	—	—	100	100	2.3	25
6	^{86}Rb	—	—	300	—	200	—	—	100	38	4.1	50
7	^{86}Rb	—	—	300	—	200	—	—	100	20	5.8	50
8*A*	^{86}Rb	—	—	300	—	200	—	—	100	24	3.2	50
8*B*	^{86}Rb	—	—	300	—	200	—	—	100	13	1.9	25
9	—	Gu*	345	—	12	20	100	525	—	4.5	0.11	50
10	—	Gu*	345	—	12	20	100	525	—	4.6	0.71	50
11*A*	—	Gu*	345	—	12	20	100	525	—	7.5	0.018	25
11*B*	—	Gu*	345	—	12	20	100	525	—	6.9	0.040	50
12*A*	—	Gu*	345	—	12	20	100	525	—	10.5	0.059	50
12*B*	—	Gu*	345	—	12	20	100	525	—	11.3	0.022	100

tween stimulation periods. The results for six axons are summarized in Table I (axons 1*A* through 3*B*). The resting efflux of potassium in these internally perfused axons was between 125 and 230 pmoles·cm^{-2}·sec^{-1}, which agrees with results reported previously (Tasaki, 1963).

When axons were subjected to repetitive stimulation there was a distinct increase in potassium efflux (Fig. 1, left, periods 4, 7, and 10). Potassium efflux during a single cycle of excitation was calculated from the "extra" potassium efflux, i.e., the flux during the period of stimulation that is greater

than the resting flux for the same period of time. The potassium efflux per impulse is then defined as the extra potassium efflux divided by the number of nerve impulses evoked during the collection period under study. Table I (column Act) shows that the extra efflux was between 11 and 15 pmoles·cm^{-2} per impulse. In the example shown in Fig. 1, left, the extra efflux was approximately 12 pmoles·cm^{-2} per impulse. At room temperature, with stimulus frequencies less than 100 impulses/sec, the extra potassium efflux was roughly independent of the frequency of stimulation.

When measured with high-frequency ac, membrane conductance rises and falls along a roughly triangular time course. At the peak of the action potential, membrane conductance is approximately 200 times as great as in the resting state (Cole and Curtis, 1939). Since variation in membrane conductance reflects the mobility and concentrations of intramembrane cations, cation fluxes are expected to follow a time course similar to the conductance variation. From these considerations it follows that the extra potassium efflux during the excitation process (11–15 pmoles·cm^{-2} per impulse) takes place in 1 msec (the duration of a single cycle of excitation), and that the peak value of the potassium efflux is approximately twice the average value during one cycle of excitation (20–30 pmoles·cm^{-2}·msec^{-1} or nmoles·cm^{-2}·sec^{-1}). On this basis the increase in potassium efflux at the peak of excitation is found to be approximately 200 times the resting efflux (Tasaki and Takenaka, 1964). Thus, the ratio of the potassium efflux at rest to the potassium efflux at the peak of excitation is roughly equal to the ratio of the membrane conductance at rest to the membrane conductance at the peak of excitation. In other words, for potassium fluxes:

$$\frac{J_a}{J_r} \approx \frac{G_a}{G_r}$$

where G_r and G_a represent the membrane conductances at rest and at the peak of excitation, respectively; J_r and J_a represent the potassium fluxes at rest and at the peak of excitation, respectively. In the absence of an electric current through the membrane, sodium influx is approximately equal to the potassium efflux (see Results, section 4, and Discussion). The approximate equality for potassium effluxes given above then applies to sodium influxes as well.

Univalent cation fluxes can be expressed in microamperes by multiplying the fluxes (expressed in moles·cm^{-2}·sec^{-1}) by the Faraday constant, F (96,500 coul/mole). In the resting state, where the membrane resistance ($1/G_r$) is approximately 1–2 kiloΩ·cm^2, fluxes of univalent cations are roughly 12–23 μa·cm^{-2}. [There is a definite tendency for cation interdiffusion to be slow in membranes with high resistance, such as the squid axon (Tasaki, 1963).]

In this series of experiments, the flux (J_r)-resistance (R_r) product in the resting state, expressed in millivolts, is estimated as: $F J_r R_r \approx$ 20–30 mv. The reliability of this estimation is limited by the fact that J_r and R_r were determined with different axons. At the peak of excitation, the flux-resistance product was roughly the same as at rest.

(2) *Efflux of Rubidium Ions at Rest and during Excitation (in Sodium-Free and Sodium-Containing External Media)*

Since it is much easier to maintain excitability in sodium-free external media when dilute cesium or rubidium is the internal cation (Tasaki, Singer, and Watanabe, 1965), the internal perfusion fluid contained a mixture of 90 mM RbF and 10 mM rubidium phosphate (pH 7.3) in these experiments. An example of the results is shown in Fig. 1, right. The sodium-free external medium contained 300 mM hydrazinium chloride (HzCl) and 200 mM $CaCl_2$ (pH 7.5–8.0). Samples of the external medium were collected at regular 5-min intervals, with three resting periods preceding and two resting periods following the period of stimulation.

The average efflux of rubidium ion (^{86}Rb) was 24 pmoles·cm^{-2}·sec^{-1} at rest. As with potassium, there was little variation in efflux among the different resting periods. However, the magnitude of rubidium efflux under these sodium-free experimental conditions was appreciably smaller than potassium efflux into sodium-containing external media. The results obtained for four axons are summarized in Table I (axons 6 through 8*B*). The efflux of rubidium at rest was between 13–38 pmoles·cm^{-2}·sec^{-1}, which is approximately one-tenth of the potassium efflux into sodium-containing medium.

The difference between potassium and rubidium effluxes might be accounted for, at least in part, by the relatively high concentration of calcium required to maintain excitability in hydrazine-containing external media; increased concentrations of external calcium are associated with increased membrane resistance (Frankenhaeuser and Hodgkin, 1957), and consequently, with small cation interdiffusion. Other possible causes for the difference in effluxes are: (*a*) the inequality of the salt concentrations in the two cases and (*b*) the difference in intramembrane mobilities and selectivities among the cations studied.

It is of interest that the extra rubidium efflux during excitation was roughly the same with or without sodium in the external medium. In sodium-containing media the extra rubidium efflux was 2.3–4.1 pmoles·cm^{-2} per impulse, and in hydrazine-containing media the extra rubidium efflux was 1.9–5.8 pmoles·cm^{-2} per impulse. These results suggest that the excitation processes (measured by efflux) that take place under sodium-free and sodium-containing external conditions are not very different.

Under the conditions of these rubidium experiments, the average duration

of the impedance loss associated with nerve excitation was found to be 2.0 msec. From this finding, it follows that the peak value of the rubidium efflux during excitation is roughly 3–6 pmoles·cm^{-2}·msec^{-1}, or roughly 0.3–0.6 ma/cm^2. (The value obtained for peak rubidium efflux will be compared with that for membrane conductance at the peak of excitation in Results, section 6.)

(3) *Influx of Guanidinium Ion during Rest and Activity*

Recently, it was shown that sodium-free external solutions containing guanidinium and calcium chlorides can be used to maintain excitability in squid giant axons perfused with dilute solutions of CsF or RbF (Tasaki, Singer, and Watanabe, 1965; Tasaki and Singer, 1965 and 1966). However, action potentials developed under these experimental conditions are of relatively long duration, and are not reproducible at high frequencies. For these reasons it is difficult to study guanidinium ion influx when guanidinium is the only external univalent cation. In this study, guanidinium influx was investigated by adding ^{14}C-labeled guanidine to an external medium containing NaCl and $CaCl_2$. The use of external sodium also permitted the use of internal potassium at relatively high concentrations (525 mM in this case).

The results are summarized in Table I, axons 9 through 12B. The concentration ratio of sodium ion to guanidinium ion in the external medium was approximately 30:1. It was found that guanidinium influx at rest was $\frac{1}{30}$ of the potassium efflux at rest (Table I, axons 1*A* through 3*B*). [Note that the univalent cation efflux roughly equals the univalent cation influx in the resting axon (see Results, section 4).]

(4) *Influxes of Sodium Ion at Rest and during Activity*

Influxes of sodium ion were traced with radioactive ^{22}Na ion in giant axons of the Chilean squid, *Dosidicus gigas*. These experiments were performed in collaboration with Dr. Adolfo Ruarte at the Marine Biological Station at Montemar, Chile, and have been reported, in part, elsewhere (Tasaki and Singer, 1966). A typical example is shown in Fig. 2, left, where the external medium consisted of 350 mM NaCl, 133 mM $MgCl_2$, and 32 mM $CaCl_2$. Samples of internal perfusion fluid were collected at regular 6-min intervals.

In the first, second, and final resting periods, the internal perfusion solution was a mixture of 350 mM sodium glutamate and 150 mM potassium glutamate. In the other resting periods, and during the period of repetitive stimulation, the internal solution contained only 500 mM potassium glutamate. No significant differences were observed in resting influxes of ^{22}Na ion under these two internal conditions. [Note that the influx of ^{22}Na ion does not trace the (net) flux of the nonradioactive species ^{23}Na when there is ^{23}Na in the axon interior (see Nims, 1959).] The influxes of sodium ion in the two resting

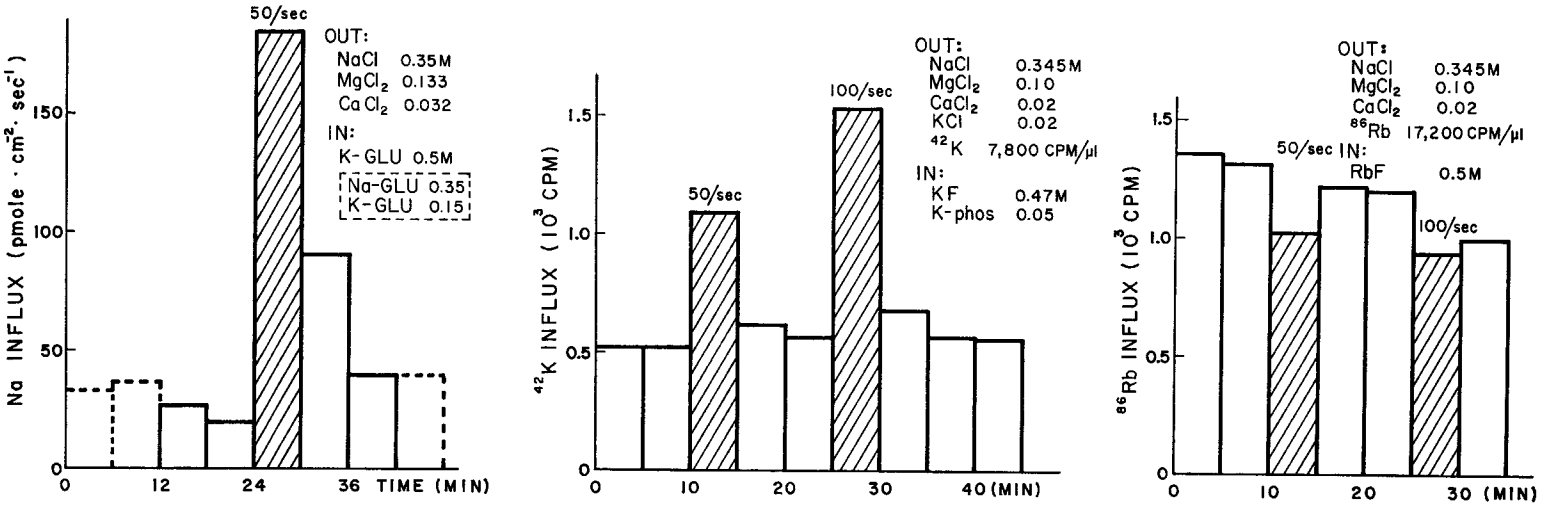

FIGURE 2. Cation influxes. The compositions of the internal and external solutions are given in the individual figures. Periods of stimulation are indicated by the cross-hatching.

Left: Sodium influx (^{22}Na). These experiments were performed at the Marine Biological Station at Montemar, Chile, with *Dosidicus gigas* giant axons. The internal medium was a mixture of 350 mM sodium glutamate and 150 mM potassium glutamate for the first, second, and final resting periods. In all other periods, the internal solution contained 500 mM potassium glutamate. Samples were collected at regular 6-min intervals in this case. With the exception of the resting period immediately following the period of stimulation, no significant variations in the resting influx were observed. The apparently increased influx in the period following the period of stimulation resulted from a delay in washout of the isotopes; therefore, the extra efflux in this resting period should be considered as part of the preceding period of stimulation.

Center: Potassium influx (^{42}K). Compositions of the internal and external media are indicated. Extra influxes were roughly proportional to the frequency of stimulation. Note the change in the units for the ordinate, and the change in scale for the abscissa.

Right: Rubidium influx (^{86}Rb). Internal and external conditions are given. The radioactivity in the medium was 2.2 times greater than in the previous case. Scales are as in the previous case. When adjusted for the difference in radioactivity, the resting influxes of rubidium were of comparable magnitude to those for potassium. However, there was no increase in influx in response to stimulation; in fact, a decrease was observed in this case, and there was little or no variation with frequency. See text for details.

periods following the stimulation period are apparently greater than the other resting periods; this results from delayed isotope washout from the relatively large amount of axoplasm remaining within the Chilean axon. The resting influx was 3–4 pmoles· cm^{-2}·sec^{-1} in the initial and final resting periods. During the period of stimulation (50 shocks/sec), there was a marked increase in sodium influx. The extra sodium influx, calculated in a manner similar to that used in the preceding sections, was approximately 0.5 pmoles· cm^{-2} per impulse. This relatively small value is due, at least in part, to the high divalent cation concentration in these experiments.

(5) *Influxes of Potassium and Rubidium Ions at Rest and during Activity*

Influxes of radioactive ^{42}K ion were studied in 17 different axons. An example is presented in Fig. 2, center. In this case, the external medium contained an artificial seawater approximating natural seawater (345 mM NaCl, 100 mM $MgCl_2$, 20 mM $CaCl_2$, and 20 mM KCl). The ^{42}K activity was 7800 CPM/mm^3. Samples of the internal perfusion fluid were collected at regular 5-min intervals. At least two resting periods preceded and followed each period of stimulation. The results indicate that ^{42}K influx was roughly 500 CPM during a 5-min resting period. The ^{42}K influx was considerably increased by stimulation, and the extra influx was roughly proportional to the frequency of stimulation. Similar results have been reported previously (Tasaki, 1963). The influxes of ^{42}K were comparable to influxes of ^{22}Na when the latter ion was used as the radioactive species under comparable internal and external conditions.

When nonradioactive ^{39}KCl in the external medium is completely eliminated, and only a tracer amount of radioactive ^{42}K is present, the results obtained were very different from those presented in Fig. 2, center, for the previous case. For a comparable level of external radioactivity, the resting rate of ^{42}K ion influx in the present case was considerably greater than in the case where there is external ^{39}KCl. Repetitive stimulation in the present case produced little or no increase in the ^{42}K influx. These results were dependent on the potassium ion concentration, the concentrations of the other external cations, and the state of the axon membrane. However, there was a definite correlation between the level of ^{42}K influx at rest and the effect of repetitive stimulation. When the ^{42}K influx is low in the resting state, there was usually a distinct increase in the ^{42}K influx during repetitive stimulation; when the resting influx was high, there was little or no increase during stimulation.

When ^{86}Rb was used instead of ^{42}K in the external medium under the same internal and external conditions, the results obtained were similar to those reported above. An example of these observations is presented in Fig. 2, right. In this case the external medium contained neither potassium nor nonradioactive rubidium. The internal solution contained the nonradioactive species

of rubidium (500 mM RbF). Conditions were otherwise similar to those presented in Fig. 2, center.

The influx of ^{86}Rb ion in the resting state was comparable to that observed in ^{42}K ion, when corrected for the initial external level of radioactivity. Repetitive stimulation did not increase the ^{86}Rb influx; in fact there was a slight decrease in the ^{86}Rb influx during the periods of high-frequency stimulation. As mentioned above, under analogous conditions for ^{42}K there is little or no increase in influx during high frequency stimulation. Similar results have been obtained for *Dosidicus* giant axons; a possible interpretation of these findings concerning ^{42}K and ^{86}Rb influxes, both in the resting state and during high frequency stimulation, has been presented elsewhere in detail (Tasaki and Singer, 1966).

(6) *Membrane Resistance and Maximum Inward Current during Excitation of Axons in Sodium-Free Media*

The voltage-clamp technique described under Methods was used to measure both membrane resistance and peak inward current in sodium-free external media. Among the sodium-free external media investigated, the solution containing 300 mM hydrazinium chloride and 200 mM $CaCl_2$ gave the largest peak inward current during excitation. The electrophysiological behavior of the axons in this hydrazine-calcium medium was similar to that observed in the usual sodium-containing media. When 500 mM KF solution was used internally, the time courses of the observed membrane currents were indistinguishable from those observed in unperfused axons immersed in sodium-containing media (Hodgkin, Huxley, and Katz, 1952; Cole and Moore, 1960). The only recognizable differences between these axons and those in "normal" internal and external media were that in sodium-free media the inward currents were slightly diminished in intensity and longer in duration than in sodium-containing media.

An example of the typical voltage-current curves obtained by this method in hydrazine-calcium media is shown in Fig. 3. In this case the internal solution contained 100 mM RbF, and the external solution contained 300 mM HzCl and 200 mM $CaCl_2$. The peak inward current observed in this case was 0.5 ma/cm^2. The ability of a puffer-fish poison, tetrodotoxin, to block the inward current under these sodium-free conditions is demonstrated; the implications of this finding are discussed elsewhere (Watanabe et al., 1967).

The results of measurements on 15 different axons under the same external conditions are presented in Table II. The different internal conditions are noted. The membrane resistance both at rest and during the process of excitation was determined by plotting the voltage-current relationship (Fig. 3), and then measuring the slopes of the approximately linear segments of the curve at their points of intersection with the voltage axis.

The maximum values of the inward currents during excitation varied with the nature and concentration of the salt in the internal perfusion solution. Dilute solutions of KF and RbF in the axon interior gave greater inward currents than dilute solutions of CsF or NaF. For a given internal medium, a variation in the nature and concentration of the univalent cation in the external fluid medium had a profound effect upon the maximum value of the inward current. For example, a mixture of 100 mM guanidinium chloride, 200

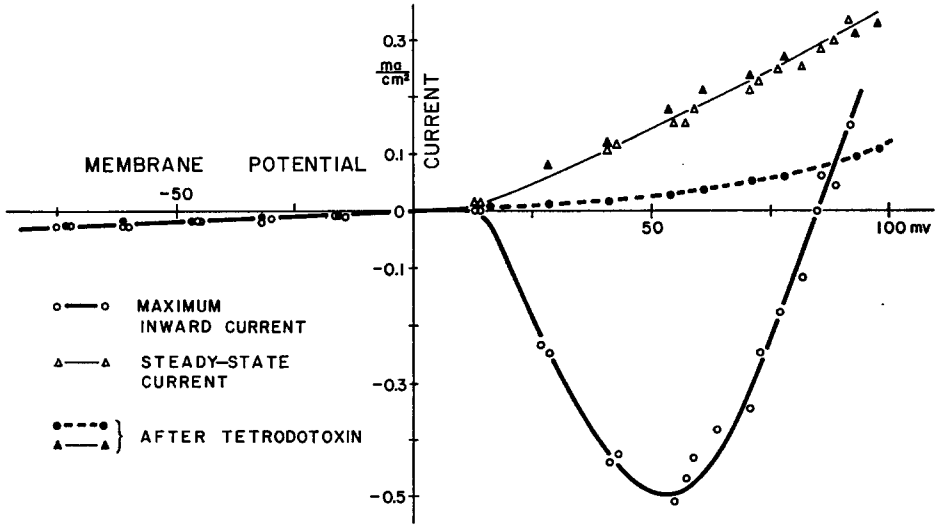

FIGURE 3. Current-voltage relationship obtained from squid giant axon immersed in sodium-free medium. The internal perfusion fluid was 100 mM RbF solution. The external fluid medium contained 300 mM hydrazinium chloride (HzCl) and 200 mM $CaCl_2$. The thick V-shaped curve represents the relationship between the peak inward current (I) and the clamping voltage (V). The thin, continuous curve shows the I–V relationship at 4 msec after the onset of the clamping voltage pulse. The broken line represents the I–V curve determined after tetrodotoxin (5×10^{-7} g/ml) was added to the external medium. The diameter of the axon was approximately 500 μ. Room temperature, 21°C.

mM TMA-Cl, and 200 mM $CaCl_2$ outside the axon produced smaller inward currents than the standard hydrazine mixture.

The results of these electrical measurements may now be compared with the magnitude of the univalent cation fluxes determined by the radiotracer technique. In axons internally perfused with 10 mM RbF solution and immersed in a medium containing 300 mM hydrazinium chloride and 200 mM $CaCl_2$, the average membrane resistance during excitation was 70 ohms·cm². The average observed value of the maximum inward current was 0.34 ma/cm², which corresponds to transfer of approximately 3.4 nmoles·cm^{-2}· sec^{-1} of univalent cations across the membrane. The average value of the peak

interdiffusion cation fluxes under these conditions (Table II) was 3.8 pmole · cm^{-2} per impulse. Since the duration of the impedance loss during excitation observed under these conditions is roughly 2.0 msec, and the impedance variation is roughly triangular, the peak value of the univalent cation inter-

TABLE II

CURRENT RESISTANCE PRODUCT

The results of the current-voltage relationships obtained for 15 axons are summarized. The internal salt is given in the second column; concentrations were generally 100 mM. The external medium contained 300 mM HzCl and 200 mM $CaCl_2$ in each case. The amplitude of the action potential (*AP*) is expressed in millivolts. The resistance values were obtained from the (positive) slopes of the three approximately linear segments (Fig. 3). Note that the membrane resistance (for a unit area) in the resting state (Rest) is expressed in kilohms whereas the resistances of the late steady state and at the peak of excitation (Act) are expressed in ohms. The peak inward current obtained in the current-voltage relationship is indicated as *I* and is expressed in milliamperes per cm^2. The *IR* product, expressed in millivolts, was obtained from the product of the membrane resistance in the active state (Act) and the peak inward current (I_{max}). The significance of this constancy is discussed in the text.

Axon	In	*AP*	Resistance			*I* (max)	*IR* (prod)
			Rest	Steady state	Act		
		mv	$K\Omega \cdot cm^2$	$\Omega \cdot cm^2$	$\Omega \cdot cm^2$	ma/cm^2	*mv*
1	CsF	72	5.0	750	200	0.16	32
2	CsF	87	3.7	410	95	0.32	30
3	CsF	74	1.2	250	88	0.39	33
4	CsF	64	2.9	600	370	0.10	37
5	RbF	64	3.1	250	110	0.18	20
6	RbF	75	2.25	170	110	0.27	30
7	RbF	73	2.5	270	65	0.37	24
8	RbF	76	1.8	210	69	0.28	19.5
9	RbF	79	3.6	210	72	0.33	24
10	RbF	93	3.3	280	60	0.46	27
11	RbF	88	2.8	250	44	0.50	22
12	KF	65	1.2	100	—	0.30	—
13	KF	87	1.6	360	17	1.25	21.5
14	NaF	52	1.2	—	260	0.08	21
15	NaF	55	1.2	290	370	0.07	26

diffusion flux is estimated to be roughly 3.8 pmoles · cm^{-2} · $msec^{-1}$ (or nmoles · $cm^{-2} \cdot sec^{-1}$). Note that cation transfer associated with the maximum value of the inward current is very close to the peak value of the univalent cation interdiffusion fluxes under the same conditions. For this reason, the product (expressed in mv) of the maximum inward current (I_{max} in the table) and the membrane resistance during excitation (Act in the table) is found to be 20–

30 mv; this *IR* product is very close to the corresponding quantity determined in sodium-containing media.

The present voltage-clamp method of determining the membrane current in the excited state can give rise to significant errors when applied to the resting membrane. Since the measuring current spreads to the axoplasm-containing unperfused zones to either side of the perfused zone under study, and since the resistivity of perfusion fluid (100 mM RbF) is greater than that of axoplasm, the measured membrane resistance tends to be somewhat smaller than the true resting value. Consequently, the flux-resistance product in the resting state also tends to be somewhat less than the true value.

DISCUSSION

(1) *Resting State*

Under continuous internal perfusion, the system consisting of the resting axon membrane and the fluid media on both sides of the membrane may be considered to be in a nonequilibrium, stationary (time-independent) state. In this state, the absence of any electric current across the membrane can be expressed by:

$$J_i + J_e + 2J_d = 0$$

where J_i, J_e, and J_d represent the fluxes (expressed in moles·cm^{-2}·sec^{-1}) of the internal univalent cation, the external univalent cation, and the external divalent cation, respectively. In internally perfused squid giant axons, each of these three cation fluxes may be measured directly. The fluxes of anions across the resting membrane are very small (Tasaki, Teorell, and Spyropoulos, 1961; Tasaki, 1963), and may be ignored.

According to current estimations, the magnitude of the influx of divalent cations, J_d, is relatively small, and is less than $\frac{1}{10}$ of the influx of the external univalent cation, J_e (Tasaki, 1963). This small contribution to the total cation influx may also be ignored within the accuracy of present radiotracer techniques, so that (approximately):

$$J_i + J_e = 0.$$

Thus, when no cation is in common on both sides of the membrane, the efflux of the internal univalent cation is of opposite direction and (approximately) of equal magnitude to the influx of the external univalent cation.

Under continuous internal perfusion, the internal cation concentration is not affected by the process of interdiffusion. In the absence of internal perfusion, however, this process produces an intracellular accumulation of external cations, e.g., sodium, accompanied by a simultaneous fall in the

internal cation, e.g., potassium, concentration. In fact, Steinbach and Spiegelman (1943) have demonstrated this potassium-sodium exchange in unperfused axons by direct chemical analysis of the cations in the axoplasm. These experimental findings are consistent with the view that there are negative fixed charges in the membrane (Tasaki, Teorell, and Spyropoulos, 1961).

The magnitude of interdiffusion flux in a negatively charged membrane is determined by the mobilities and concentration of the interdiffusing cations in the membrane (Helfferich, 1962, p. 357). The same factors determine the membrane conductance. Consequently, a direct proportionality exists between the membrane conductance and the rate of interdiffusion flux. The resistance, i.e. the reciprocal of conductance, of a cation-exchange membrane varies with the chemical nature of the interdiffusing cations; but, the product of the resistance and the interdiffusion flux is independent of the chemical species of the cations involved and of the nature of the membrane. In uniform membranes, the resistance (R_r) flux (J_r) product is given by

$$R_r J_r = \frac{RT}{F^2}$$

where the symbols in the right-hand member of the equation have the usual thermodynamic meanings. The validity of this equation originates in the Nernst-Einstein equation relating the diffusion coefficient to the mobility (Spiegler and Coryell, 1953). (In highly nonuniform membranes, this equation is only approximate.) In nonbiological ion-exchange membranes, the validity of this equation has been shown experimentally by Gottlieb and Sollner (personal communication) and by Kobatake and Tasaki (in preparation). The equation above can be written in the form

$$R_r F J_r = \frac{RT}{F} \ (\approx 29 \text{ mv}).$$

The experimental findings described under Results suggest that this equation is applicable to the squid axon membrane in the resting state. (Note that interdiffusion flux J_r cannot be defined in normal, unperfused axons in which mixtures of many salts are present on both sides of the membrane.)

As stated above, divalent cation influx, J_d, is small as compared with univalent cation influx, J_e, and has been ignored in the present discussion. However, it should be noted that divalent cations are essential for the maintenance of the resting state of the membrane.

(2) *Excited State*

It is impossible to consider the system consisting of the perfused axon membrane in the excited state and the solutions on both sides of the membrane as

being in a stationary state because the various properties of the membrane change with time during excitation. But, in the excited state of the membrane, the time constant (or relaxation time) is far shorter than the duration of the action potential. Therefore, the membrane may be regarded as being in a quasi-stationary state, where membrane properties change very slowly from one stationary state to another. It is known that the anion fluxes are unmeasurably small during excitation, and may be ignored as before. Under the conditions in which the transmembrane current during excitation is very weak, the relationship $J_i + J_e \approx 0$ should be valid. With this assumption, the equation

$$R_a F J_a \approx \frac{RT}{F}$$

can be applied to the membrane in the excited state, where J_a and R_a denote interdiffusion flux and the membrane resistance, respectively, during excitation.

The results of the present investigation clearly indicate that the rate of interdiffusion flux of internal and external univalent cations increases greatly during repetitive stimulation. This increased flux also has been shown to be directly related to the increased membrane conductance (or decreased membrane resistance) during excitation. Hence, the flux-resistance product does remain roughly constant during the excitation period (when the membrane resistance decreases greatly). This relationship also holds for the excited state in both sodium-containing and sodium-free media, as was true for the resting state.

(3) *Implications for the Excitation Process*

It has been demonstrated both in the present series of experiments and elsewhere (Cole and Moore, 1960; Hodgkin, 1964; Tasaki and Singer, 1966) that the peak inward current during excitation of a "normal" axon is of the order of 1–5 ma/cm^2 when measured by voltage-clamp techniques. In the equivalent circuit model, it is assumed that the inward current is a measure of the sodium influx during excitation (Hodgkin, 1964). However, the peak interdiffusion flux is of the same order as the maximum inward current. Therefore, in axons immersed in sodium-rich medium and internally perfused with a pure potassium salt solution, the presence of a (net) inward current through the membrane in the excited state may represent either a decrease in potassium efflux or an increase in sodium influx. Since there is significant interdiffusion both at rest and during excitation, the observed inward current represents an imbalance in the interdiffusion flux, most likely due to a combination of these possibilities. Because of the limited time-resolution of the

isotope measurements, it is not possible, at present, to determine the transport numbers of individual ions during excitation.

When either the external or the internal medium of the axon contains more than one univalent cation species, the relationship between the cation fluxes and the membrane resistance becomes very complex. In this case, the ion selectivities (Eisenman, 1963) and the mobilities of all the cations involved have to be taken into consideration.

The effect of a puffer-fish poison, tetrodotoxin, on the voltage-clamp behavior of axons has been discussed by Narahashi, Moore, and Scott (1964) and by Tasaki and Singer (1966). The results of further studies on the effect of this poison on internally perfused squid giant axons are discussed elsewhere (Watanabe, Tasaki, Singer, and Lerman, 1967).

Received for publication 19 April 1966.

REFERENCES

COLE, K. S., and H. J. CURTIS. 1939. Electric impedance of the squid giant axon during activity. *J. Gen. Physiol.* **22:** 649.

COLE, K. S., and J. W. MOORE. 1960. Ionic current measurements in the squid giant axon. *J. Gen. Physiol.* **44:** 123.

EISENMAN, G. 1963. The influence of Na, K, Li, Rb, and Cs on cellular potentials and related phenomena. *Bol. Inst. Estud. Med. Biol. (Mex.).* **21:** 155.

FRANKENHAEUSER, B., and A. L. HODGKIN. 1957. The action of calcium on the electrical properties of squid giant axon. *J. Physiol., (London).* **137:** 218.

HELFFERICH, F. 1962. Ion Exchange. McGraw-Hill Book Company, N. Y. 357.

HODGKIN, A. L. 1964. The Conduction of the Nervous Impulse. Liverpool University Press, Liverpool, Great Britain.

HODGKIN, A. L., A. F. HUXLEY, and B. KATZ. 1952. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo. J. Physiol., (London),* **116:** 424.

NARAHASHI, T., J. W. MOORE, and W. R. SCOTT. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* **47:** 965.

NIMS, L. F. 1959. Membranes, tagged components and membrane transfer coefficient. *Yale J. Biol. Med.* **31:** 373.

OIKAWA, T., C. S. SPYROPOULOS, I. TASAKI, and T. TEORELL. 1961. Methods for perfusing the giant axon of *Loligo pealii. Acta Physiol. Scand.* **52:** 195.

SINGER, I., I. TASAKI, A. WATANABE, and Y. KOBATAKE. 1966. Interdiffusion flux, ionic currents, membrane resistance and impedance in squid giant axons. *Federation Proc.* **25:** 569.

SPIEGLER, K. S., and C. S. CORYELL. 1953. Electromigration in a cation exchanger resin. III. Correlation of self-diffusion coefficients of ions in a cation-exchanger membrane to its electrical conductance. *J. Phys. Chem.* **57:** 687.

STEINBACH, H. B., and S. SPIEGELMAN. 1943. The sodium and potassium balance in squid nerve axoplasm. *J. Cellular Comp. Physiol.* **22:** 187.

TASAKI, I. 1963. Permeability of squid axon membrane to various ions. *J. Gen. Physiol.* **46:** 755.

TASAKI, I., and I. SINGER. 1965. A macromolecular approach to the excitable membrane. *J. Cellular Comp. Physiol.* **66** (Suppl. 2): 137.

TASAKI, I., and I. SINGER. 1966. Membrane macromolecules and nerve excitability: A physico-chemical interpretation of excitation in squid giant axons. *Ann. N.Y. Acad. Sci.* **137:** 792.

TASAKI, I., I. SINGER, and T. TAKENAKA. 1965. Effects of internal and external ionic environment on excitability of squid giant axon: A macromolecular approach. *J. Gen. Physiol.* **48:** 1095.

TASAKI, I., I. SINGER, and A. WATANABE. 1965. Excitation of internally perfused squid giant axons in sodium-free media. *Proc. Nat. Acad. Sci.* **54:** 763.

TASAKI, I., I. SINGER, and A. WATANABE. 1966. Observations of excitation of squid giant axons in sodium-free external media. *Am. J. Physiol.* **211:** 746.

TASAKI, I., and T. TAKENAKA. 1964. Ion fluxes and excitability in squid giant axon. *In* The Cellular Functions of Membrane Transport. J. F. Hoffman, editor. Prentice-Hall, Inc., Englewood Cliffs, N.J. 95.

TASAKI, I., T. TEORELL, and C. S. SPYROPOULOS. 1961. Movement of radioactive tracers across squid axon membrane. *Am. J. Physiol.* **200:** 11.

TASAKI, I., A. WATANABE, and T. TAKENAKA. 1962. Resting and action potential of intracellularly perfused squid giant axon. *Proc. Natl. Acad. Sci.* **48:** 1177.

WATANABE, A., I. TASAKI, I. SINGER, and L. LERMAN. 1967. Effects of tetrodotoxin on excitability of squid giant axons in sodium-free media. *Science.* **155:** 95