Sodium Movements in Perfused Squid Giant Axons

Passive fluxes

EDUARDO ROJAS and MITZY CANESSA-FISCHER

From the Laboratorio de Biofísica, Facultad de Ciencias, Universidad de Chile, Clasificador 198, Correo Central, Santiago, Chile

ABSTRACT Sodium movements in internally perfused giant axons from the squid *Dosidicus gigas* were studied with varying internal sodium concentrations and with fluoride as the internal anion. It was found that as the internal concentration of sodium was increased from 2 to 200 mM the resting sodium efflux increased from 0.09 to 34.0 pmoles/cm²sec and the average resting sodium influx increased from 42.9 to 64.5 pmoles/cm²sec but this last change was not statistically significant. When perfusing with a mixture of 500 mM K glutamate and 100 mM Na glutamate the resting efflux was 10 ± 3 pmoles/cm²sec and 41 ± 10 pmoles/cm²sec for sodium influx. Increasing the internal sodium concentration also increased both the extra influx and the extra efflux of sodium due to impulse propagation. At any given internal sodium concentration the net extra influx was about 5 pmoles/cm²impulse. This finding supports the notion that the inward current generated in a propagated action potential can be completely accounted for by movements of sodium.

INTRODUCTION

In 1961 two different groups of investigators (Baker, Hodgkin, and Shaw; Oikawa, Spyropoulos, Tasaki, and Teorell) independently developed techniques to replace the axoplasm of squid giant axons by artificial solutions without altering axonal membrane potentials. Since then this preparation has been widely used to study further the origin of these potentials and many experiments have been performed under a variety of intra- and extraaxonal conditions (Narahashi, 1963; Tasaki and Takenaka, 1964; Rojas and Ehrenstein, 1965; Adelman, Dyro, and Senft, 1965; Chandler and Hodgkin, 1965). Sodium transport has been well-studied in intact axons (Keynes, 1951; Shanes and Berman, 1955; Hodgkin and Keynes, 1955; Caldwell, Hodgkin, Keynes, and Shaw, 1960) but only preliminary work has been reported on the effects of axoplasm removal on sodium movements. As internally perfused axons provide an ideal preparation for studying not only the physical properties of the excitable membrane but also the role of membrane metabolism in active sodium transport, we considered it mandatory to determine first what

fraction of the sodium fluxes was independent of axonal membrane metabolism in this preparation. It was found that when axons were perfused with either fluoride or glutamate in the absence of "energy-rich" phosphate compounds, the resting efflux was much smaller than that measured in unperfused axons. Sodium influx on the other hand, was of the same order of magnitude as that found in intact axons. As sodium influx in intact axons appears to be mainly a passive flux (Shanes and Berman, 1955; Caldwell et al., 1960) and as sodium efflux in the present research was not affected by the sodium transport inhibitor, ouabain, it seems reasonable to regard the sodium fluxes studied in this work as passive reflecting only the diffusion properties of the membrane. Thus, a base line is established for studying the effect of energy-rich phosphate compounds in the reactivation of the so-called active transport of sodium (Brinley and Mullins, 1966).

One fundamental consequence of the sodium hypothesis in the Hodgkin and Huxley formulation (1952 *b*) was the prediction of the time course of the sodium current during a propagated action potential. With this computed time course the net entry of sodium was calculated to be 4.33 pmoles/cm^2 impulse for a temperature of 18.5°C. Keynes (1951) measured a net entry of sodium equal to 3.7 pmoles/cm^2 per impulse in *Sepia* axons and Keynes and Lewis (1951) measured 3.5 pmoles/cm^2 impulse in *Loligo* axons. The net entry determined in the present paper from *Dosidicus gigas* perfused axons was 5.0 pmoles/cm^2 impulse. These values obtained in intact axons and in perfused fibers are in agreement with that calculated by Hodgkin and Huxley to explain the inward current generated by a net influx of sodium ions during a propagated impulse. However, the predicted values for the extra influx and efflux were not in agreement with the values actually measured previously (Keynes, 1951) but are in agreement with our experimental curves. In the present work it was found that both the extra influx and the extra efflux of sodium due to impulse propagation increased with the internal sodium concentration, the difference or net entry being constant at 5 pmoles/cm^2 per impulse. Some of the previous discrepancy might have been due to differences in internal sodium concentration between the model nerve fiber and the fibers used by Keynes (1951).

A preliminary report of this paper was presented at the Second International Biophysical Congress in Vienna, Austria, in 1966.

METHODS

The experiments were carried out on the giant axon of the Chilean squid, *Dosidicus gigas*, at the Laboratorio de Fisiología Celular, Montemar, Chile. These squid were caught and killed a few miles offshore. The mantles were kept in iced seawater during the trip to shore. Within 2 hr of capture of the animal, the mantle nerves were removed and the giant axons were then dissected from these bundles and prepared for experiment.

Fig. 1 shows a simplified diagram of the experimental arrangement used in the present experiments for measuring ^{22}Na fluxes as a function of electrochemical potential. The details of the method have been described before (Rojas and Ehrenstein, 1965; Rojas and Atwater, 1967) and can be summarized as follows: A cleaned giant fiber, 900 μ average diameter, with both ends tied is mounted on a chamber as illustrated in the diagram. An outlet glass pipette, 600–700 μ in diameter, is slowly introduced through one end for about 35 mm. To prevent stretching of the membrane, a plastic tube is connected to the outlet cannula and some of the axoplasm is removed by gentle mouth suction. Another glass tube, the inlet pipette, 250–350 μ

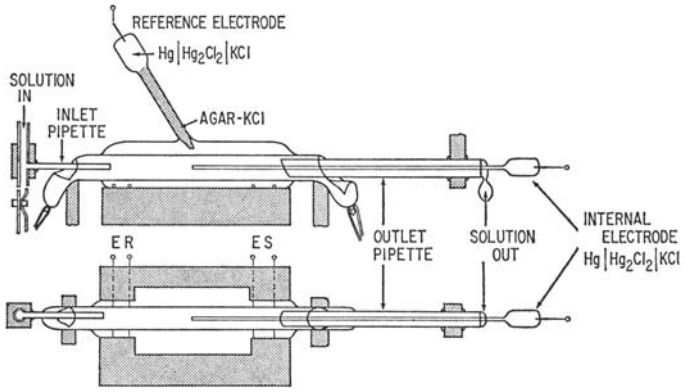

FIGURE 1. Simplified diagram of the experimental arrangement used for internal perfusion. The upper diagram represents a section through the perfusion chamber with cannulas and electrodes in position. The lower diagram represents a top view of the chamber. *ER* represents two platinum wires used for external recording. *ES* represents two platinum wires used for external stimulation. The reference and the internal electrodes were connected to the input of a differential P-6 Grass amplifier. The inlet and the outlet cannulas were mounted on micromanipulators not shown in the diagram.

in diameter, is then introduced through the opposite end and placed in the lumen of the first pipette. A small volume of perfusing solution is driven from the inlet pipette through the outlet pipette to remove the remaining axoplasm. Then, the outlet pipette is withdrawn about 23 mm, leaving an average perfused area of 0.6 cm^2. Finally the internal electrode is introduced through the outlet cannula to its final position in the middle of the axon. During these procedures, the axons are stimulated at ES and action potentials are recorded externally at ER; after the internal electrode is in place, only records of the internal action potentials are made. Standard electronic equipment is used for stimulation and recording (S-5 Grass stimulator, P-6 Grass differential amplifier, and 502 Tektronix oscilloscope).

With the above technique, perfusion rates are high, as much as 100 μl/min, and the dead space of the perfusing system is small, less than 10 μl. This facilitates a rapid change of the internal medium and also allows more precise measurements, especially of extra influxes.

Criteria for Successful Perfusion

Before sodium flux experiments were begun, perfused fibers were tested in the following manner for possible damage which might have occurred during the manipulations described above. (*a*) The resting potential was measured and its time course was followed for about 15 min (minimum value −50 mv). (*b*) Action potentials were measured with either the internal electrode shown in Fig. 1, or with a clean platinum wire extended over the entire length under perfusion. Again the time course was followed for at least 15 min. Axons with action potentials smaller than 90 mv were rejected. In some fibers in which a high biphasic action potential was externally recorded (1 mv), the internally recorded action potential was not up to standard, and sometimes was as low as 40 mv. (*c*) If full size action potentials could not be elicited by stimulating the axon 50 times per sec, the fiber was rejected. This test was particularly important for those experiments in which changes in sodium fluxes during activity were studied.

Flux Measurements

The ^{22}Na outflow as a function of time was measured in the following way. (*a*) 5–10 min after the onset of perfusion, the solution inside the axon fiber was replaced by one containing the isotope (see Table I). (*b*) After 10 min (or in some experiments 20 min) the first sample of about 1 ml of external seawater was taken. The chamber was washed once in order to collect the total radioactivity. The two collections were made in less than 30 sec and, (*c*) all following samples were taken as described in (*b*). Action potential height was internally recorded at least once during each flux determination.

The ^{22}Na inflow as a function of time was measured in the following manner. (*a*) 5 to 10 min after the onset of perfusion, the external seawater was replaced by seawater containing ^{22}Na (see Table I). (*b*) Perfusate samples were collected every 10 or 20 min. The volume of collected perfusate ranged from 0.8 to 2.0 ml.

Sodium fluxes were computed by multiplying the measured flow of radioactivity in counts per minute per square centimeter second (cpm/cm^{2}sec) by the reciprocal of the specific activity of the solution measured in picomoles, 10^{-12} M, per cpm (pmole/cpm).

The experiments to be described here were performed during three consecutive squid seasons. During the 1965 Chilean season only a fraction of the part of the fiber bathed in seawater was under intracellular perfusion. During the 1966 and 1967 seasons the experiments were performed perfusing the complete fiber bathed in seawater. As the computed sodium fluxes are proportional to the area, in the former case it was difficult to decide what length of the axon membrane was participating in the sodium exchange study. Average sodium fluxes computed by the use of an area equal to $\pi\ d \cdot l$ (d is axon diameter, l is the separation betwen the tips of the cannulas) as the area of exchange were larger for the partially perfused axons than for the completely perfused ones. However, the difference was not statistically significant.

The data to be examined are the average of the data obtained in both groups of experiments.

Radioactivity of the samples was measured with either a manual or an automatic 3 inch crystal scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Solutions used were prepared with distilled water passed through an ion exchange resin and redistilled in glass. They were kept at 4°C and were not stored for more than 5 days. All solutions containing fluoride were kept in Nalgene containers. All salts used were of analytical grade (Merck, Sharp & Dohme, West Point, Pa.). Tris(hydroxymethyl)aminomethane was obtained from Sigma Chemical Co., St. Louis, Mo., glutamic acid (grade A) was obtained from Calbiochem, Los Angeles, Cal., and ^{22}Na was obtained from New England Nuclear Corporation, Boston, Mass.

TABLE I
INTERNAL AND EXTERNAL ^{22}Na-CONTAINING SOLUTIONS FOR FLUX MEASUREMENTS

	Cations				Anions			Radioactivity	
Solution	Na^+	K^+	Ca^{++}	Mg^{++}	F^-	Glutamate, aspartate	Cl^-		
		mM				mM		cpm/cm³	
22a	2	600	—	—	600	—	2	12.0	10^6
22b	10	600	—	—	600	—	5	12.8	10^6
19	100	500	—	—	600	—	5	12.8	10^6
23	200	300	—	—	600	—	5	12.0	10^6
1	100	500	—	—	—	600	5	9.2	10^6
50	100	445	—	—	—	545	5	10.5	10^6
17	100	500	—	—	100	—	505	5.1	10^6
6	430	10	10	50	—	—	565	22.4	10^6

Internal solutions were brought to pH 7.3 with 5 mM Tris Cl. External solution (No. 6) was brought to pH 8.0 with 5 mM Tris Cl.

RESULTS

Resting Sodium Fluxes As a Function of Internal Sodium Concentration

Fig. 2 shows part of the time course of a typical sodium efflux experiment. Intracellular perfusion was begun with 600 mM KF; after 13 min, this solution was replaced by 500 mM KF, 100 mM NaF and then, after 23 min, by a solution of the same composition, but with ^{22}Na added. (See solution 19 in Table I.) In this figure the vertical axis represents the sodium efflux measured during a 10 min period. Each column represents one determination. It can be seen that the sodium efflux remained almost constant for long periods. For example, determination 5 is not different from determinations 12 and 13, although 70 min had elapsed between the measurements. This figure also demonstrates that the resting outward movement of sodium is increased by impulse propagation. Determination 14 represents the efflux during external

stimulation of the fiber. As there were 50 propagated action potentials per sec recorded on the oscilloscope, the average extra efflux per impulse in this particular experiment is seen to be about 6 pmoles/cm².

Fig. 3 shows part of the time course of a sodium influx experiment. In this case the perfusion was again started with 600 mM KF and 2 mM NaF. After 20 min the external seawater was replaced by ^{22}Na seawater (see solution 6 in Table I). It is clear from this figure that the sodium influx again remained almost constant during the internal perfusion with a given sodium concentration. For example, with 100 mM, determination 8 is not different from

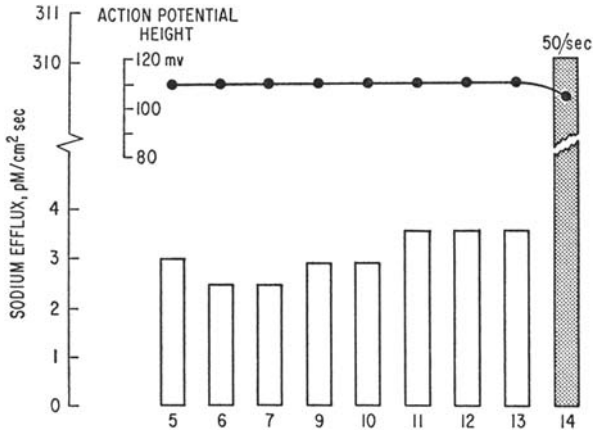

FIGURE 2. Resting sodium efflux in internally perfused giant axon. Vertical axis is efflux measured in pmoles/cm² sec. The number under each column represents the determination number. Determinations were made every 10 min. Internal solution contained 500 mM KF, 100 mM NaF, and 5 mM Tris Cl at pH 7.3. Axons bathed in artificial seawater at room temperature at about 20°C. Determination 14 was measured under electrical stimulation pulsing the fiber 50 times per sec during 10 min. The recording of the action potential height has been inserted on the upper part of the figure. It was internally measured once during each determination. Axon diameter 875 μ.

the later determination 12, although 50 min have elapsed. Tables II and III summarize the data on resting sodium fluxes obtained by internal perfusion with different concentrations of sodium. The data summarized in these tables were obtained in experiments of two types (*a*) data obtained from axons in which only one internal sodium concentration was used and (*b*) data obtained from axons in which different internal concentrations of sodium were used in the same fiber. In this case the measurements obtained during the change of solutions were not considered. As shown in Table II the resting sodium efflux is affected by the internal sodium concentration. There is an increase in sodium efflux from 0.1 pmole/cm² sec to 34 pmoles/cm² sec as the internal sodium concentration is increased from 2 to 200 mM. This increase was observed in all fibers in which the internal concentration was increased

in three consecutive steps, from 2 or 10 mM to 100 mM and finally to 200 mM. Therefore the average values given in Table II also include what was observed in some of the fibers in which several concentrations were used. The resting potential varied from −53 mv measured at 2 mM internal sodium to −47 mv measured at 200 mM internal sodium. On the other hand, although the resting sodium influx as shown in Table III seems to be increasing as the internal sodium concentration is raised, this change is not statistically significant, because of the large variances shown in Table III. Those fibers for which

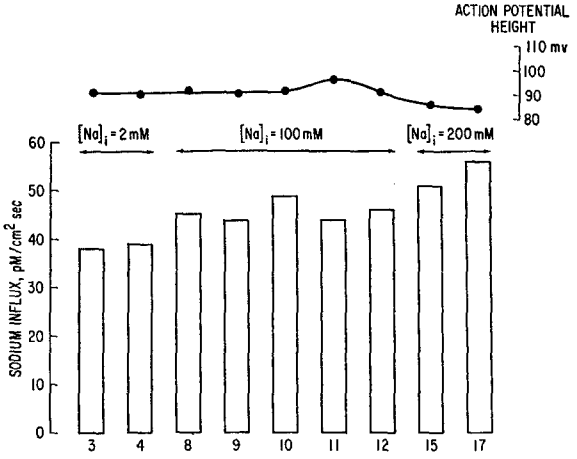

FIGURE 3. Resting sodium influx in internally perfused giant axon. This figure summarizes the results obtained when the internal sodium concentration was increased from 2 to 200 mM and when fluoride was used as internal anion. The internal sodium concentration for each determination is given at the top of each column. The insert on the upper part of the figure represents as before the action potential height. Axon diameter 945 μ; area for sodium exchange 0.34 cm². Experiment performed at room temperature of about 20°C.

all three concentrations given in Table III were examined showed this increase in the resting sodium influx with increased internal sodium concentration. Again this increase seen in individual axons was not statistically significant.

Effect of Impulse Propagation on Sodium Fluxes

The resting sodium efflux is increased by impulse propagation. Fig. 4 shows the data obtained in two different fibers in which the resting efflux is compared to the efflux measured during activity, with stimulation at three different frequencies. The vertical axis represents an average efflux in pmoles/cm² sec measured during a 10 min period. The internal concentration of sodium was changed in order to compare resting effluxes. The resting effluxes at both internal concentrations were comparable in these two experiments

TABLE II
SODIUM EFFLUX AS A FUNCTION OF THE INTERNAL SODIUM CONCENTRATION WITH FLUORIDE IN THE PERFUSATE

Internal sodium concentration	Perfused area	Average resting potential for each fiber	Average efflux for each fiber
	cm²	*mv*	*pmoles/cm² sec*
2 mM	0.29 (2)	−50	0.09
	0.30 (3)	−56	0.08
	0.26 (2)	−52	0.07
	0.36 (2) (Fig. 4)	−56	0.12
	0.45 (2) (Fig. 4)	−52	0.09
	Average ± SD	−53.2	0.09 ± 0.02
10 mM	0.31 (4)	−56	0.18
	0.26 (2)	—	0.10
	Average	−56	0.14
100 mM	0.32 (10)	−50	4.4
	0.27 (11) (Fig. 2)	−50	3.1
	0.36 (2) (Fig. 4)	−53	11.0
	0.45 (3) (Fig. 4)	−51	14.3
	0.55 (4)	—	3.9
	0.60 (4)	−52	3.3
	0.60 (5)	−52	2.9
	0.43 (2)	−51	3.0
	Average ± SD	−51.3	5.7 ± 3.6
200 mM	0.37 (2)	—	36.3
	0.40 (2)	—	41.6
	0.26 (2)	−47	24.0
	Average ± SD	−47	34.0 ± 9.0

The number in parentheses indicates the number of determinations using the same fiber. Experiments for which the time course of the sodium efflux is given in the figures have been labeled with the figure number. All experiments were performed at room temperature of about 20°C. External solution: 430 mM NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl, 5 mM Tris Cl at pH 8.0. Internal solutions with fluoride as the main anion.

(determinations 2, 3, and 7 in Fig. 4 are comparable to determinations 8 and 9 in Fig. 4 B; determinations 2, 5, and 7 of Fig. 4 B are comparable to 8 and 9 in Fig. 4 A). The extra efflux of sodium was computed by subtracting the resting level measured in pmoles/cm² sec from the efflux value obtained during activity; this difference was divided by the number of impulses per sec to obtain pmoles/cm² impulse. For 2 mM internal sodium an extra efflux of 0.08 pmole/cm² impulse was obtained when stimulating 10 times per sec, 0.075 pmole/cm² impulse when stimulating 20 times per sec, and 0.06 pmole/cm²

impulse stimulating 30 times per sec. The average is then 0.07 pmole/cm² impulse. For an internal concentration equal to 100 mM, the extra effluxes were 2.6, 2.9, and 2.2 pmoles/cm² impulse for 10, 20, and 30 stimulations per sec, respectively. The average for the experiment shown in Fig. 4 B is thus 2.6

TABLE III
SODIUM INFLUX AS A FUNCTION OF THE INTERNAL SODIUM CONCENTRATION WITH FLUORIDE IN THE PERFUSATE

Internal sodium concentration	Perfused area	Average resting potential for each fiber	Average influx for each fiber
	cm²	*mv*	*pmoles/cm² sec*
2 mM	0.31 (2)	—	54.6
	0.27 (5)	—	34.3
	0.34 (6) (Fig. 3)	−53	39.9
	Average ± SD		42.9±10.5
10 mM	0.64 (3) (Fig. 5)	−54	39.0
	0.60 (10)	−52	41.0
	Average		40.0
100 mM	0.30 (1)	—	49.0
	0.30 (5)	—	61.6
	0.34 (5) (Fig. 3)	−50	44.8
	0.35 (2)	—	45.2
	0.49 (11)	—	49.4
	0.38 (2)	—	49.4
	0.64 (3) (Fig. 5)	−51	41.5
	Average ± SD		48.7±6.0
200 mM	0.30 (3)	—	81.1
	0.27 (5)	—	56.7
	0.34 (4) (Fig. 3)	−45	55.6
	Average ± SD		64.5±15.0

The number in parentheses indicates the number of determinations using the same fiber. Experiments for which the time course of the sodium influx is represented by a figure have been labeled with the number of the figure. All experiments were performed at room temperature of about 20°C. External solution: 430 mM NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM KCl, 5 mM Tris Cl at pH 8.0. Internal solutions with fluoride as the main anion.

pmoles/cm² impulse. But there is some spread from axon to axon. The highest value of the extra efflux was approximately 6 pmoles/cm² impulse which was obtained for the experiment described in Fig. 2 (50 pulses per sec). Finally, considering the data from two other fibers (4 pmoles/cm² impulse and 3.7 pmoles/cm² impulse) the extra efflux for 100 mM internal sodium, weighting the four axons equally, is 4 ± 1 pmoles/cm² impulse.

Fig. 5 shows the effect of stimulation and the resting sodium influx. This figure shows the data obtained in one fiber in which the resting influx deter-

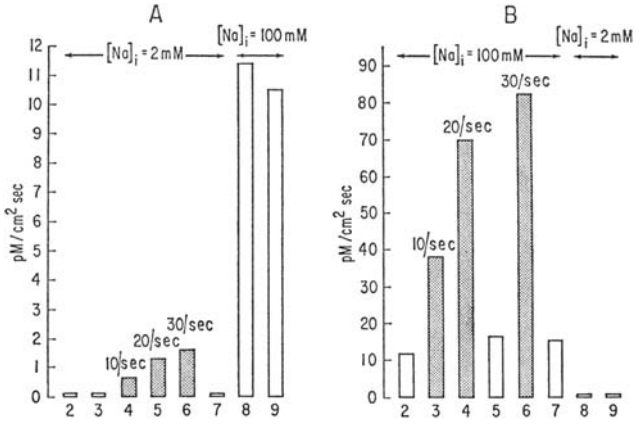

FIGURE 4. Effect of impulse propagation on sodium efflux. Vertical axis represents the efflux in pmoles/cm^2 sec measured at rest and during external stimulation (shaded columns). The frequency of stimulation is given at the top of each column. In Fig. 4 A the internal sodium concentration was augmented from 2 to 100 mM (as indicated in the upper part of the figure) with fluoride as the internal anion. In Fig. 4 B the internal sodium concentration was decreased from 100 to 2 mM. Both experiments were carried out at room temperature, about 18°C. The areas for sodium exchange were 0.36 cm^2 for axon 4 A and 0.45 cm^2 for axon 4 B.

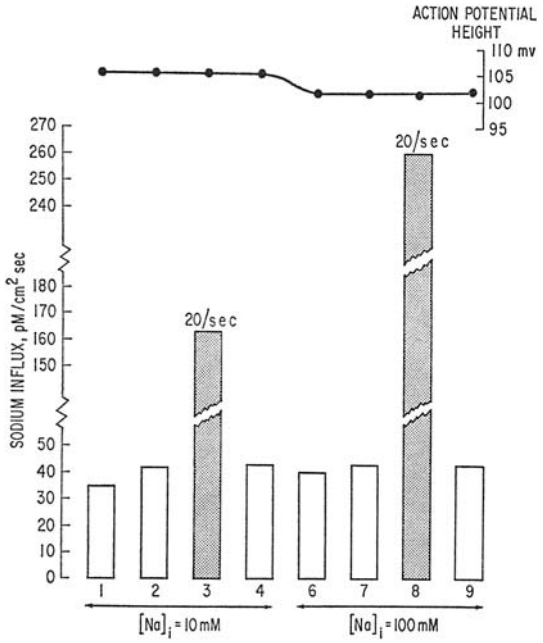

FIGURE 5. Effect of impulse propagation on sodium influx. This figure compares the influx during rest and during activity. Axon diameter 900 μ, area for sodium exchange, 0.64 cm^2. Experiment performed at room temperature of 17°C. Frequency of stimulation is indicated at the top of each column.

mined at 10 and 100 mM internal sodium is compared with the influx measured during activity stimulating 20 times per sec. The extra influx was computed as before for the extra efflux.

Fig. 6 summarizes the data on extra fluxes due to activity. In this figure the

vertical axis represents the net extra flux during a propagated action potential. The horizontal axis represents the internal sodium concentration. There is one insert in this figure, the values measured by Keynes in intact axons (1951) inserted as two horizontal dashed lines. Although both the extra efflux and the extra influx increased as the internal sodium concentration was raised, the difference seems to be constant.

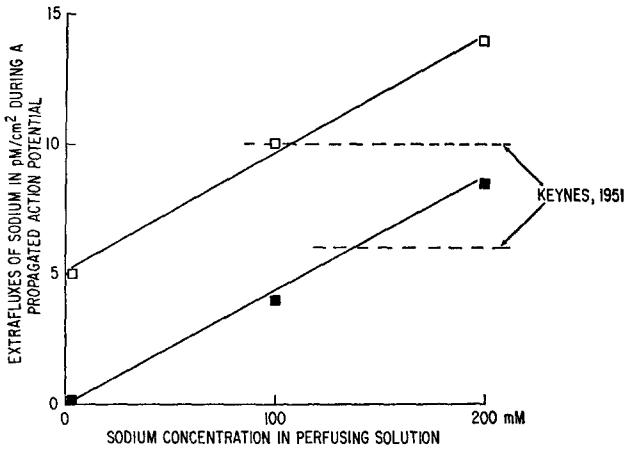

FIGURE 6. Net sodium movements during a propagated action potential. Vertical axis represents extra flux of sodium in pmoles/cm² during a propagated impulse. Horizontal axis represents the internal sodium concentration in mM per liter. Open squares indicate extra influx. Each point represents the average extra influx obtained with several fibers (seven fibers in all). Values for individual fibers represent averages of several determinations at different frequencies of stimulation. Filled squares indicate extra efflux. Again each point represents the average of more than two fibers (five fibers in all). In all fibers more than one internal sodium concentration was used and several determinations were made while stimulating at different frequencies. There is one insert in this figure. Dashed lines indicate determination in *Sepia* axons (Keynes, 1951). Average action potential height for 2 or 10 mM was 109 mv; for 100 mM, 100 mv and for 200 mM, 75 mv.

Effect of Anion on Sodium Fluxes

Different anions can be used for intracellular perfusion with different effects upon electrophysiological conditions (Tasaki, Singer, and Takenaka, 1965). In fibers from *Dosidicus gigas*, we have found that anions can be ordered in a series in terms of the duration of constant normal resting and action potentials during perfusion with them. This series is: fluoride, aspartate, glutamate, sulfate, and chloride. The perfusing anion that gives the longest survival time is fluoride, and chloride is the one that gives the shortest survival time. In five experiments we observed that after less than 30 min of perfusion with chloride solutions, action potentials could not be elicited and resting potentials were decreased from -53 to -30 mv.

To determine whether the resting sodium fluxes were also altered by a change in internal anion, sodium fluxes were measured while varying the internal anion. Fig. 7 shows one of six experiments, in which the effect of replacement of the internal fluoride with chloride upon resting sodium influx was studied. In this fiber, determination 6 was made while there was still a small action potential present. The influx for this determination was 120 pmoles/cm² sec. After this determination, the fiber was in poor physiological condition and could not be restored by perfusion with fluoride. Measuring

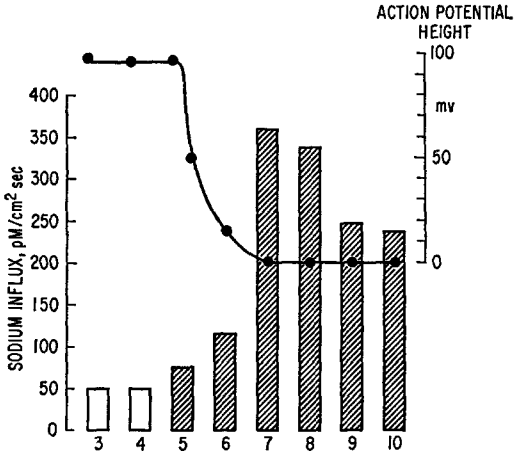

FIGURE 7. Effect of substitution of fluoride for chloride on sodium influx. This figure shows one of six experiments perfusing with a mixture of 500 mM KCl and 100 mM NaF. Vertical axis represents sodium influx. After determination 4 the internal solution No. 19 (500 mM KF, 100 mM NaF, 5 mM Tris at pH 7.2) was changed for one containing 84% chloride (No. 17 in Table I). After determination 5 the resting potential decreased from −52 mv to −26 mv. This fiber did not recover after return to an internal perfusion solution of 600 mM KF. Axon diameter 950 μ. Temperature 18°C.

the efflux under the same conditions, replacement of internal fluoride with chloride increased the efflux up to 290 pmoles/cm² sec after no more than 40 min of perfusion with chloride.

Eight experiments were carried out with glutamate as the internal anion. Average sodium fluxes obtained with each fiber are given in Table IV. All these fibers were excitable and the resting potentials measured were constant during the determination of sodium fluxes. When perfusing with a mixture of 500 mM K glutamate and 100 mM Na glutamate the resting efflux was 10 pmoles/cm² sec with this flux remaining constant for 120 min. When perfusing with the same glutamate solution the resting influx was 45 pmoles/cm² sec, again almost constant for 80 min. The large variances shown in Table IV are probably due to a rather poor determination of the area for sodium exchange. Most of these fluxes were obtained by perfusing only that part of the

fiber bathed in seawater. The computed area for sodium exchange is smaller than the true area of exchange because labeled sodium will diffuse to the unperfused region and the membrane of the unperfused region will be incorporated into the sodium exchange. In general, the flux measured was greater for fibers of greater diameter.

TABLE IV
SODIUM FLUXES WITH GLUTAMATE IN THE PERFUSATE

	Perfused area	No. of determinations	Resting potential	
	cm²		*mv*	*pmoles/cm² sec*
A. Efflux	0.22	2	−50	7
	0.30	12	−50	11
	0.41	7	−53	8
	0.63	8	−49	13
	Average ± SD		−50.5±3	10±3
B. Influx	0.28	4	−54	42
	0.55	5	−51	53
	0.55	8	−50	45
	0.60	3	−50	22.5
	Average ± SD		−51±2	40.6±11

Experiments performed at room temperature of about 20°C. Internal solution 500 mM K glutamate, 100 mM Na glutamate, 5 mM Tris Cl, pH 7.2. Resting potential recorded with Ag-AgCl-KCl cells and not corrected for the junction potential.

TABLE V
SODIUM EFFLUX WITH ASPARTATE IN THE PERFUSATE

Perfused area	Determination number	Action potential height	Sodium efflux
cm²		*mv*	*pmoles/cm² sec*
0.60	1	100	11.4
	2	95	11.6
	3	95	15.6
	4	95	13.5
	5	90	11.8
0.55	1	100	8.7
	2	90	9.0
	3	95	12.8
	4	95	9.7
	8	110	12.8
0.60	1	110	12.1
	2	90	15.6
	3	90	15.0
Average ± SD		99.4±7	12.5±2

Experiments performed at room temperature of about 20°C. Internal solution: 445 mM K aspartate, 100 mM Na aspartate, 5 mM Tris Cl, pH 7.2. Action potential height recorded with an axial platinum wire extended over the whole length under perfusion.

Three experiments were performed perfusing with aspartate as the main internal anion. Sodium effluxes during each determination and for each fiber are given in Table V. The resting efflux perfusing with a mixture of 445 mM K aspartate and 100 mM Na aspartate is 12.5 pmoles/cm² sec. These values of resting sodium effluxes measured while perfusing with aspartate are not statistically different from those effluxes measured using glutamate in the perfusate.

DISCUSSION

Resting Sodium Fluxes in Perfused Fibers

It has been determined that resting sodium influxes in intact axons vary between 32 pmoles/cm² sec for *Loligo forbesi* (Hodgkin and Keynes, 1955) and 52 pmoles/cm² sec for *Dosidicus gigas* (Canessa et al., 1968). Our results in perfused axons, summarized in Table III, fall within the same range values, perfusing with either fluoride or glutamate (40.0 to 64.5 pmoles/cm² sec). Influx measurements carried out in perfused fibers from *Loligo pealei* and *Loligo forbesi* by other investigators gave extremely high values ranging from 131 ± 25 pmoles/cm² sec (Shaw 1966) to 300 pmoles/cm² sec (Tasaki, 1963) when perfusing with sulfate. We obtained such high values of influx when we perfused with chloride solutions. These high values were seen even when the perfused fibers were still excitable. It is clear, then, that in the presence of internal sulfate or chloride the excitable membrane becomes extremely permeable to sodium ions. Thus, those anions which have been shown to increase the survival time of axons (fluoride, glutamate, aspartate) give resting sodium influxes comparable to those measured for intact axons; the high rates of influx are observed with the other anions (chloride and sulfate). Thus we have seen that the resting sodium influx is critically dependent on the internal anion. Furthermore, there seems to be a correlation between the sequence of survival times of axons perfused with different anions and the resting sodium influx.

We might ask whether the large resting sodium permeabilities measured with chloride and sulfate are caused by some sort of unspecific flux or "leakage." Evidence for this idea can be found in the voltage-clamp measurements of the ohmic component of the voltage-clamp currents (leakage). This leakage has been shown to be much higher in chloride-perfused axons than in fluoride-perfused ones (Adelman, Dyro, and Senft, 1966). Furthermore, membrane selectivity as examined with the voltage-clamp procedure is lower perfusing with sulfate than with fluoride. Chandler and Meves (1966) measured a P_{Na}/P_K ratio of 12 perfusing with sulfate, whereas with internal fluoride we have obtained a ratio of 25 (Rojas and Atwater, 1967).

Let us now ask why the fluoride or glutamate causes the resting fluxes of sodium to be smaller. An explanation has been given in terms of the known

effects of calcium on membrane permeability. That is to say, calcium ions which permeate the membrane (Luxoro and Yañez, 1968) will affect the permeability to other ions unless the calcium is trapped inside the fiber. It is known that the dissociation constant of CaF_2 is several orders of magnitude smaller than the dissociation constant of $CaCl_2$. We have observed, however, that the presence of up to 16% fluoride in the chloride perfusing solution does not prevent the increase in fluxes and the decrease in potentials (see Fig. 7). Furthermore, the addition of ethylenediaminetetraacetate (EDTA) to the chloride perfusing solution does not prevent the deterioration observed with this solution. Therefore it is unlikely that the role of the fluoride is merely to precipitate calcium. It is possible that anions present in the axoplasm have high association affinities for positive sites on the structural molecules of the excitable membrane. If the anions used in the perfusing solution do not have such high association affinities for positive sites, then there may be a decrease in the effective negative charge. This would result in a decrease in cationic selectivity (Helfferich, 1962) and an increase in the leakage conductance, if indeed there is an anionic component of leakage. From our observations it would seem that the association affinities of fluoride, glutamate, and aspartate are comparable with those of the naturally existing anions.

The resting sodium efflux in intact axons has been reported to be from 31 pmoles/cm² sec in *Sepia* (Keynes, 1951) to 67 pmoles/cm² sec (Canessa, Zambrano, and Rojas, 1968) in *Dosidicus gigas*, a rather wide variation in values. As the efflux in intact fibers is dependent on internal sodium concentration and metabolic activity, the variation is probably due to a breakdown in the metabolic apparatus of the axon after it has been cut off from the cell body. Once the metabolism is disrupted there will be an uncontrolled increase in internal sodium. Sodium concentration increases rather rapidly in isolated unperfused fibers from about 50 to about 100 mM (Steinbach and Spiegelman, 1943; Keynes and Lewis, 1951). It is not surprising, therefore, that the efflux measured from perfused axons with either 2 or 10 mM internal sodium (i.e. 0.09 or 0.14 pmole/cm² sec) is much smaller than the values reported for sodium efflux in intact fibers. At 100 mM internal sodium, a concentration comparable to that in intact fibers, the efflux measured in this work (i.e. 5.7 pmoles/cm² sec) is also lower than all values reported for intact fibers. This is most probably because the active transporting system is not operating in this preparation. We have used a transport inhibitor, ouabain, in all efflux determinations perfusing with either fluoride or glutamate. Although 10^{-5} M ouabain dissolved in seawater is a potent inhibitor of sodium efflux in intact axons from *Dosidicus gigas*, it does not affect the resting sodium efflux in perfused fibers. This information is given in more detail elsewhere (Canessa, 1965; Canessa et al., 1968). In experiments to be reported elsewhere we (Canessa

et al., 1968) have reactivated the sodium pump of perfused axons using aspartate or glutamate as the main internal anion. We have shown in the present experiments that these anions maintain the passive sodium fluxes at a level comparable to that obtained with fluoride. Fluoride cannot be used as the perfusing anion when studying the reactivation of the sodium transport system because fluoride inhibits the ATP hydrolysis, as has been seen in vitro for isolated membrane fractions having ATPase activity (Canessa et al., 1968; Opit, Potter, and Charnock, 1966).

Changes in Sodium Permeability Caused by Excitation in Perfused Axons

For a fiber bathed in seawater (450 mM NaCl) at 18.5°C Hodgkin and Huxley calculated a net entry of sodium of 4.3 pM/cm² per impulse. The net entry of sodium determined by us in perfused axons was 5.0 pM/cm² per propagated action potential. Sodium efflux and sodium influx were also computed by Hodgkin and Huxley for a propagated action potential. The extra efflux was computed using the computed time course of the sodium current, I_{Na}, during a calculated impulse and using the equation $I_{Na}/(\exp(V - V_{Na})F/RT - 1)$ (Hodgkin and Huxley, 1952 *b*). The computed extra efflux was 1.09 pM/cm² per impulse. To account for this extra efflux our experimental curve (see Fig. 6) predicts an internal sodium concentration of about 20 mM. On the other hand, the extra influx per impulse was obtained by Hodgkin and Huxley by adding the computed net entry to the computed net efflux; i.e., $4.33 + 1.09 = 5.42$ pM/cm² impulse. To account for this value our experimental curve predicts an internal sodium concentration of about 20 mM. It seems clear, therefore, that for this internal sodium concentration the three computed values (net entry, extra efflux, and extra influx) fit well the experimental curve. We have inserted in our experimental curve as two horizontal dashed lines the values measured by Keynes. The internal sodium concentration predicted from our curve in this case is of the order of 100 mM. This is a reasonable figure for axons which were cut off from the cell body and kept in seawater for several hours (Steinbach and Spiegelman, 1943). Thus there is an excellent agreement between the predicted individual changes in sodium permeability and the values measured by us in perfused axons.

We have shown that as the internal sodium concentration is increased both the extra efflux and the extra influx per impulse are increased but the net flux remains approximately constant. The action potential height is lower, as expected from the changed chemical driving force as the internal concentration of sodium is increased, but it is somewhat prolonged so that the total net flow of charge is roughly constant. The probable reason for this prolonged action potential and the increase in extra influx is the incomplete sodium inactivation which occurs when axons are perfused with high concentrations of internal sodium (Adelman and Senft, 1966; Chandler and Hodgkin, 1965). To

see whether this explanation holds up in detail would require solving the Hodgkin and Huxley equations for a propagated action potential with conditions of incomplete sodium inactivation; i.e., when perfused with high internal sodium. This further analysis is beyond the scope of this manuscript.

The authors are pleased to thank Drs. K. S. Cole and R. E. Taylor for reading and commenting on this paper. They also thank Dr. H. Lecar for many valuable discussions when preparing the final version of this paper.

The technical assistance of F. Zambrano and I. Atwater is acknowledged.

This research was supported by the University of Chile and by grants from the United States National Institutes of Health (NB-06503-01) and the United States Air Force (AFOSR-788-66).

Received for publication 3 August 1967

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