

# Penetration of Non-Electrolyte Molecules in Resting and Stimulated Squid Nerve Fibers

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The experiments to be described deal with the penetration of  $C^{14}$ -labeled erythritol, mannitol, and sucrose in resting and stimulated axons of the squid *Doryteuthis plei*. The results indicate an increase in the penetration of the non-electrolytes in the stimulated axons. This increase may be due either (a) to a change in the structure of the axolemma or (b) to a drag effect of the sodium ions entering the axon during the nerve impulse, since sodium entry is larger during stimulation. Experiments on the effects of high potassium and low sodium concentrations in the extracellular fluid on the penetration of  $C^{14}$ -erythritol also are described. Some recent sodium entry measurements are reported (1).

## *Diffusion Barriers at the Nerve Fiber Surface*

As shown in Fig. 1, the main barriers for diffusion at the surface of a well-cleaned nerve fiber of the squid *D. plei* are considered as being formed by the axolemma, a membrane 80 Å thick perforated by pores with an effective radius of 4 to 4.5 Å in the resting fiber, and the Schwann cell layer crossed by 60 Å wide-slit channels with a length of 4 to 5  $\mu$ , connecting the extracellular space with the 80 Å wide axolemma-Schwann cell space (2, 3). It has been calculated for the resting fiber that the pores occupy  $7.6 \times 10^{-5}$  cm<sup>2</sup> per cm<sup>2</sup> of axolemma, and the slit channels  $4.1 \times 10^{-3}$  cm<sup>2</sup> per cm<sup>2</sup> of Schwann cell layer surface (4).

The restriction offered by each barrier to diffusion depends critically on the ratio of the area for diffusion to the length of the pathways crossing the barrier and on the size of the diffusing molecule. Thus, the restriction to the diffusion of the molecules offered by the channels crossing the Schwann cell layer is mainly due to their length, and that offered by the pores in the axolemma of the resting fiber, to their small radius (4). The significance of

the different layers around the axon as diffusion barriers for non-electrolyte molecules is expected to be quantitatively different in other nerve fibers; *i.e.*, with a thicker Schwann cell layer or basement membrane or with a thicker layer of endoneurium left after isolation from the animal (5).

In the resting nerve fiber of *D. plei*, as utilized in the present work, the most significant diffusion barrier for molecules with radii greater than 3 Å is the axolemma (4). This allowed us to investigate a possible change produced by stimulation in the permeability of the axolemma to the penetration of some non-electrolytes. The radii of erythritol, mannitol, and sucrose, the test molecules used, are 3.1, 4.0, and 4.5 Å, respectively.

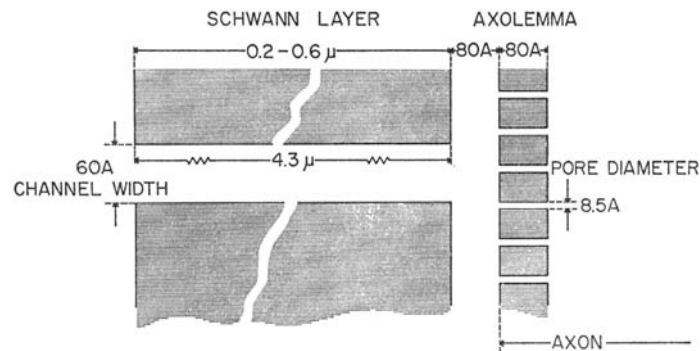


FIGURE 1. Arrangement of the barriers, Schwann layer, and axolemma, between the extracellular fluid and the axoplasm. A labeled molecule in the extracellular fluid has to pass the channels in the Schwann layer and the pores in the axolemma to reach the axoplasm. The dimensions in the diagram are those obtained from the electron microscopy observations (2), the water diffusion and filtration permeability studies (2), and the measurements of the sieving properties of the axolemma to non-electrolytes (3). Reproduced from *J. Gen. Physiol.* (4).

#### *Permeability of Resting and Stimulated Axons to the Penetration of Non-Electrolytes*

A total of 32 pairs of well cleaned giant nerve fibers isolated from the squid *D. plei* were equilibrated for 5 minutes in artificial sea water (6), and then immersed during 21 minutes in artificial sea water to which  $C^{14}$ -labeled, erythritol, mannitol, or sucrose had been added. Concentrations of the  $C^{14}$ -labeled solutes were about 5 millimols per liter. One fiber of each pair was stimulated at a frequency of 25 stimuli per second during 20 minutes. At the end of the 21 minute period in the radioactive sea water, both the resting and the stimulated fibers were taken out, soaked for about 15 seconds in non-radioactive sea water, blotted carefully with filter paper, and their axoplasms extruded and weighed. The concentrations of  $C^{14}$ -labeled solute in the axoplasms and the radioactive sea water baths were determined by liquid scintillation counting (4).

Table I shows the results expressed as the amount of  $C^{14}$ -labeled substance that reached the axoplasm per unit of concentration in the radioactive sea water bath, per  $cm^2$  of axolemma, per second. These results indicate that the penetration of the three test molecules used is significantly larger in the stimulated than in the resting axons. The ratios of the permeabilities of the axons stimulated at a frequency of 25 stimuli per second to those of the paired resting axons are:  $1.8 \pm 0.2$  (mean  $\pm$  SE) for erythritol,  $2.0 \pm 0.2$  for mannitol and  $2.2 \pm 0.3$  for sucrose. In seven pairs of nerve fibers stimulated at 50 per second, the ratio of the permeability to the penetration of erythritol of the stimulated axons to that of the paired resting axons is  $2.4 \pm 0.4$ . The increase

TABLE I  
PENETRATION OF  $C^{14}$ -ERYTHRITOL, MANNITOL, AND SUCROSE  
IN RESTING AND STIMULATED SQUID AXONS

Molecule	No. of nerve fiber pairs	Permeability in $10^{-7}$ cm/sec.*				P of net increase†	Calculated permeability during activity‡
		Resting axon Axon a	Stimulated at 25/sec. Axon b	Net increase 25 stim./sec. (paired data) (b - a)			
Erythritol	10	$3.6 \pm 0.4$	$6.1 \pm 1.0$	$2.5 \pm 0.8$	$<0.02$	110	
Mannitol	10	$2.3 \pm 0.4$	$4.0 \pm 0.5$	$1.7 \pm 0.3$	$<0.001$	75	
Sucrose	12	$0.9 \pm 0.1$	$1.8 \pm 0.3$	$0.9 \pm 0.3$	$<0.02$	40	

\* The values are mean  $\pm$  standard error.

† Statistical significance calculated according to Student's method for correlated samples.

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

in permeability to the penetration of non-electrolytes caused by stimulation is more evident if it is taken into account that the nerve fibers were stimulated at frequencies of 25 or 50 stimuli per second and it is assumed that the permeability change of the axolemma occurred only during 1 millisecond per stimulus (Table I).

#### *Control Experiments*

The penetration of  $C^{14}$ -erythritol was studied in six pairs of nerve fibers, both fibers of each pair at rest. The ratio of the permeabilities of paired axons is  $1.0 \pm 0.1$  (mean  $\pm$  SE).

The penetration of  $C^{14}$ -erythritol was determined in six pairs of nerve fibers, both fibers of each pair stimulated at a frequency of 25 stimuli per second. As expected, the ratio of the permeabilities of paired stimulated axons is  $0.97 \pm 0.08$ , thus indicating that the increase in penetration caused by stimulation is the same in both fibers of each pair.

*Reversibility of the Change in Permeability Caused by Stimulation*

A group of five nerve fiber pairs was utilized to explore whether aftereffects of stimulation on the non-electrolyte permeability could be observed. Both fibers of each pair were first kept in non-radioactive sea water during 20 minutes, one at rest and the other stimulated at 50 per second, and then transferred to  $C^{14}$ -erythritol sea water where both fibers were kept at rest during 21 minutes. The ratio of the permeability of the resting axons previously stimulated to that of the paired axons always kept at rest is  $0.93 \pm 0.07$ . Thus, the change in permeability observed in the stimulated axons is reversible and not due to a permanent alteration of the structure.

TABLE II  
PENETRATION OF  $C^{14}$ -ERYTHRITOL IN  
RESTING SQUID AXONS IMMERSSED IN MEDIA WITH  
DIFFERENT POTASSIUM CONCENTRATIONS

No. of nerve fiber pairs	Potassium concentration*		Permeability in $10^{-7}$ cm/sec†		Axon a/Axon b (paired data)
	Axon a	Axon b	Axon a	Axon b	
6	1.0	1.0	$3.3 \pm 0.9$	$3.4 \pm 0.8$	$1.0 \pm 0.1$
11	3.0	1.0	$3.6 \pm 0.3$	$3.7 \pm 0.4$	$1.0 \pm 0.2$
5	15.0	1.0	$2.5 \pm 0.3$	$3.6 \pm 1.0$	$0.85 \pm 0.18$
5	30.0	1.0	$3.1 \pm 0.7$	$3.6 \pm 0.7$	$0.91 \pm 0.14$

\* Potassium concentrations in the media are expressed as the ratio of the concentration in the bath to that in artificial sea water (1.0 = 10 millimols per liter). Potassium chloride replaced sodium chloride.

† Values are mean  $\pm$  standard error.

*External Potassium Concentration and Penetration of a Non-Electrolyte in Resting Axons*

A total of 27 pairs of nerve fibers were used to study the relation between external potassium concentration and the permeability of the resting axon to  $C^{14}$ -erythritol penetration. In addition to the normal artificial sea water containing 10 millimols of potassium per liter, three other media containing 3, 15, and 30 times the normal sea water potassium concentration were used. The fibers of each pair were equilibrated for 10 minutes, one in normal sea water and the other in normal sea water or in one of the media with high potassium concentration. At the end of the equilibration time the fibers were transferred and kept during 20 minutes in  $C^{14}$ -erythritol sea water with the same potassium concentration as that of the corresponding non-radioactive bath.

As shown in Table II the permeability to the non-electrolyte of the axons in media with a high potassium concentration tends to decrease. However, these values are not statistically different from those of the paired axons in

normal potassium concentration. Tasaki and Spyropoulos (7) also have found no clear effect of high potassium concentration in the extracellular fluid on the efflux of thiourea, urea, sucrose, and starch from the squid axon.

*External Sodium Concentration and Penetration of a Non-Electrolyte in Stimulated Axons*

A total of 15 pairs of nerve fibers were used to study the relation between external sodium concentration and the permeability of stimulated fibers to the penetration of C<sup>14</sup>-erythritol. In addition to normal artificial sea water containing 444.5 millimols of sodium per liter, two other media in which the sodium concentration was reduced to one-half and one-third were used.

TABLE III  
PENETRATION OF C<sup>14</sup>-ERYTHRITOL IN SQUID  
AXONS STIMULATED AT 25/SEC. IN MEDIA WITH  
DIFFERENT SODIUM CONCENTRATIONS

No. of nerve fiber pairs	Sodium concentration*		Permeability in 10 <sup>-7</sup> cm/sec. ‡		Axon a/Axon b (paired data)
	Axon a	Axon b	Axon a	Axon b	
6	1.0	1.0	5.3±0.8	5.6±0.9	0.97±0.08
5	0.50	1.0	3.9±0.6	5.3±0.4	0.74±0.11
4	0.33	1.0	3.7±0.7	6.7±1.0	0.57±0.10

\* Sodium concentration expressed as fraction of normal concentration in artificial sea water (1.0 = 444.5 millimols per liter). Choline chloride was used to replace sodium chloride.

‡ Values are mean ± standard error.

Choline chloride was used to replace sodium chloride. The fibers of each pair were equilibrated during 10 minutes, 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 during 21 minutes in C<sup>14</sup>-erythritol sea water with the same sodium concentration as the corresponding non-radioactive bath. Both fibers of the pairs were stimulated at a frequency of 25 stimuli per second for the last 20 minutes of immersion in the radioactive bath.

As shown in Table III, the penetration of the non-electrolyte in the stimulated axons in the low sodium media is smaller than that in the paired stimulated axons in sea water with normal sodium concentration. The ratios of the permeability of the axons immersed in the media containing one-half and one-third the normal sodium concentration to that of the paired axons in sea water with its normal sodium concentration, are: 0.74 ± 0.11 (mean ± SE) and 0.57 ± 0.10, respectively. Dr. Francisco C. Herrera and the present authors (1) have found that the magnitude of the sodium influx also depends on the sodium concentration in the extracellular fluid. The influx of sodium was studied in 4 pairs of nerve fibers, one fiber of each pair in sea water con-

taining one-third of the normal sodium concentration and the other in sea water with its normal sodium concentration. Both fibers of each pair, as for those of the non-electrolyte experiments, were stimulated at a frequency of 25 stimuli per second. The ratio of the sodium influx of the axons in low sodium to that of the paired axons in normal sodium is  $0.35 \pm 0.06$  (mean  $\pm$  SE). The decrease of the sodium entry in low sodium medium might represent a decrease of the sodium influx both at rest and during stimulation.

The results of the present work suggest that sodium and non-electrolyte penetration are related. It is possible that some of the sodium ions and non-electrolyte molecules share a common pathway to cross the axolemma. One rather attractive possibility is that, at least part of the downhill sodium and non-electrolyte movements, occur through the pores that were proposed by Mullins (8, 9) and by us (2) to explain the ionic selectivity of the axonal membrane. These pores were located later by us in the axolemma when studying the non-electrolyte permeability of the nerve fiber (3, 4).

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#### REFERENCES

1. VILLEGAS, R., BLEI, M., VILLEGAS, G. M., and HERRERA, F. C., unpublished.
2. VILLEGAS, R., and VILLEGAS, G. M., *J. Gen. Physiol.*, 1960, **43**, No. 5, pt. 2, 73.
3. VILLEGAS, R., and BARNOLA, F. V., *J. Gen. Physiol.*, 1961, **44**, 963.
4. VILLEGAS, R., CAPUTO, C., and VILLEGAS, L., *J. Gen. Physiol.*, 1962, **46**, 245.
5. VILLEGAS, G. M., and VILLEGAS, R., *Biochim. et Biophysica Acta*, 1964, **88**, 231.
6. HODGKIN, A. L., and KATZ, B., *J. Physiol.*, 1949, **108**, 37.
7. TASAKI, I., and SPYROPOULOS, C. S., *Am. J. Physiol.*, 1961, **201**, 413.
8. MULLINS, L. J., in *Molecular Structure and Functional Activity of Nerve Cells*, (R. G. Grenell and L. J. Mullins, editors), Washington, D.C., American Institute of Biological Sciences, Symp. No. 1, 1956, 123.
9. MULLINS, L. J., *J. Gen. Physiol.*, 1960, **43**, No. 5, suppl., 105.