

Nonelectrolyte Penetration and Sodium Fluxes through the Axolemma of Resting and Stimulated Medium Sized Axons of the Squid *Doryteuthis plei*

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ABSTRACT The penetration of ^{14}C -labeled erythritol, mannitol, and sucrose through the axolemma was determined in medium sized paired axons, one at rest and the other stimulated 25 times per sec. The resting permeabilities, in 10^{-7} cm/sec, are erythritol, 2.9 ± 0.3 (mean \pm SEM); mannitol, 2.3 ± 0.4 ; and sucrose 0.9 ± 0.1 . In the stimulated axons they are: erythritol, 5.2 ± 0.3 ; mannitol, 4.0 ± 0.5 ; and sucrose, 1.8 ± 0.3 . Thus, the calculated permeabilities during activity (1 msec per impulse), in the same units, are: 100, 75, and 38, respectively. These changes in permeability are reversible. The effects of external potassium and sodium concentrations on erythritol penetration were also studied. At rest, erythritol penetration is independent of potassium and sodium concentrations. In the stimulated axons, erythritol penetration decreases when the extracellular sodium is diminished. Sodium influx (not the efflux) decreases during rest and activity when the extracellular sodium is diminished. The diminution during activity of erythritol and sodium entries in low sodium solutions may be related to a decrease of a drag effect of sodium ions on the nonelectrolyte molecules or to independent effects of the sodium concentration on sodium influx and the nonelectrolyte pathways. The axolemma discriminates among erythritol, mannitol, sucrose, and the different ionic species during rest and activity.

It has been shown in the resting nerve fiber of the squid, *Doryteuthis plei*, that the axolemma (excitable membrane of nerve fibers, 1) is the most significant barrier (2, 3) for the diffusion of nonelectrolyte molecules with radii greater than 3 Å and smaller than 60 Å, the width of the channels that cross the Schwann cell layer. The present work studies the effects of experimentally

produced changes in the permeability of the axolemma on the penetration of small (3 to 4.5 Å radius) nonelectrolyte molecules.

The present experiments deal with: (I) the penetration of erythritol, mannitol, and sucrose labeled with ^{14}C in resting and stimulated *D. plei* axons and the reversibility of the change in permeability caused by stimulation; (II) the effect of different external potassium concentrations on the permeability of the axolemma to the penetration of erythritol; and (III) the effect of different external sodium concentrations on the penetration of erythritol and the movements of sodium in resting and stimulated axons. Studies which are described also in section I, indicate that the radioactive molecules found in the axoplasm samples are the same ^{14}C -labeled molecules added to the sea water baths.

A preliminary report of some of these experiments has been presented (4).

EXPERIMENTAL METHOD

General Procedure

Living squids, *Doryteuthis plei*, were utilized. A pair of nerve fibers, obtained from the hindmost stellar nerves of the mantle, was used from each squid. The isolation of each fiber from the mantle, immersed in natural sea water, took about 10 min. Most of the small fibers and the endoneurium were removed. Immediately after isolation the fiber was equilibrated for 5 min in artificial sea water while its length and the axon diameter were measured.

(a) For the sodium influx and nonelectrolyte penetration measurements the axon pairs were transferred to an artificial sea water bath containing ^{22}Na or one of the following ^{14}C -labeled solutes: erythritol, mannitol, or sucrose. Both axons of each pair were immersed in approximately 5 ml of radioactive bath for the same period in two separate Lucite holders. (The composition of the sea water baths is given in the text and tables.) The Lucite holders had four platinum electrodes above the sea water level, two for stimulation and two for recording. A fifth platinum electrode immersed in the bath was used to ground the preparation. The electrodes for stimulation were connected via a stimulus isolation unit (Grass SIU-4B) to a stimulator (Grass S4E) and the recording electrodes were connected to an oscilloscope (Tektronix 532; plug in unit D) via an ac preamplifier (Grass P5R). About 3 mm at both ends of the nerve fiber were placed on the electrodes and were kept wet with hanging drops of radioactive sea water. At the end of the immersion period the fiber was taken out, soaked for about 15 sec in nonradioactive sea water, blotted with filter paper, and then placed lengthwise on a strip of filter paper. The nerve fiber was cut about 5 mm from one end and the axoplasm extruded from the nerve by gently pressing on the axon with a finger covered with Parafilm paper. At least 5 mm of the other end of the axon were left unextruded. The drop of extruded axoplasm was collected on a piece of about 0.25 cm² of filter paper and weighed in a balance (Mettler S6), with a precision of $\pm 2 \mu\text{g}$, successively at 60, 90, 120, 150, and 180 sec after extrusion. The weight of collected axoplasm at zero time was calculated by extrapolation. The small piece of filter paper with the axoplasm sample was finally transferred to a glass test tube for ^{22}Na counting or to a Tri-Carb glass flask for ^{14}C counting.

(b) For the sodium efflux measurements the axon pairs were immersed, in order to label the axoplasm sodium, in a 2 ml bath of artificial sea water containing 25 μc of ^{22}Na per ml for 30 min. Then the extraaxonal ^{22}Na was washed out by soaking the fibers in a series of three nonradioactive baths. The length of time in each bath was successively: 10, 20, and 1 min. At the end of the last soaking period both fibers of each pair were transferred and kept in two separate Lucite holders, each containing 5 ml of nonradioactive bath, for 15 min. One fiber of each pair was kept at rest and the other was stimulated for 14 min at a frequency of 25 stimuli per sec. At the end of the 15 min the amount of ^{22}Na collected in the baths was determined. To calculate the efflux a concentration of 52 mmoles of Na per liter of axoplasm was used (52 ± 8 is the mean \pm SEM of the sodium concentration, determined by flame photometry, in the axoplasm of 5 axons kept 50 min in normal sea water), and the ^{22}Na remaining in the axon was measured.

Composition of the Solutions

Artificial sea water (5), called normal sea water in the present work, was used as normal medium. The concentration of its components in millimols per liter was as follows: NaCl, 442; KCl, 10; CaCl_2 , 11; MgCl_2 , 53; NaHCO_3 , 2.5.

Sea water in which one-half of its normal sodium chloride concentration was replaced, mole per mole, by choline chloride, and solutions in which two-thirds of the normal sea water sodium concentration were replaced by choline or lithium, were used. All other constituents of the normal sea water were kept at their normal concentrations. These solutions are named (0.50 Na:0.50 Ch)-sea water, (0.33 Na:0.66 Ch)-sea water, and (0.33 Na:0.66 Li)-sea water.

Solutions containing 3, 15, 20, and 30 times the potassium concentration in normal sea water were used also. These media were prepared by replacing, mole per mole, sodium chloride by potassium chloride.

The baths containing radioactive nonelectrolytes were prepared by adding to the media one of the following ^{14}C -labeled solutes: (a) 3.3 mmoles of erythritol per liter, specific activity, 3.00 mc per mmole; (b) 4.7 or 18 mmoles of mannitol per liter, specific activities, 1.10 and 1.38 mc per mmole, respectively; (c) 2.0 or 3.2 mmoles of sucrose per liter, specific activities 3.02 and 3.09 mc per mmole, respectively. At these concentrations the osmotic effects of the nonelectrolytes are negligible. The purity of the ^{14}C -labeled solutes was tested by paper chromatography and radiochromatography.

The ^{22}Na -labeled baths were prepared by adding the isotope to the media in the following amounts (a) 0.9 or 1.3 μc per ml for the influx experiments and (b) 25 μc per ml for the bath used, in the efflux experiments, to label the axoplasm sodium. ^{22}Na was obtained as the chloride salt dissolved in distilled water, specific activity 0.6 mc per mmole.

Sample Analysis

The concentration of ^{14}C -labeled solute in the samples of axoplasm and in the radioactive sea water baths was determined by a liquid scintillation counter (Tri-Carb spectrometer, 314). To the sample of axoplasm diluted in 1 ml of distilled water or to

1 μ l of radioactive sea water bath, also diluted in 1 ml of distilled water, were added 20 ml of the following scintillation solution (reference 6): 2,5-diphenyloxazole, 3.00 g; 1,4-di-2-(5-phenyloxazolil)-benzene, 0.10 g; naphthalene, 50.00 g; made up to 1 liter with dioxane. All counting flasks contained a piece of filter paper of about 0.25 cm^2 similar to that on which the samples of axoplasm were collected. Counting was performed at 4°C, with the pulse height discriminator set to receive pulses of 10 to 100 v, and a photomultiplier voltage of 1100 v. The standard deviation of net counting was less than 2%. The counting flasks were discarded after each experiment.

The ^{22}Na in the axoplasm samples diluted in 1 ml of distilled water and in samples of radioactive baths also diluted in 1 ml of water, was counted in a scintillation well counter (Nuclear Chicago, DS-5) connected via a radiation analyzer (Nuclear Chicago, 1810) to a scaler (Nuclear Chicago, 186 A). All test tubes contained a piece of filter paper of about 0.25 cm^2 . The standard deviation of net counting was less than 3%.

Expression of Results

For the nonelectrolytes the results are given in terms of the permeability of the axolemma to the penetration of the test molecules in cm/sec , and indicate the amount of substance that reached the axoplasm per unit concentration in the radioactive sea water bath, per cm^2 of axolemma, per second. The results are given as mean \pm SEM.

For sodium the results are expressed in terms of the influx or efflux through the axolemma in $\text{pmole}/\text{cm}^2 \cdot \text{sec}$ or in $\text{pmole}/\text{cm}^2 \cdot \text{impulse}$. The results are given as mean \pm SEM.

RESULTS AND DISCUSSION

I. Penetration of Nonelectrolytes during Rest and Activity

(a) CHANGES IN NONELECTROLYTE PENETRATION CAUSED BY STIMULATION

Fifty-two pairs of nerve fibers were utilized to determine the permeability of resting and stimulated axons to the penetration of erythritol, mannitol, and sucrose.

20 pairs were used for erythritol, 10 for mannitol, and 12 for sucrose. Both fibers of each pair were immersed for the same period, in the same radioactive bath, but in two separate Lucite holders, one at rest and the other stimulated for a period 1 min less than the total immersion time. The times of immersion in the media were: 21 min in the baths containing ^{14}C -erythritol or ^{14}C -sucrose, and 11, 21, or 31 min in those containing ^{14}C -mannitol.

Table I summarizes the results and shows the increase in permeability to the penetration of the nonelectrolytes caused by stimulation. The permeabilities of the axons stimulated 25 times per sec, in $10^{-7} \text{ cm}/\text{sec}$, are: 5.2 ± 0.3 for erythritol, 4.0 ± 0.5 for mannitol, and 1.8 ± 0.3 for sucrose. The values for the paired resting axons, in the same units, are: 2.9 ± 0.3 for erythritol, $2.3 \pm$

TABLE I
 PERMEABILITY OF RESTING AND STIMULATED AXONS*
 TO THE PENETRATION OF NONELECTROLYTES
 Temperature 21° to 23°C

Molecules	No. of axon pairs	Axon a Rest		Axon b Stimulated		Calculated permeability during activity‡	
		Diameter μ	Permeability 10^{-7} cm/sec	Diameter μ	Stimuli/sec Permeability 10^{-7} cm/sec		
Erythritol	15	253±4	2.9±0.3	252±3	25	5.2±0.3	100±18
	5	258±12	2.6±0.4	256±12	50	6.2±0.5	77±14
Mannitol	10	257±8	2.3±0.4	258±7	25	4.0±0.5	75±27
Sucrose	12	254±8	0.9±0.1	255±8	25	1.8±0.3	38±13

* Values are mean \pm SEM.

‡ Calculated permeability during activity obtained by considering that the permeability change per impulse lasts 1 msec.

0.4 for mannitol, and 0.9 ± 0.1 for sucrose. The erythritol permeability of the axons stimulated 50 times per sec is 6.2 ± 0.5 and that of the paired resting axons is 2.6 ± 0.4 .

These results deserve some comments and criticisms, since Hodgkin and Martin (personal communication) have determined the permeabilities to mannitol and sucrose in four pairs of giant nerve fibers of *Loligo forbesi* (two for each substance; one axon of each pair at rest and the other stimulated 50 times per sec) and they found resting permeabilities which are about two-thirds lower than ours and with no increase caused by stimulation. The axons used by Hodgkin and Martin have diameters about two to three times larger than the *D. plei* axons used by us and their nerve fibers, except for a short distance near their ends, were only lightly cleaned. It is worthy of note that we also found some experimental results similar to those of Hodgkin and Martin, as illustrated by the group of sucrose experiments shown in Table II. It is also possible that the increase in nonelectrolyte permeability due to activity might be observed only in axons of the size used by us and smaller.

It should be noticed that the influx of sodium in the resting axons of *D. plei* is about three times larger (see Appendix) than in those of *Sepia officinalis* (7), *Loligo pealii* (8, 9), and *L. forbesi* (10). It may be relevant to point out that the temperature of the Venezuelan coastal waters in which the *D. plei* live is 25° to 28°C.

The increase in permeability to the penetration of the nonelectrolytes caused by stimulation in the *D. plei* axons is more evident if it is taken into account that the axons were stimulated at frequencies of 25 or 50 per sec and it is as-

TABLE II
PENETRATION OF ¹⁴C-SUCROSE IN SQUID AXONS*
Temperature 21° to 23°C

Squid No.	Axon <i>a</i> Rest			Axon <i>b</i> Stimulated 25/sec			Permeability ratio Axon <i>b</i> /Axon <i>a</i>
	Diameter	Axoplasm weight	Permeability	Diameter	Axoplasm weight	Permeability	
	μ	μg	10 ⁻⁷ cm/sec	μ	μg	10 ⁻⁷ cm/sec	
40	232	694	1.0	231	806	2.9	2.9
41	226	1035	0.3	230	655	0.6	2.0
42	230	793	0.2	228	1137	0.8	4.0
43	211	512	1.2	210	665	1.1	0.92
44	288	1940	0.7	292	1743	0.9	1.3
45	278	1170	1.0	274	447	1.3	1.3
46	270	530	1.0	269	1060	1.3	1.3
47	244	473	1.2	248	425	3.2	2.7
48	246	700	1.6	245	794	4.0	2.5
49	279	857	1.5	282	712	1.0	0.67
50	283	1036	1.0	284	1105	3.0	3.0
51	260	955	0.6	262	854	2.0	3.3
Mean±SEM	254±8		0.9±0.1	255±8		1.8±0.3	2.2±0.3

* The concentration of ¹⁴C-sucrose in the bath was 2.0 mmoles per liter, specific activity 3.02 mc per mmole, for pairs 40-42 and 49-51; and 3.2 mmoles per liter, specific activity 3.09 mc per mmole for pairs 43-48.

sumed that the permeability change during activity occurred only during 1 msec per impulse. Under this assumption it is calculated, as shown in Table I, that the permeabilities during activity in 10⁻⁷ cm/sec, are 80 to 100 for erythritol, 75 for mannitol, and 38 for sucrose. Thus, the permeability of the axolemma to these nonelectrolytes increases about 30 to 45 times during activity.

It may be observed in Table I that for resting and stimulated axons the permeability values for erythritol, mannitol, and sucrose decrease with increasing molecular radius. The radii of the nonelectrolytes used in the present work, as obtained from constructed molecular models, are: 3.1 Å for erythritol, 4.0 Å for mannitol, and 4.5 Å for sucrose. The relation between the permeability values and the molecular radii found in the present study agrees with the results of previous work on the permeability of the nerve fibers of *D. plei* to water and nonelectrolyte molecules (2, 11).

(b) ANALYSIS OF THE NONELECTROLYTE MOLECULES AFTER PENETRATION IN THE AXOPLASM

The following experiments were carried out to explore whether or not, under our experimental conditions, the penetrating test molecules are transformed into another molecular species by the resting or stimulated nerve fibers.

(a) Since various cells and cell extracts have been found to carry out di-phosphopyridine nucleotide (DPN)-linked conversion of polyols to ketoses, the possible oxidation of erythritol and mannitol by *D. plei* nerve fibers was explored. Erythritol and mannitol DPN-linked dehydrogenase activities were determined photometrically by measuring the increase in absorption due to the formation of DPNH, the reduced form of DPN. Sorbitol was tested also.

Six isolated nerve fibers were homogenized in 0.25 ml ice cold 0.02 M KCl with an all-glass Potter-Elvehjem microapparatus and centrifuged at $38000 \times g$ for 10 min. After homogenizing, the material was kept at 0° to 2°C until used. The clear supernatant, nerve fiber extract, was used directly for the assay. The standard reaction mixture (1.14 ml total volume) contained the following constituents: tris(hydroxymethyl)aminomethane (pH 8.0), 32.4 μ mole/ml; nerve fiber extract equivalent to 3 or 6 mg of tissue; DPN, 0.9 μ mole/ml; polyol (erythritol, mannitol, or sorbitol), 150 μ mole/ml. The first three components were added in the order listed and incubated at 22°C for 15 min. Then the polyol was added to initiate the reaction. Readings were obtained at 15 sec intervals for 15 min at 366 m μ with an Eppendorf photometer.

The squid nerve fiber extracts did not show erythritol, mannitol, or sorbitol dehydrogenase activities. Similar experiments with rat liver extracts showed that dehydrogenase activities could be detected by the method described above using liver samples one hundred times smaller by weight than the nerve fiber samples.

(b) To obtain direct evidence that the test molecules remain unaltered after passing the diffusion barriers of the resting and stimulated nerve fiber, the nature of the radioactive molecules obtained in the axoplasm was determined by paper radiochromatography.

Six nerve fibers, immersed in sea water baths containing ^{14}C -labeled erythritol (5.0 mmoles per liter; 2.26 mc per mmole), mannitol (10.0 mmoles per liter; 2.07 mc per mmole), or sucrose (10.0 mmoles per liter; 3.00 mc per mmole), were stimulated 50 times per sec for 30 min and their axoplasms collected in 1 ml of distilled water. The resulting solutions were placed in 1.5 cm diameter cellulose tubes and ultrafiltered by centrifugation at 2°C for 16 hr. The ultrafiltrates were spotted on Whatman No. 1 paper for chromatography. The solvent system consisted of *n*-butanol:acetic acid:water (4:1:5 v/v). The descending technique was employed. Simultaneously and under identical conditions samples of the radioactive compounds from the sea water baths were chromatographed also. The length of solvent flow was limited to 50 cm from the starting line. The radioactivity in the chromatograms was localized and measured with the aid of an automatic scanner (Vanguard, Low Background Auto-scanner, Model 880). The chromatograms of the axoplasm samples from each substance show only one radioactive spot with an R_f equal to that of the corresponding substance from the respective sea water bath. The R_f values obtained are: erythritol, 0.37; mannitol, 0.18; and sucrose, 0.12.

These experiments indicate that, under our experimental conditions, the ^{14}C radioactivity measured in the axoplasm of nerve fibers immersed in sea water baths containing ^{14}C -labeled erythritol, mannitol, or sucrose, is in the same ^{14}C -labeled molecule added to the respective sea water bath.

(c) CONTROL EXPERIMENTS AND REVERSIBILITY OF THE CHANGE IN PERMEABILITY CAUSED BY STIMULATION

Three types of experiments were carried out as controls and to explore the reversibility of the change in nonelectrolyte permeability caused by stimulation.

First, the penetration of erythritol was studied in pairs of axons kept at rest for 21 min in ^{14}C -erythritol sea water. Second, the penetration of the same substance was studied in pairs of axons kept for 21 min in ^{14}C -erythritol sea water and stimulated 25 times per sec for the last 20 min of immersion. Third, the penetration of erythritol was determined in pairs of axons, both axons of each pair first kept in nonradioactive sea water for 20 min, one at rest and the other stimulated at 50 per sec, and then transferred to ^{14}C -erythritol sea water in which both axons were kept at rest for 21 min.

Table III summarizes the results of the experiments described above. The ratio of the erythritol permeabilities of the paired resting axons is 1.0 ± 0.1 , that of the paired stimulated axons is 0.97 ± 0.08 , and that of the resting axons previously stimulated to that of the paired axons always kept at rest is 0.93 ± 0.07 . These ratios indicate that the resting permeability is the same in both axons of the pairs, that the increase in permeability caused by stimulation is the same in both axons of a pair, and that the change in permeability observed in the stimulated axons is reversible and not due to a permanent alteration of the axolemma structure.

II. *Effect of Different External Potassium Concentrations on Nonelectrolyte Penetration in Resting Axons*

Thirty-three pairs of nerve fibers were used to study the relation between the permeability of the axon to the penetration of erythritol and external potassium concentration.

In addition to normal sea water, four other solutions containing 3, 15, 20, and 30 times the normal sea water potassium concentration were used. The axon pairs were equilibrated for 10 min, one in normal sea water and the other in normal sea water or in one of the solutions with high potassium concentration. At the end of the equilibration time the fibers were transferred and kept at rest for 21 min in ^{14}C -erythritol sea water with the same potassium concentration as the corresponding nonradioactive bath.

TABLE III
 REVERSIBILITY OF THE CHANGE IN NONELECTROLYTE
 (ERYTHRITOL) PERMEABILITY DUE TO STIMULATION*
 Temperature 21° to 23°C

No. of axon pairs	Axon a			Axon b			Axon a/Axon b	
	Diameter	Experimental condition	Erythritol permeability	Diameter	Experimental condition	Erythritol permeability	Erythritol permeability ratio	Permeability ratio (paired data)
	μ		10^{-7} cm/sec	μ		10^{-7} cm/sec		
6	236 ± 7	Rest	3.3 ± 0.9	240 ± 8	Rest	3.4 ± 0.8		1.0 ± 0.1
6	238 ± 6	Stimulated at 25/sec	5.3 ± 0.8	239 ± 7	Stimulated at 25/sec	5.6 ± 0.8		0.97 ± 0.08
5	236 ± 6	Rest (after 20 min of stimulation at 50/sec)	2.6 ± 0.4	236 ± 6	Rest (after 20 min at rest)	2.8 ± 0.4		0.93 ± 0.07

* Values are mean \pm SEM.

Table IV shows the ratios of permeabilities of the resting axons in normal sea water and the solutions with 3, 15, 20, and 30 times the normal potassium concentration to those of their paired axons in normal sea water. These ratios are respectively 1.0 ± 0.1 ; 1.0 ± 0.2 ; 0.85 ± 0.18 ; 0.98 ± 0.20 ; and 0.91 ± 0.14 .

These results indicate that the permeability to the penetration of erythritol is independent of the external potassium concentration. An increase of 30 times in the external potassium concentration produces a displacement of the membrane potential to near zero in *D. plei* axons as observed also in other squid species (5, 12, 13). Thus, the mechanism of penetration of erythritol in the resting axon does not appear to be related, at least closely, to the electrical

TABLE IV
PERMEABILITY TO THE PENETRATION OF ERYTHRITOL OF
RESTING SQUID AXONS IMMERSSED IN ARTIFICIAL SEA WATER
WITH DIFFERENT POTASSIUM CONCENTRATIONS*
Temperature 21° to 23°C

No. of axon pairs	Axon <i>a</i> Rest			Axon <i>b</i> Rest			Axon <i>a</i> /Axon <i>b</i>
	Diameter	[K] _o	Erythritol permeability	Diameter	[K] _o	Erythritol permeability	Permeability ratio (paired data)
	μ	mM	10 ⁻⁷ cm/sec	μ	mM	10 ⁻⁷ cm/sec	
6	236±7	10	3.3±0.9	240±8	10	3.4±0.8	1.0±0.1
11	250±8	30	3.6±0.3	252±7	10	3.7±0.4	1.0±0.2
5	238±10	150	2.5±0.3	236±8	10	3.6±1.0	0.85±0.18
5	354±11	200	2.3±0.6	345±6	10	2.4±0.4	0.98±0.20
5	246±6	300	3.1±0.7	242±9	10	3.6±0.7	0.91±0.14

* Values are mean ± SEM. Potassium chloride replaced sodium chloride, mole per mole, in the artificial sea water.

potential difference across the axolemma. This conclusion seems to establish a difference between the ionic and nonelectrolyte permeabilities of resting axons, since the dependence of the ionic permeabilities on the membrane potential is well known (14, 15). An alternative explanation would be that the effect of the electrical potential, if any, is canceled by the changes in ionic fluxes observed in media with high potassium concentrations (13).

III. *Effect of Different External Sodium Concentrations on Nonelectrolyte Penetration and Sodium Movements in Resting and Stimulated Axons*

(a) EXPERIMENTS ON NONELECTROLYTE PENETRATION

The experiments to be described were performed to investigate whether the penetration of a nonelectrolyte in resting and stimulated axons could be related to the external sodium concentration.

Penetration in Resting Axons Fifteen pairs of nerve fibers were used to study the relation between the permeability of resting axons to the penetration of erythritol and external sodium concentration.

In addition to normal sea water containing 445 mmoles of sodium per liter, media in which part of the sodium concentration was replaced by choline were used. The axon pairs were equilibrated for 10 min, one in normal sea water and the other in normal sea water or in one of the media with diminished sodium concentration. At the end of the equilibration time the fibers were transferred and kept at rest for 21 min in ^{14}C -erythritol sea water with the same sodium concentration as in the corresponding non-radioactive bath.

TABLE V
PERMEABILITY TO THE PENETRATION OF ERYTHRITOL OF RESTING AND STIMULATED (25/SEC) SQUID AXONS* IMMERSSED IN ARTIFICIAL SEA WATER WITH DIFFERENT SODIUM, CHOLINE (Ch), AND LITHIUM CONCENTRATIONS
Temperature 21° to 23°C

No. of axon pairs	Axon a Rest				Axon b Rest				Axon a/Axon b Permeability ratio (paired data)	
	Diameter	Na _o	Ch _o	Li _o	Erythritol permeability	Diameter	Na _o	Ch _o		Erythritol permeability
	μ	mM			10^{-7} cm/sec	μ	mM		10^{-7} cm/sec	
6	236±7	445	0	0	3.2±0.8	240±8	445	0	3.3±0.9	1.0±0.1
4	352±6	223	223	0	2.2±0.4	320±5	445	0	2.0±0.2	1.1±0.1
5	311±8	147	298	0	2.2±0.3	316±8	445	0	2.2±0.2	0.98±0.05
	Axon a Stimulated 25/sec				Axon b Stimulated 25/sec				Axon a/Axon b	
	Diameter	Na _o	Ch _o	Li _o	Erythritol permeability	Diameter	Na _o	Ch _o		Erythritol permeability
	μ	mM			10^{-7} cm/sec	μ	mM		10^{-7} cm/sec	
6	238±6	445	0	0	5.3±0.8	239±7	445	0	5.6±0.8	0.97±0.08
5	239±7	223	223	0	3.9±0.6	242±8	445	0	5.3±0.4	0.74±0.11
7	277±17	147	298	0	3.1±0.5	275±14	445	0	5.3±0.9	0.65±0.08
6	344±13	147	0	298	2.0±0.2	350±18	445	0	2.8±0.5	0.72±0.06
7	383±6	147	0	298	2.5±0.3	382±7	147	298	2.5±0.1	1.1±0.1

* Values are mean ± SEM.

Table V shows the ratios of the permeabilities of resting axons in normal sea water, (0.50 Na:0.50 Ch)-, and (0.33 Na:0.66 Ch)-sea water to those of their paired axons in normal sea water. The values of these ratios are respectively: 1.0 ± 0.1 ; 1.1 ± 0.1 ; and 0.98 ± 0.05 .

The results of these experiments do not show that the permeability of the resting axon to the penetration of erythritol depends on the external sodium concentration.

Penetration in Stimulated Axons Thirty-one pairs of nerve fibers were used to study the relation between the permeability of stimulated fibers to the penetration of erythritol and external sodium concentration.

Normal sea water and media in which part of the normal sodium concentration was replaced by choline or lithium were used. The axon pairs were equilibrated for 10 min, one in normal sea water and the other in normal sea water or in one of the media with diminished sodium concentration. At the end of the equilibration time the fibers were transferred for 21 min to ^{14}C -erythritol sea water with the same sodium concentration as the corresponding nonradioactive bath, where they were stimulated 25 times per sec for the last 20 min of immersion. The penetration of erythritol was also studied in pairs of stimulated axons, one axon of each pair in (0.33 Na:0.66 Li)-sea water and the other in (0.33 Na:0.66 Ch)-sea water.

Table V shows the ratios of the permeabilities to the penetration of erythritol of the stimulated axons immersed in normal sea water, (0.50 Na:0.50 Ch)-, (0.33 Na:0.66 Ch)-, and (0.33 Na:0.66 Li)-sea water to those of their paired axons stimulated in normal sea water. The values of these ratios are respectively 0.97 ± 0.08 ; 0.74 ± 0.11 ; 0.65 ± 0.08 ; and 0.72 ± 0.06 . In agreement with the latter two ratios it was found that the ratio of the permeability of stimulated axons in (0.33 Na:0.66 Li)-sea water to that of the paired axons in (0.33 Na:0.66 Ch)-sea water is 1.1 ± 0.1 .

The results of these experiments indicate that the penetration of the non-electrolyte during activity decreases when the external sodium concentration is diminished.

We have investigated in this squid species and under our experimental conditions the effects of the sodium-deficient solutions on the resting and action potentials of the axon. It has been confirmed that the replacement of external sodium by choline reduces the action potential without affecting the resting potential, the size of the action potential being a function of the sodium concentration in the extracellular fluid (5). It has been found also that the axons maintained resting and action potentials of normal size during the experimental period in the (0.33 Na:0.66 Li)-sea water (5).

(b) EXPERIMENTS ON THE SODIUM MOVEMENTS

To determine whether the permeability to the penetration of erythritol could be related to sodium movements, measurements of the sodium fluxes in resting and stimulated axons were carried out in the same media used for the nonelectrolyte penetration experiments.

Sodium Influx in Resting Axons Nineteen pairs of nerve fibers were used to study the relation between sodium influx in resting axons and external sodium concentration.

The axon pairs were equilibrated for 10 min, one in normal sea water and the other in normal sea water or in one of the media with diminished sodium concentration. At the end of the period of equilibration the fibers were transferred and kept at rest for 21 min in ^{22}Na -labeled sea water with the same sodium concentration as the nonradioactive bath.

Table VI shows the influx values and the ratios of the influxes in normal sea water, (0.50 Na:0.50 Ch)- and (0.33 Na:0.66 Ch)-sea water to those of their paired axons in normal sea water. These ratios are respectively 0.98 ± 0.13 ; 0.45 ± 0.06 ; and 0.39 ± 0.04 .

These ratios indicate that the influx of sodium in the resting axons decreases when the external sodium concentration is diminished. Resting influx values are given in Appendix.

Sodium Influx in Stimulated Axons Nineteen pairs of nerve fibers were used to investigate the relation between sodium influx in stimulated axons and external sodium concentration.

TABLE VI
SODIUM INFLUX IN RESTING AND STIMULATED (25/SEC) SQUID AXONS* IMMERSED IN ARTIFICIAL SEA WATER WITH DIFFERENT SODIUM, CHOLINE (Ch), AND LITHIUM CONCENTRATIONS
Temperature 21° to 23°C

No. of axon pairs	Axon a Rest					Axon b Rest			Axon a/Axon b
	Diameter	Na _o	Ch _o	Li _o	Na influx	Diameter	Na _o	Na influx	Na influx ratio (paired data)
	μ	mM			pmole/cm ² sec	μ	mM	pmole/cm ² sec	
5	324±10	445	0	0	141±18	326±10	445	149±24	0.98±0.13
6	345±17	223	223	0	57±7	348±16	445	147±35	0.45±0.06
8	316±8	147	298	0	56±6	315±8	445	147±14	0.39±0.04
Axon a Stimulated 25/sec									
Axon b Stimulated 25/sec									
Axon a/Axon b									
5	302±7	445	0	0	277±20	294±7	445	297±40	0.96±0.07
4	256±6	223	223	0	163±19	255±9	445	257±35	0.66±0.10
5	260±5	147	298	0	124±15	261±3	445	306±15	0.40±.030
5	316±15	147	0	298	100±18	319±13	445	285±51	0.42±0.12

* Values are mean ± SEM. pmole = 10⁻¹² mole.

After isolation the axon pairs were equilibrated for 10 min, one in normal sea water and the other in normal sea water or in one of the media with diminished sodium concentration. After equilibration the fibers were transferred for 21 min to ²²Na-labeled sea water with the same sodium concentration as the corresponding nonradioactive bath in which they were stimulated 25 times per sec during the last 20 min of immersion.

Table VI shows the influx values and the ratios of the influxes in normal sea water, (0.50 Na:0.50 Ch)-, (0.33 Na:0.66 Ch)-, and (0.33 Na:0.66 Li)-sea water to those of their paired axons in normal sea water. The values are respectively 0.96 ± 0.07 ; 0.66 ± 0.10 ; 0.40 ± 0.03 ; and 0.42 ± 0.12 .

These ratios indicate that the influx of sodium in the stimulated axons de-

creases when the external sodium concentration is diminished. Influx values during activity in $\text{pmole/cm}^2 \cdot \text{impulse}$ are given in Appendix.

Sodium Efflux in Resting and Stimulated Axons Thirteen pairs of nerve fibers were used to investigate the relation between efflux of sodium and external sodium concentration in resting and stimulated axons.

After labeling the axoplasm sodium with ^{22}Na , the efflux of sodium was determined as described under the Experimental Method, in normal sea water and in the media with low sodium concentration. Both axons of each pair were immersed, to determine the efflux, in the same nonradioactive medium in two separate Lucite holders for 15 min, one at rest and the other stimulated 25 times per sec.

TABLE VII
SODIUM EFFLUX IN RESTING AND STIMULATED (25 STIMULI/SEC)
SQUID AXONS* IMMERSed IN ARTIFICIAL SEA WATER WITH DIFFERENT
SODIUM AND CHOLINE (Ch) CONCENTRATIONS
Temperature 21° to 23°C

No. of axon pairs	Axon a Rest			Axon b Stimulated 25/sec			Axon a/Axon b		
	Diameter	Na _o	Ch _o	Na efflux	Diameter	Na _o	Ch _o	Na efflux	Net increase 25 stim./sec (paired data)
	μ	mM		$\text{pmole/cm}^2 \text{ sec}$	μ	mM		$\text{pmole/cm}^2 \text{ sec}$	$\text{pmole/cm}^2 \text{ sec}$
5	275±5	445	0	133±17	277±2	445	0	182±20	49±5
4	285±12	223	223	132±11	290±13	223	223	178±6	46±10
4	278±3	147	298	139±24	279±4	147	298	185±27	47±14

* Values are mean ± SEM.

The results shown in Table VII indicate that the sodium efflux from resting and stimulated axons appears to be unaffected by changes in external sodium concentration. Efflux values at rest and during activity are given in Appendix.

(c) ON NONELECTROLYTE PENETRATION AND SODIUM MOVEMENTS

The results of the experiments carried out in resting axons when the external sodium concentration is diminished show that the erythritol penetration and the sodium efflux remain unchanged and the sodium influx decreases.

The results of the experiments with stimulated axons described in the present section show that the increase in the entries of erythritol and sodium caused by stimulation depends on the external sodium concentration. This dependence may be due to a drag effect on the nonelectrolyte molecules produced by the sodium ions which enter the axon during activity.

The results of the experiments performed in stimulated axons in which lithium was used to replace two-thirds of the normal sodium concentration may be compared to those performed using the normal sodium concentration

The ratio of the sodium flux to sodium concentration in the stimulated axons is the same in both solutions, as is the size of the action potentials during activity. Therefore, the permeability to sodium is the same in both solutions. However, the penetration of erythritol into the stimulated axons immersed in the lithium solution is lower than that in normal sea water. These observations suggest that sodium permeability (not the sodium influx) and nonelectrolyte penetration during activity are independent. On the other hand, the present results are compatible with a drag effect of the sodium ions on the nonelectrolyte molecules during activity, since nonelectrolyte penetration and sodium influx in the stimulated axons decrease when the external sodium concentration is diminished. Lithium ions have less dragging effect than sodium ions, since nonelectrolyte penetration is lower when sodium is replaced by lithium, though it tends to be larger when sodium is replaced by lithium than when it is substituted by choline. This drag effect of sodium ions, if it exists, could be mediated by water movement.

An alternative explanation of the dependence of the erythritol and sodium entries on external sodium concentration in the stimulated axons is that sodium (and lithium less than sodium) may have simultaneously independent effects on the sodium influx and on the pathways used by the nonelectrolytes to enter the axon during activity.

It should be pointed out also that the increase in permeability to the penetration of nonelectrolytes caused by stimulation in medium sized axons, as already noticed in section I, diminishes when the size of the probing molecules increases, thus indicating a finite size for the pathways through the axolemma. To be able to discriminate among erythritol, mannitol, sucrose, and the different ionic species, the size of the pathways during activity should not be very much greater than the sucrose diameter, as it is in the case of the resting axolemma (2). Therefore, the increase in penetration during activity should be due more probably to an increase in the number of the pathways in the axolemma than to their change in size.

Further experiments are underway to determine whether the increase in nonelectrolyte permeability caused by stimulation, found by us in medium sized axons of *D. plei*, a phenomenon not observed by Hodgkin and Martin in axons of *L. pealii* with diameters two to three times as large (see Section I of the present paper), is a species peculiarity or is due to structural or physiological characteristics of the nerve fiber related to the axon diameter.

APPENDIX I

On the Sodium Fluxes in Doryteuthis plei Axons Immersed in Normal Sea Water and in Solutions with Different Sodium Concentrations

The sodium fluxes of the resting axons, in pmole/cm². sec, are: 148 ± 44 for the influx and 135 ± 31 for the efflux in normal sea water; 57 ± 7 for the influx and 132

± 11 for the efflux in (0.50 Na: 0.50 Ch)-sea water; and 56 ± 6 for the influx and 139 ± 24 for the efflux in (0.33 \pm 0.66 Ch)-sea water.

(The values in normal sea water are the mean \pm SEM of axons *b* in Table VI for influx and of axons *a* in Table VII for the efflux. The values for influx and efflux in the media with low sodium concentration correspond to axons *a* in Tables VI and VII, respectively.)

The influx and efflux values in normal sea water are approximately equal, thus indicating that these axons appear to be in steady state. The influx is lower than the efflux in the axons immersed in the solutions with low sodium concentrations. The observed magnitude of the fluxes of *D. plei* axons in normal sea water is greater than that of the axons of *S. officinalis* and *L. forbesi* determined by Keynes (7, 10) and *L. pealii* determined by Brinley and Mullins (9) and Shanes and Berman (8). It may be due to species differences. The temperature of the Venezuelan coastal water in which the *D. plei* live is 25° to 28°C.

The measurements of the sodium fluxes in the media with different sodium concentrations show that only the resting influx is affected by the changes in the external concentration of this ion. Keynes (7) has reported similar findings. This observation supports the proposed independence of the influx and efflux mechanisms in the resting axons.

The sodium fluxes during activity in pmole/cm². impulse are: 5.4 ± 1.1 for the influx and 2.0 ± 1.0 for the efflux in normal sea water; 4.2 ± 0.8 for the influx and 1.8 ± 0.5 for the efflux in (0.50 Na:0.50 Ch)-sea water; and 2.7 ± 0.6 for the influx and 1.8 ± 1.4 for the efflux in (0.33 Na:0.66 Ch)-sea water.

(The influx per impulse was calculated subtracting the value in Table VI for axons *a* stimulated 25 times per sec from that in the same table for the resting axons *a* and then dividing the net increase in influx by 25, the frequency of stimulation. The efflux per impulse was calculated similarly from the value in Table VII for the stimulated axons *b* and that of the paired resting axons *a* in the same table.)

The net sodium entries during activity in pmole/cm². impulse are: 3.4 in normal sea water; 2.4 in (0.50 Na:0.50 Ch)-sea water; and 0.8 in (0.33 Na:0.66 Ch)-sea water. It should be noticed that the net sodium entry in the *D. plei* immersed in normal sea water is equal to that determined by Keynes and Lewis (16) in *S. officinalis* (3.6 pmole/cm². impulse) and *L. forbesi* (3.5). It is interesting to point out also that the net entries in all the media are more than enough to account for the sizes of the action potentials in the different sodium concentrations.

The results of the sodium influx and efflux measurements in the media with different sodium concentrations indicate that only the influx is affected by changes in the external concentrations of this ion. This observation indicates the independence of the influx and efflux mechanisms during activity.

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REFERENCES

1. VILLEGAS, R., VILLEGAS, L., GIMÉNEZ, M., and VILLEGAS, G. M., Schwann cell and axon electrical potential differences: squid nerve structure and excitable membrane location, *J. Gen. Physiol.*, 1963, **46**, 1047.
2. VILLEGAS, R., and BARNOLA, F. V., Equivalent pore radius in the axolemma of the giant axon of the squid, *Nature*, 1960, **188**, 762.
3. VILLEGAS, R., and BARNOLA, F. V. Characterization of the resting axolemma in the giant axon of the squid, *J. Gen. Physiol.*, 1961, **44**, 963.
4. VILLEGAS, R., BLEI, M., and VILLEGAS, G. M., Penetration of non-electrolyte molecules in resting and stimulated squid nerve fibers, *J. Gen. Physiol.*, 1965, **48**, No. 5, pt. 2, 35.
5. HODGKIN, A. L., and KATZ, B., The effect of sodium ions on the electrical activity of the giant axon of the squid, *J. Physiol.*, 1949, **108**, 37.
6. VILLEGAS, R., and VILLEGAS, G. M., Characterization of the membranes in the giant nerve fiber of the squid, *J. Gen. Physiol.*, 1960, **43**, No. 5, pt. 2, 73.
7. KEYNES, R. D., The ionic movements during nervous activity, *J. Physiol.*, 1951, **114**, 119.
8. SHANES, A. M., and BERMAN, M. D., Kinetics of ion movement in the squid giant axon, *J. Gen. Physiol.*, 1955, **39**, 279.
9. BRINLEY, F. J., and MULLINS, L. J., Ion fluxes and transference numbers in squid axons, *J. Neurophysiol.*, 1965, **28**, 526.
10. KEYNES, R. D., cited by Brinley, F. J., and Mullins, L. J., reference 9, Table 7.
11. VILLEGAS, R., CAPUTO, C., and VILLEGAS, L., Diffusion barriers in the squid nerve fiber. The axolemma and the Schwann layer, *J. Gen. Physiol.*, 1962, **46**, 245.
12. CURTIS H. J., and COLE, K. S., Membrane resting and action potentials from the squid giant axon, *J. Cell. and Comp. Physiol.*, 1942, **19**, 135.
13. HODGKIN, A. L., and KEYNES, R. D., The potassium permeability of a giant nerve fibre, *J. Physiol.*, 1955, **128**, 61.
14. HODGKIN, A. L., HUXLEY, A. F., and KATZ, B., Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*, *J. Physiol.*, 1952, **116**, 424.
15. HODGKIN, A. L., and HUXLEY, A. F., Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*, *J. Physiol.*, 1952, **116**, 449. The components of membrane conductance in the giant axon of *Loligo*, *J. Physiol.*, 1952, **116**, 473. The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*, *J. Physiol.*, 1952, **116**, 497. A quantitative description of membrane current and its application to conduction and excitation in nerve, *J. Physiol.*, 1952, **117**, 500.
16. KEYNES, R. D., and LEWIS, P. R., The sodium and potassium content of cephalopod nerve fibres, *J. Physiol.*, 1951, **114**, 151.