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Water Fluxes in Nerve Fiber*

C.S. Spyropoulos

Department of Biophysics and Theoretical Biology, The University of Chicago, Chicago, Illinois 60637*

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Summary. The hydrostatic (L_p) and osmotic (L_{PD}) filtration coefficients and the efflux rates of tritiated water were measured in the giant axon of *Loligo vulgaris*. The L_p was 8 to 14×10^{-8} cm/sec/cm H_2O and the L_{PD} was two orders of magnitude smaller (3 to 6×10^{-10} cm/sec/cm H_2O). In axons whose diameter was $\sim 500\,\mu$, the time (t 1/2) required for a reduction in the axonal labeled water activity to one half its initial value was 38 to 48 sec. The rate limiting structure for solute flux was made ineffective by (1) storing the axon in isosmotic KF at 0–2 °C for one month to one year or by (2) fixing the axon in 2–4% glutaraldehyde for 3 to 7 hr. The criteria of ineffectiveness of the rate limiting structure for solute flux were (1) a reduction of L_{PD} to immeasurably low values, (2) the absence of electrical properties characteristic of a plasmalemma, and (3) a marked increase in the rate of efflux of Na_{22}. In such impaired axons the L_p and the t 1/2 of tritiated water efflux were unaffected. This independence of solute and solvent flux in conjunction with the finding that the hydraulic conductivity determined by bulk osmotic and hydrostatic pressure gradients is not equivalent (i.e., $L_{PD}/L_P \ll 1$) indicate that the rate limiting structures for solute and solvent flux are in series. Solvent fluxes appear to be surface-limited, not bulk-limited. We have been unable to resolve whether the surface structure involved in limiting solvent flux is the sheath (Schwann layer and adhering connective tissue) and/or the cortical layer of the axoplasmic gel.

It is generally thought that the diffusional and pressure-driven flow of solute and solvent is limited in the surface of the cell. Dick [4], Ling [6] and Løvthrup [7] have taken exception and suggested that the rate limiting structure for solvent flux may be the bulk of the cytoplasm.

In this paper we will present experiments on the giant axon of *Loligo vulgaris* that indicate that, although both solute and solvent fluxes are surface-limited, the respective rate-limiting structures are separate and in series. Our flux measurements were prompted by an attempt to ascertain the role of the osmotic swelling pressure of the axoplasmic gel. These experiments are presented in the adjoining paper [11].

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* The early experiments were undertaken in the Stazione Zoologica, Naples, and the Democritus Nuclear Research Center, Athens, Greece; the more recent experiments were undertaken in facilities provided by Prof. N. Nikolaou, Aegina, Greece.

Materials and Methods

The giant axon of *Loligo vulgaris* was used. The methods of isolating, cleaning, internally perfusing the whole axon, and of measuring the electrical properties of the axolemma were modifications of the ones used previously by the author (e.g., [10]).

Measurement of Hydrostatic Filtration Coefficient

The method is illustrated in Fig. 1*a*. The perfusion tube was a syringe needle whose wall was thinned and perforated (for 3 cm) by electrolytic etching. Two tubes were used. The diameter of one was 373–394 μ and that of the other 303–316 μ. A photograph of one of the tubes is seen in Fig. 2*h* of the adjoining paper [11]. The porosity was ~75%. The resolution of Fig. 2*h* is insufficient to show this clearly. The tube was insulated in the nonperforated regions so that it may also function as a current electrode. The internal stirrer was a plastic coated iron ball that was moved back and forth along the lumen of the perfusion tube with

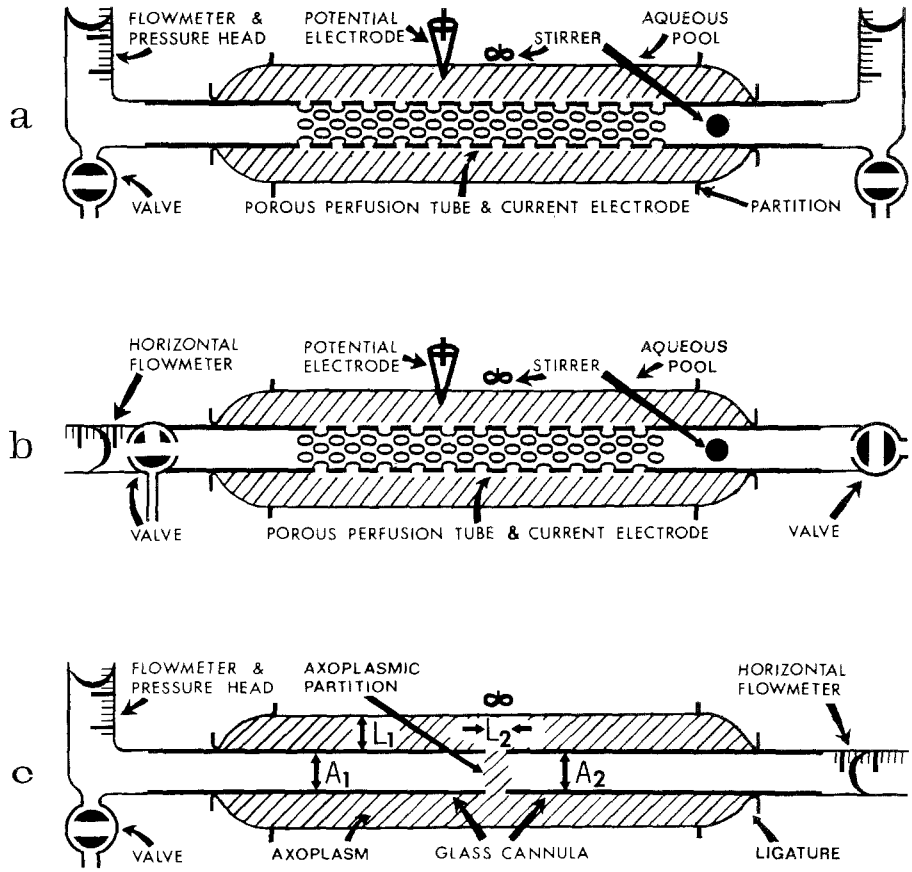

Fig. 1. (*a*) Method of measurement of radial hydrostatic filtration coefficient of axon. (*b*) Method of measurement of osmotic filtration coefficient of axon. (*c*) Method of measurement of longitudinal filtration coefficient of axoplasm

an external magnet attached to a pendular device. Internal and external stirring in conjunction with frequent intermittent fluid renewal assured that concentrations did not change by ultrafiltration. The external stirrer was a massage vibrator. Since the ball was seen to vibrate in the internal compartment, the external vibrator stirred the interior as well by slightly vibrating the entire axon. The internal solution was isotonic KF and the external sea water. The diameter of the axon was monitored as the pressure was increased and we allowed for this slight increase in surface area at higher pressures when the filtration coefficient was calculated. The excitability of the axon was checked at the beginning and periodically throughout each experiment. The ligatures that were applied around the axon and the perfusion tube were at least 1.5 cm from the edges of the perforated region of the perfusion tube.

Since the measurement of the hydrostatic filtration coefficient turned out to be crucial, a concerted effort to control sources of artifact was warranted. Since water fluxes in the axon appeared to be surface-limited, in order that the cut or ligated ends do not act as leaks, the axoplasmic tube (more than 1.5 cm long) interposed between the edge of the perfusion tube and the damaged and potentially leaky ligated region of the axon must offer some hydraulic resistivity in order to act as a sealant. This appears to be so from measurements of the Ks, the water permeability of axoplasm (*cf. Results*). Leaks are also possible in otherwise damaged regions of the axon (e.g., cut branches). Finally it is conceivable that a high internal hydrostatic pressure may make the membrane leaky (i.e., increase the porosity). We controlled for the presence of these various leaks in *normal* axons as follows:

The fluid bathing the axon was divided into three compartments. The width of the middle pool was 3.5 cm. The partitions were made of prophylactic rubber. The axon was threaded through holes in the rubber. The diameter of the holes was slightly smaller than the diameter of the axon. This resulted in a snug fitting and a minimization of leakage between the pools. The holes in the rubber were punched or drilled. Drilling of the holes was made feasible by solidifying the rubber with dry ice or running liquid nitrogen. The perforated region of the tube was centered in the middle pool. Chlorophenol red (0.05 %–0.1 %) tritiated water (THO) or $Na_{22}Cl$ were introduced into the lumen of the perfusion tube in the region of the perforations. Chlorophenol red with and without applied pressure (40 cm of H_2O) stained the axoplasm preferentially that is in the region of the perforations. With applied pressure there was no streaking toward the ligated region between the axoplasmic gel and the tube. The outside fluid was then replaced with a medium containing 1 % bovine fibrinogen. After clotting was induced by spiking with thrombin, 40 cm of H_2O pressure was applied to the interior of the axon for 30 min. The external gel at the interface of the gel and the axon was not stained. The surface of the axon was then pricked with a needle whose tip diameter was 15–30 μ. The clot in the vicinity of the hole was stained. Without the clot in the voluminous outside medium it is very difficult to visualize leaks of comparable size.[1] The activity of THO or Na_{22} appearing in the middle pool after injection was compared to the activity appearing in the lateral pools. Within the first five min after injection, with or without 30 cm of H_2O pressure, the activity of either Na_{22} or THO in the middle pool was 15–50 times the activity appearing in the lateral pools. Within the first five min practically all of the injected THO appeared in the external medium *(see Results)*. Within five min only about 0.8 to 2.8 % of the injected Na_{22} appeared in the outside medium. This rate of loss of axonal Na_{22} to the middle pool was not affected markedly by pressure (i.e., it increased by <10 % at 30 cm of H_2O pressure). This last result supports the idea that pressure does not produce gross leaks in the surface of the axon. All the control experiments mentioned above show that the flow path of most of the pressure driven water flow is radial. The excitability of the axon was tested periodically throughout the experiment. In normal axons and glutaraldehyde-fixed axons the

1 The dimensions of the hole made are unknown. They would depend upon the depth of penetration of the pipette and the sealing and retractive characteristics of the axonal surface.

L_P was determined with isotonic KF in the perfusate and sea water in the external medium. With KF-stored axons both the perfusate and the bathing medium were isotonic KF.

Measurement of Osmotic Filtration Coefficient

The method is illustrated in Fig. 1*b*. The perfusion tube and the stirring and fluid renewal arrangement to maintain constant solute concentrations were the same as those used for the measurement of the hydrostatic filtration coefficient. The internal solution was isotonic KF. The capillary that functioned as a flowmeter was horizontal so that the internal hydrostatic pressure was maintained near atmospheric. The axon was tested in normal sea water for action potentials at the beginning and the end of each experiment.

Measurement of the Balance Between the Mechanical and Osmotic Pressure Difference that Sustains a Zero Net Water Flow

The arrangement illustrated in Fig. 1*a* for measurement of the hydrostatic filtration coefficients was used. Periodically during the experiment the axon was bathed in normal sea water, perfused with isotonic KF and its excitability was checked. Data from axons that at the *end* of the experiment failed to show an action potential whose amplitude was more than 100 mv were discarded.

Measurement of Longitudinal Hydrostatic Filtration Coefficient of Axoplasm

The method is illustrated in Fig. 1*b*. The internal diameter, A_2, of the distal cannula (right of the figure) and L_2 give the area and the path length for calculating the axoplasmic filtration coefficient. Either $A_1 = A_2$ and $L_1 = L_2$ or $A_1 > A_2$ and $L_1 > L_2$. The external medium was sea water. The pressure head was isotonic KF. The flow path was controlled with added chlorophenol red (0.05 %).

Measurement of Efflux of Tritiated Water (THO)

The cleaned axon was loaded with THO either by soaking or by intraxonal injection. The former method is described in [8] and [14] and the latter in [12]. Axons were soaked in THO media for 30 min. With the intraxonal injection method the injection time was 1.0–1.5 sec. The time required to change external solutions was 1.0–1.5 sec. Our injection method differed from the previous method [12] as follows: The injection pipette (160–180 μ) had two parallel slits, one on each side. The slits were 50–80 μ wide and 2.5–3.5 cm long. The injection fluid was advanced to the slitted region by applying gentle suction to the tip of the pipette emerging from the cut end of the axon. Our soaking method differed from previous methods [8, 14] in that in order to avoid end effects the effluxes from the terminal cut ends of the axon were kept apart from the effluxes of the middle (5 cm long) portion by dividing the bathing medium in to 3 compartments. The temperature was kept at 22 °C. The fluxes of normal and glutaraldehyde-fixed axons were measured in sea water. In KF-stored axons the fluxes were measured in isotonic KF. The external fluid in injected axons was run past the axon and fractionated to resolve the effluxes temporally.

pH of Solutions

With the exception of sea water or when otherwise indicated the pH was 7.35. Apparently this is the normal pH of axoplasm [9].

Control of Osmotic Pressure of Solutions

The osmotic pressure of sea water was varied by varying the NaCl component. Reference [2] should be consulted for this method. Isotonic solutions of other substances were matched against sea water using a Fiske osmometer. Anisotonic solutions were also checked with a Fiske osmometer and in a few instances using the freezing point depression method.

In measuring the L_{PD} of normal and glutaraldehyde-fixed axons the external osmotic pressure was varied by NaCl enrichment of sea water. The internal osmotic pressure was kept constant with isotonic (to sea water) KF. The L_{PD} of axons stored in KF was measured upon varying the external osmotic pressure by increasing the concentration of a salt or sucrose above the isosmotic concentration. The external neutral salts employed were NaCl, KCl or KF. The internal fluid was isotonic KF. In the experiments where the balance between the hydrostatic and osmotic pressure sustaining zero net flow was measured, the osmotic pressure difference was controlled by NaCl depletion of the external sea water combined with KF and sucrose enrichment of the internal medium. This combination of slight external dilution and internal enrichment was deemed advisable since the axon becomes inexcitable in more hypotonic external media.

Temperature

Except when indicated otherwise the experiments were carried out at room temperature (20–23 °C).

Results

Osmotic and Hydrostatic Filtration Coefficients

A static and a dynamic method were used in measuring water flux coefficients. The static method involves a determination of ΔP, the steady state hydrostatic pressure that sustains a zero net solvent flow at a given difference in bulk osmotic pressure, $\Delta\Pi_{\text{th}}$. In this method $(L_{PD}/L_p)\Delta\Pi_{\text{th}} = -(\Delta P)_{J_v=0}$, where L_{PD} is the osmotic filtration coefficient, L_p the hydrostatic filtration coefficient and J_v is the rate of total volume transport. The dynamic method involves a separate determination of L_P and L_{PD}. In this method $L_p \Delta P = (J_v)_{\Delta\pi_{\text{th}}=0}$ and $L_{PD}\Delta\Pi_{\text{th}} = (J_v)_{\Delta P=0}$.

Our results using the static method are presented in Fig. 2 and Table 1. $\Delta P/\Delta\Pi_{\text{th}}$ varied from 0.003 to 0.006. Our results using the dynamic method are presented in Fig. 3 and Tables 2 and 3. The L_p varied from 8 to 14×10^{-8} cm/sec/cm H_2O. The L_{PD} varied from 3 to 6×10^{-10} cm/sec/cm

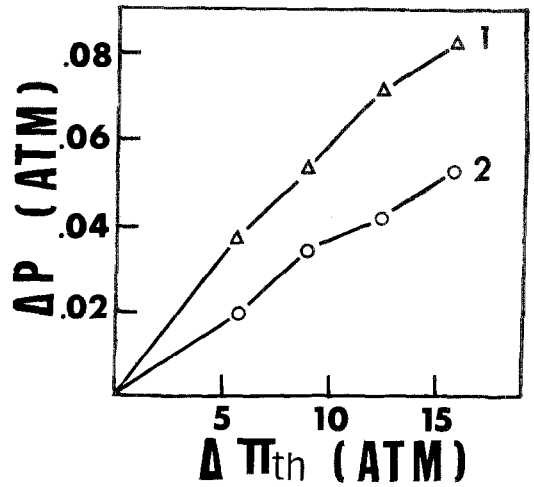

Fig. 2. The relation of the bulk osmotic pressure difference between the inside and the outside of the axon ($\Delta \Pi_{th}$) to the hydrostatic pressure difference (ΔP) required to sustain zero net volume flow. The axons in curves 1 and 2 showed the highest and lowest $\Delta P/\Delta \Pi_{th}$ seen. $\Delta \Pi_{th}$ was attained as follows: (a) 6 atm were obtained by NaCl depletion of external sea water. The internal perfusate was KF isosmotic to normal sea water. (b) 9 and 12.5 atm were obtained by using the same external medium as in (a) but the internal medium of (a) was enriched with KF. (c) 16 atm were obtained by using the same media as in (b) except that the internal was further enriched with sucrose

Table 1. Ratio of hydrostatic to bulk osmotic pressure difference to sustain zero net volume flow in axon[a]

Axon code	Axon diameter (μ)	$(\Delta P/\Delta \Pi_{th}) \times 10^3$
1	645–657	5.8
2	668–677	3.3
3	690–702	5.4
4	708–716	4.8
5	720–726	4.4

[a] Axons coded 1 and 2 are also depicted in Fig. 2. In this and the remaining Tables the diameter given constitutes the distance measured between the diametrically apposed *inner* surfaces of the sheath. It does not include the sheath. Usually this coincides with the diameter of the axoplasmic tube, but not always; for example, in alcohol treated axons the axoplasm detaches from the sheath. The values given in the tables apply to the fresh axon, *not* the treated axon.

H_2O. The L_{PD}/L_P ratio varied from 0.002 to 0.007. This ratio, as expected, is close to the ratio of $\Delta P/\Delta \Pi_{th}$ determined with the static method. We were unaware when we undertook this work that Vargas [13] had already found in the Dosidicus axon a comparable $L_{PD}/L_P \sim 0.01$. Vargas appeared to favor the interpretation that this ratio is due to the presence of one or

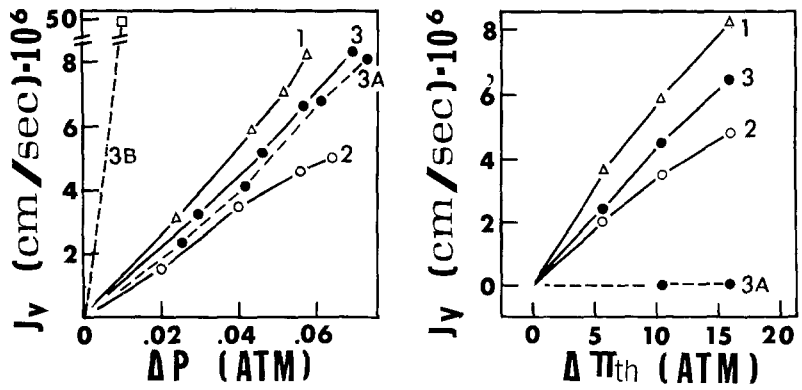

Fig. 3. A comparison of hydrostatic and osmotic filtration coefficients. *Left frame:* The relation of the rate of total volume transport (J_v) to the hydrostatic pressure difference (ΔP) between the inside and the outside of the axon. The axons in curves 1 and 2 showed the highest and lowest flow rates per unit surface area and pressure difference seen. Curves 3, 3*A* and 3*B* are from the same axon. In curve 3 the axon was normal, in curve 3*A* the axon had been stored in isotonic KF at 0–2 °C for 36 days and in curve 3*B* the axoplasm in the axon was extruded so that only the sheath remained. It can be seen that extended storage in KF does not affect L_p markedly. The diameter of the axon in curve 1 was 685–698 μ, in curve 2, 712–722 μ and in curves 3, 3*A* and 3*B*, 697–702 μ. *Right frame:* The relation of the rate of total volume transport (J_v) to the theoretical (bulk) osmotic pressure difference ($\Delta \Pi_{th}$) between the inside and the outside of the axon. The axons in curves 1 and 2 showed the highest and lowest flow rates per unit surface area and bulk osmotic pressure difference seen. Curves 3 and 3*A* were obtained from the same axon. In curve 3 the axon was normal and in curve 3*A* the axon had been stored in isotonic KF at 0–2 °C for 35 days. It can be seen that after extended storage in KF there is no detectable steady state osmotic flow. The diameter of the axon in curve 1 was 654–663 μ, in curve 2, 648–656 μ, and in curves 3 and 3*A*, 686–693 μ

more large holes with a low reflection coefficient. He is effectively invoking the presence of a leak; but our experiments as well as those of Vargas do not favor this interpretation. Leaks through cut branches, cut or ligated ends, and otherwise damaged or even "intact" regions were carefully controlled (*cf. Methods*). The cross sectional annular area of the axoplasmic tube interposed between the edge of the perforated region of the perfusion tube and the ligature is sufficiently small, its length sufficiently long (i.e., >1.5 cm) and the length of the perforated region of the tube sufficiently long that even with an axoplasmic water permeability, $Ks \sim 5 \times 10^{-13}/\text{cm}^2$ (*see* later section) a potential outlet at the end of the axon should be sealed by the intervening axoplasm. That axoplasm is incompletely swollen in the axon (*see* adjoining paper [11]) may account for the observation (*see Materials and Methods*) that there was no leakage between the gel and the perfusion tube. The system appears to be self-sealing. Since moderate internal hydrostatic pressures do not markedly

Table 2. L_p, Hydrostatic filtration coefficient of axon[a]

Axon code	Axon diameter (μ)	L_p(cm/sec/cm H_2O) × 10^8		Treatment
		Normal	Treated	
1	705–716	11.2	13.6	*S*(42 days)
1	705–716	–	155	*S*(42 days) scrambled
2	706–720	12.4	9.5	*S*(43 days)
2	706–720	–	82	*S*(43 days) scrambled
3	723–732	13.9	16.4	*S*(44 days)
3	723–732	–	810	*S*(44 days) extruded
4	688–702	11.2	180	Scrambled
5	682–696	13.6	770	Extruded
6	605–617	–	680	Extruded
7	621–629	–	290	Extruded
8	732–745	9.2	6.4	*F*(3 hours)
9	738–750	8.1	6.1	*F*(3 hours)
10	641–652	12.3	9.3	*F*(7 hours)
11	645–654	10.3	9.8	*F*(3 hours)
11	645–654	–	8.6	*F*(3 hours) *S*(67 days)
12	682–695	13.2	17.2	*F*(3 hours)
12	682–695	–	15.1	*F*(3 hours) *S*(68 days)
13[b]	678–692	10.2	13.4	*F*(7 hours)
14[b]	695–717	8.5	11.6	*F*(7 hours)

[a] The experiments depicted in the figures are not included in the Table. In axons coded 6 and 7 the L_p on the normal axon was not measured. In axons where the code is repeated means two experiments were done on the same axon. Treatment *S* means storage in isotonic KF at 0–2 °C. Treatment *F* means fixation in glutaraldehyde. The axoplasm was scrambled using a rubber roller. Extruded means that all of the axoplasm was extruded, the L_p measured being that of the sheath alone. The time of storage and of fixation is indicated in the parentheses. In axons 6, 7, 10 and all of the perfusion tube the diameter was 303–316 μ. In the remaining axons the diameter was 383–394 μ. *Note* footnote Table 1.

[b] These were the first two axons used. The technique was not as well developed. The internal concentrations were kept constant merely by intermittent perfusion.

Table 3. L_{PD}, osmotic filtration coefficient of axon[a]

Axon code	Axon diameter	L_{PD} (cm/sec/cm H_2O) × 10^{10}		Treatment
		Normal	Treated	
1	658–667	3.7	0	*S*(28 days)
2	692–702	5.0	0	*S*(17 days)
3	698–706	4.4	0	*S*(16 days)
4	697–709	4.7	0	*S*(19 days)
5	705–712	4.9	0	*S*(20 days)
6	712–720	5.1	0	*F*(7 hours)
7	718–732	4.6	0	*F*(7 hours)

[a] The experiments presented in Fig. 3, right frame, are not included in the table. *S* means storage at 0–2 °C in isotonic KF. *F* means fixation in glutaraldehyde. The storage and fixation times are given in parentheses. 0 L_{PD} means less than 1/50 the normal L_{PD}. *Note* footnote Table 1.

affect (1) the resting potential and membrane resistance [1, 13 and *our observations*], (2) the efflux of Na_{22} or chlorophenol red (*see Materials and Methods*) or (3) the efflux of glycerol [13] it is unlikely that such pressures increase porosity of the *solute* barrier significantly. That the $J_v - \Delta P$ relation is linear, i.e., the L_p determined at 5 cm pressure is the same as that at 60 cm H_2O pressure, supports the idea that the porosity of the *solvent* barrier is not increased by pressure. Vargas felt that 1.3×10^{10} pores of 4.25 Å radius per cm^2 of the *solute* rate-limiting structure determine L_{PD} so that a hole 0.4 μ in radius in the same structure could serve as a leak and determine L_P. Probably we could not have detected such a small leak. However Vargas' reasoning is invalid if, as it will be shown, neither pressure-driven nor diffusional H_2O flow is limited in the structure limiting solute flux.

Diffusional Water Fluxes (Efflux of THO)

These data are presented in Fig. 4 and Fig. 5 (bottom) and in Table 4.

With the soaking method of THO loading the curve of the logarithm of remaining THO fraction *vs.* efflux time consists of an initial rapid phase followed by a slower linear phase. The first portion represents primarily diffusion from the extraxonal connective tissue and aqueous annular film. The second phase presumably represents efflux from the intraxonal compartment. The intercept at $t=0$ of the extrapolated linear portion gives an indication of the relative volumes of the intraxonal and extraxonal compartments. It varied from 0.51 to 0.78. The t 1/2, the time required for 50% reduction in intraxonal THO activity (estimated from the linear portion of the curve) is given in Table 4. At 22 °C and in axons whose diameter varied from 480 to 525 μ, the t 1/2 was 38 to 46 sec. These results are similar to those of Nevis [8] and Villegas and Villegas [14].

With the injection method of THO loading, the efflux curve of THO was linear from $t=0$ (i.e., the intercept at $t=0$ of the extrapolated straight line was unity). This is true within the limits of the resolution of our technique. At 22 °C and in axons whose diameter varied from 485 to 520 μ, the t 1/2 was 39 to 48 sec. As expected the t 1/2 obtained using the injection method is the same as that obtained using the soaking method. In Table 4 axons coded as 9 and 10 were loaded both by the injection and the soaking techniques. The t 1/2 seen with the two methods was the same. The linearity of the THO efflux curve was not made an issue in previous studies [12] using the injection method. The reason is that the temporal resolution in these early experiments was not as good as the present one.

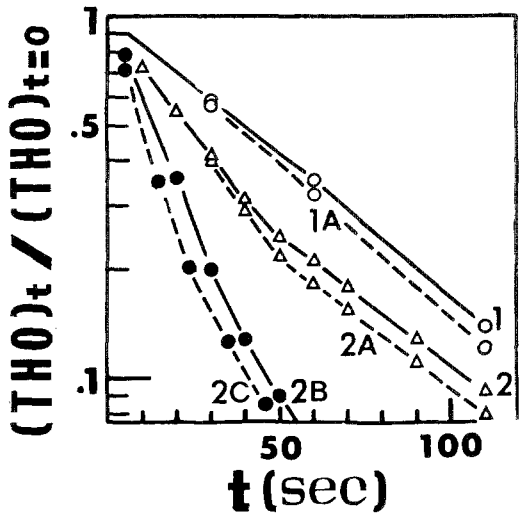

Fig. 4. The logarithm of the fraction of tritiated water remaining in the axon and sheath $[(THO)_t/(THO_{t=0}]$ plotted against efflux time (t). Curves 1 and 1*A* were obtained from the same axon. In curve 1 the axon was normal and in curve 1*A* the axon had been stored in isotonic KF at 0–2 °C for 29 days. The axon was loaded with THO by injection. It can be seen that the efflux rate is affected only slightly by extended storage in KF. Note alo that the curve is a straight line (i.e., with an intercept at $t=0$, close to unity). Curves 2, 2*A*, 2*B* and 2*C* were obtained from the same axon. The axon was loaded with THO by soaking. The axon in curve 2 was normal. In 2*A* the axon was stored in isotonic KF at 0–2 °C for 28 days. In 2*B* the axoplasm in the axon was scrambled by moving it back and forth with a roller. In 2*C* the axoplasm was extruded and replaced with 2 % gelatin gel. Note that the rate of efflux of THO was not affected appreciably by extended storage in KF. Note also in 2 and 2*A* that the curve is characterized by an initial rapid phase and a delayed slower linear phase and that the intercept of the straight line at $t+0$ is slightly different in curve 2*A* than it is in curve 2. Since the total counts of the axon at $t=0$ in the experiment of curve 2 were 8 % higher than those in the experiment of curve 2*A*, this difference in intercepts may be due to a difference in the amount of adhering annular fluid during soaking (e.g., due to variable blotting). The axon in curves 1 and 1*A* was obtained from the same squid as the axon in curves 2, 2*A*, 2*B* and 2*C*. The diameter of the axon in curves 1 and 1*A* was 495–510 μ and of the axon in curves 2, 2*A*, 2*B* and 2*C*, 490–510 μ

The t 1/2 of THO efflux expressed as the diffusion permeability coefficient $P_d = 1.5 - 2 \times 10^{-4}$ cm/sec. From $P_d = D_{H_2O} A/\Delta x$, where D_{H_2O} is the diffusion constant of water, A the fraction of membrane area available for diffusion and Δx the path length Villegas and Villegas found that Δx for THO is in the range of the length of the Schwann channel. However, they thought that the rate of pressure-driven water flow is limited by the solute rate-limiting structure; accordingly, they calculated from L_{PD} and P_d an equivalent cylindrical pore radius $r \leqq < 8.5$ A, thereby equating the pores restricting solute flux to those limiting solvent flux. Since we think

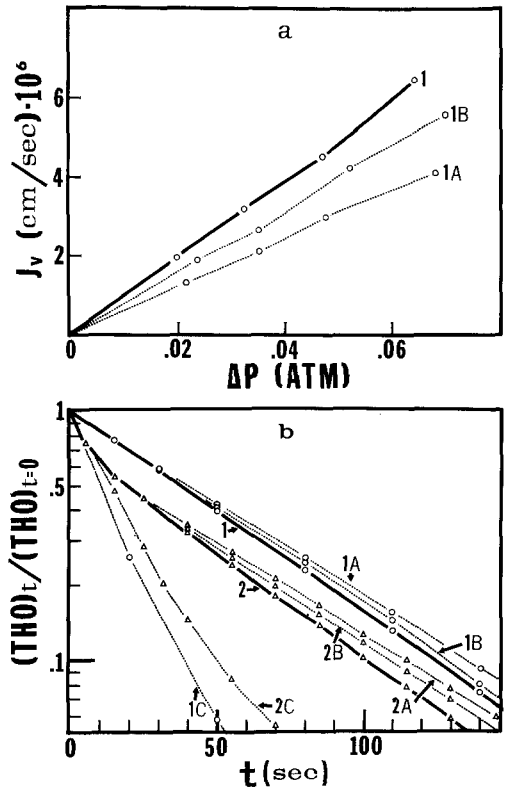

Fig. 5. The effects of glutaraldehyde fixation on the hydrostatic filtration coefficient and on the efflux rate of tritiated water. *Top frame:* The relation of the rate of total volume transport (J_v) to the hydrostatic pressure difference (ΔP) between the inside and the outside of the axon. Curves 1, 1*A* and 1*B* were obtained from the same axon. In curve 1 the axon was normal. In curve 1*A* it had been fixed in 2 % glutaraldehyde for 7 hr. In curve 1*B* the fixed axon had been stored in isotonic KF for 345 days at 0–2 °C. *Bottom frame:* Semilogarithmic plot of fraction of initial labelled water remaining in axon and sheath $[(THO)_t/(THO)_{t=0}]$ against washout (efflux) time (t). Curves 1, 1*A*, 1*B* and 1*C* were obtained from the same axon. The axon was loaded with THO by injection. In curve 1 the axon was normal. In curve 1*A* the axon had been fixed in 2 % glutaraldehyde for 7 hr. In curve 1*B* the fixed axon was stored in isotonic KF at 0–2 °C for 348 days. In curve 1*C* the fixed and stored axon was dehydrated in 100 % ethanol, rehydrated and finally loaded with THO. Note that curves 1, 1*A* and 1*B* are straight lines. The temporal resolution in the experiments in Fig. 5, top, is better than that in the experiment of Fig. 4, curve 1. Curves 2, 2*A* and 2*B* were obtained from the same axon. The axon was loaded with THO by soaking. The axon in curve 2 was normal. In curve 2*A* the axon had been fixed for 7 hr in 2 % glutaraldehyde. In curve 2*B* the fixed axon had been stored for 350 days in isotonic KF at 0–2 °C. In curve 2*C* the axon, initially 5 cm long, was cut in 7 mm segments. Curve 2*C* represents the combined efflux of the seven segments. The diameter of the axon in curves 1, 1*A*, 1*B* and 1*C* was 489–495 μ. The diameter of the axon in curves 2, 2*A*, and 2*B* was 487–495 μ; in 2*C* it decreased by the dehydration procedure to 434–446 μ. This axon was from the same squid as the axon of curves 1, 1*A*, 1*B* and 1*C*

Table 4. t 1/2, half time of efflux of THO from axon[a]

Axon code	Axon diameter (μ)	Loading method	t 1/2 (sec)		Treatment
			Normal	Treated	
1	480–492	Soaking	40	41	*S*(34 days)
2	482–495	Soaking	38	36	*S*(34 days)
3	505–514	Soaking	43	40	*S*(34 days)
4	502–516	Soaking	45	44	*S*(92 days)
5	495–510	Soaking	41	42	*S*(92 days)
6	485–597	Injection	41	38	*S*(35 days)
7	486–495	Injection	39	41	*S*(93 days)
8	500–511	Injection	44	42	*S*(93 days)
9	502–513	Injection	45	47	*S*(326 days)
9	502–513	Soaking	43	44	*S*(325 days)
10	503–520	Soaking	46	43	*S*(325 days)
10	503–520	Injection	47	44	*S*(326 days)
11	496–505	Injection	48	51	*F*(3 hr)
12	494–502	Injection	41	45	*F*(3 hr)
13	512–525	Soaking	44	52	*F*(3 hr)
14	514–525	Soaking	46	41	*F*(3 hr)
15	508–520	Soaking	45	41	*F*(7 hr)
16	506–518	Soaking	43	45	*F*(7 hr)
17	510–524	Soaking	45	53	*F*(3 hr)
17	510–524	Soaking	–	47	*F*(3 hr) *S*(76 days)
18	512–524	Soaking	43	51	*F*(3 hr)
18	512–524	Soaking	–	45	*F*(3 hr) *S*(76 days)
19	497–512	Soaking	42	46	*F*(7 hr)
19	497–512	Soaking	–	40	*F*(7 hr) *S*(407 days)
20	510–520	Injection	42	50	*F*(3 hr)
20	510–520	Injection	–	43	*F*(3 hr) *S*(77 days)
21	510–516	Injection	40	49	*F*(7 hr)
21[b]	510–516	Injection	–	42	*F*(7 hr) *S*(408 days)
22[b]	492–510	Injection	47	42	*F*(7 hr)
23[b]	487–502	Injection	48	41	*F*(7 hr)
24[b]	492–508	Injection	46	44	*F*(7 hr)
25[b]	497–515	Injection	45	20	Axoplasm Liquefied
26[b]	502–520	Injection	47	12	Axoplasm Liquefied
27[b]	504–518	Injection	48	~4	Axoplasm Liquefied
28[b]	495–516	Injection	45	11	Dehydrated (15 min)
29[b]	508–518	Injection	46	13	Gelatin filled
30[b]	504–517	Injection	47	12	Scrambled
31[b]	488–506	Injection	46	15	Scrambled
32[b]	494–513	Injection	48	10	Scrambled

[a] The temperature was 22 °C. Treatment *S* means storage in KF. Treatment *F* means glutaraldehyde fixation. The period of treatment is indicated in parentheses.

[b] Axons 22–32 were injected with THO using the method given in reference 12, not the method used in the remaining fibers (*cf.* Methods section). With the old method, especially when the axoplasm is liquefied, the efflux initially may be pressure driven (due to the injection pressure). This may explain the result of axon 27. The axoplasm in axons 25–27 was liquefied by immersion for 7–10 hr in sea water containing 80 mM KCl. The efflux curves of *treated* axons 25–32 was *nonlinear*. The efflux curve of the untreated was linear. In these axons $1/2t$ was calculated from the initial portion of the curve (from $t=0$). It is important to note footnote, Table 1.

(*cf.* next section) that pressure-driven flow is not rate-limited in the solute barrier we roughly estimated, for what it is worth, from L_P and P_d an $r \sim 100$ Å. An equivalent 1/2 width of a *slit* channel would be less. The width of the Schwann channels is thought to be ~ 60 Å.

A $L_{PD}/L_P < 1$ not explicable in terms of Staverman's reflection coefficient we regarded as negative anomalous osmosis. Electrophysiological and tracer studies in the axon in conjunction with the finding that volume changes upon varying $\Delta\Pi_{th}$ are *steady state* imply that the reflection coefficient for the solutes varied in our experiments is close to unity. In inanimate systems various mechanisms can account for a negative anomalous osmosis: (1) inherent or induced leaks; (2) electric field gradients, either existing or due to the applied pressure; (3) local circulating electrosmotic currents due to heterogeneity in pore geometry or pore composition (mosaic membrane); (4) interaction or selective absorption involving solute and membrane; (5) in series (to the solute barrier) resistances due to boundary film diffusion or due to juxtaposed structures and (6) others. In the perfused axon the material interposed between the aqueous compartments is complex (i.e., endoplasm-ectoplasm-solute barrier-Schwann layer-connective tissue). We considered the possibility that the structures sandwiching the solute barrier may act as in series resistances. We tested this by measuring diffusional and pressure conjugated water fluxes in preparations where the solute barrier was destroyed but the adjacent structures presumed to be intact.

(a) We had already found that in certain media (*cf.* [11]) not only the structure of extrudates was preserved indefinitely but also that of the axoplasm in the axon and, at the light microscope level, that of the sheath. The (1) lack of any measurable axolemmal resistance or resting potential (in isotonic NaCl), (2) immeasurably low L_{PD} (curve 3*A*, Fig. 3, *Right*, and Table 3), and (3) pronounced increase in efflux of injected Na_{22} in these stored axons imply that the solute barrier is nonfunctional. With our setup we would have detected easily an L_{PD} that was 1/50 the normal. Attempts at measuring L_{PD} in KF-stored axons were made by controlling $\Delta\Pi_{th}$ by increasing the concentration of NaCl, KCl, KF, NaF and sucrose in the external medium. $Na_{22}Cl$ was injected in seven fresh axons varying in diameter from 530 to 740 μ. In the normal axon the loss of Na_{22} in a period of five min was 0.8 to 2.8% of the initial radioactivity in the axon. The loss of Na_{22} within the same period of time after 37 to 43 days storage of the axon in isotonic KF was 92% to more than 99%. In KF-stored axons neither the efflux of THO nor the L_P were affected. These data are presented in Fig. 4, curve 1*A* and 2*A*, Fig. 3, left frame, curve 3*A*, and Tables 2 and 4.

(b) In the second method we felt that we could "fix" the solvent barrier in normal sea water with protein cross-linking agents and simultaneously, if the concentration of such agents was high enough, make the solute barrier nonfunctional. We used 2–4% glutaraldehyde as a fixative. This is at least two orders of magnitude higher concentration than that at which changes in the electrical properties of the axon are observed. The data on the effects of glutaraldehyde fixation on pressure driven and diffusional fluxes are presented in Tables 2 and 4 and Fig. 5. It can be seen that neither the L_p nor the efflux rate of THO were increased significantly by fixation of the axon. Glutaraldehyde was increased stepwise over a period of 3 or 7 hr, respectively, to a concentration of 2% or 4%. The mixture was kept isosmotic to sea water. The fixative was washed out in sea water or isosmotic KF. Fixed axons appeared "brittle" and unless extreme caution was exercised the observed t 1/2 was low. Bending and twisting were particularly injurious.

Bulk-Limited vs. Surface-Limited Solvent Flux

Having shown that water flux may not be limited across the surface structure limiting solute flux, we attempted to ascertain whether it is limited by the bulk of the cytoplasm or some surface structure.

If water fluxes were bulk-limited the data presented above with the soaking method of determining THO efflux would infer a diffusion coefficient of THO, $\bar{D}_{\mathrm{THO}} \sim 1.5-2.0\times10^{-6}\,\mathrm{cm^2/sec}$ and the data on L_P a H_2O permeability coefficient, $Ks \sim 1-2\times10^{-14}\,\mathrm{cm^2}$, where $Ks=\dfrac{vLn}{tA\,\Delta P}$ and in turn where volume, v, is in $\mathrm{cm^3}$, the path length, L, in cm, the viscosity, η, in poises, the time, t, in seconds, the area, A, in $\mathrm{cm^2}$ and the pressure differential, ΔP, in dynes/$\mathrm{cm^2}$. Such a $\bar{D}_{\mathrm{THO}}$ would be ~ 10 times smaller than that found in inanimate gels with comparable polymer volume fractions. Similarly, such a Ks would be two orders of magnitude smaller than that of agar and silica gels, one order smaller than that of gelatin gels and of the same order as that of certain acrylamide gels [15]. This comparison is based on using comparable polymer volume fractions, i.e., 2–3%.

If water fluxes were surface-limited the structure involved may be the sheath (Schwann layer and adhering connective tissue) and/or an ectoplasmic layer of axoplasmic gel. The structure of the sheath has been studied (e.g., [14]) and seems to be sufficiently complex. Bear, Schmitt

and Young [3] mentioned briefly the existence of an insoluble surface film in extruded axoplasm. We confirmed the presence of a cortical axoplasmic film in both stained and unstained preparations. In isotonic nondispersing anions (*cf.* Table 1 of [11]) we could peel the cortex off of the extrudate (Fig. 2*g* of [11]). In a dispersing anion (i.e., isotonic KCl) we observed that the endoplasm of the extrudate is dispersed but a ghost-like cylindrical cortex remained. In isotonic nondispersing anions we *extruded* mechanically the endoplasm from *extrudates*. The residual cytoplasmic material was insoluble in isotonic KCl while the extruded endoplasmic material dispersed.

Our evidence in favor of axoplasmic surface-limited pressure-driven flow is the following:

(a) We scrambled the axoplasm by rolling it in the axon until although the bulk was still gelatinous the cortical organization was clearly disrupted. It is very likely that the scrambling procedure increased the porosity of the sheath. The extrudate from scrambled axons is amorphous. The L_p in three such axons was increased by a factor of 8–16. These data are presented in Table 2.

(b) In three normal axons we measured the *longitudinal* Ks (*cf.*, method of Fig. 1*c*) and found it to be 18×10^{-14}, 21×10^{-14} and 27×10^{-14} cm^2. This longitudinal Ks is 10–20 times the radial Ks across the *normal* axoplasmic wall and of the same order as the radial Ks in preparations with "*scrambled*" axoplasm.

(c) We measured the L_p in 5 sheaths devoid of axoplasm. The procedure of extruding the axoplasm (i.e., a rubber roller) may very well have increased the porosity of the sheath. One experiment is presented in curve 3*B* Fig. 3. In four other sheaths devoid of axolemma and axoplasm the L_p was $2.9-8 \times 10^{-6}$ cm/sec/cm H_2O. This L_p is greater than even the L_p seen in axons whose axoplasm was scrambled. These data are presented in Table 2. An alternate interpretation of these experiments is that without the gel interposed between the perforated region of the perfusion tube and the ligated ends of the axon there is no sealant action and the ends may act as effective leaks. Inadvertently we did not control these experiments using the 3-pool arrangement with injected Na_{22} mentioned in *Methods*.

Our evidence for surface-limited THO efflux is the following:

(a) When the logarithm of the amount of *injected* THO remaining in the axon at time t, expressed as a fraction of the initial amount, was plotted against t, a straight line was obtained (*cf.* Fig. 4 and 5, bottom frame). The intercept of this straight line is close to unity. Clearly this efflux curve shows the characteristics of a surface-limited diffusion process.

(b) If the efflux of THO were bulk-limited, cutting the axon in small pieces should increase the efflux rate far less than if the efflux were surface-limited. The cut in fresh axons immersed in sea water is ill defined; in the region of the cut, axoplasm disperses and the sheath constricts. The cut in fresh axons immersed in an anion that does not disperse axoplasm (*cf.* Table 1 in [11]) is also ill defined on account of the protrusion (the swelling of the axoplasm) through the cut end (*cf.* Fig. 2*e* of [11]). Axons that are fixed in glutaraldehyde or axons stored in isotonic KF upon being cut either do not show a protrusion or the protrusion is slight. When the guillotine method of cutting (*cf. Materials and Methods* of [11]), the cut was clean, the lips of the sheath are open and the axoplasm at the cut region visually is indistinguishable from the remaining encapsulated portion. An example out of four such experiments is presented in the bottom frame of Fig. 5, curve 2*C*. The remaining data are consistent. The axon intitially was 5 cm long. It was cut in 7 mm long segments. With a 500 μ axon this should result in a 3% increase in the surface area of the axoplasmic gel and if the efflux were bulk-limited the increase in efflux rate should be somewhat less than 3%. It can be seen, however, from curve 2*C*, that the increase is far greater. This would be expected from a surface-limited diffusion process.

(c) We scrambled the axoplasm by rolling it in the axon until the axoplasmic cortex was clearly disrupted. The sheath may very well have been damaged. In four such axons the rate of efflux of THO was markedly increased. An example is given in curve 2*B*, Fig. 4 and the remaining experiments are presented in Table 4.

(d) The efflux of injected THO was measured in 4 axons whose axoplasm was liquefied by prolonged immersion in sea water containing 80 mM KCl. The concentration of divalent ions was normal. The efflux curve of injected THO was nonlinear. The t 1/2 of the initial phase (from $t=0$) was much lower than that seen in the normal axon. These results are presented in Table 4. There is no assurance that the sheath was not damaged also.

(e) In 2 axons the axoplasm was extruded with a rubber roller and replaced with a 2% gelatin gel. The gelatin was introduced into the axon while still liquid (warm). One of these experiments is presented in curve 2*c*, Fig. 4, and the other in Table 4. Again the rolling procedure or elevated temperatures 30 °C may have very easily damaged the sheath. There was a marked increase in THO efflux rate.

In order to show that the THO efflux of these hardy axons can be affected by something other than by such mechanical means as scrambling, chopping up and liquefaction we subjected 2 glutaraldehyde-fixed and

KF-stored axons to ethanol dehydration. The dehydration procedure was stepwise. Effluxes were measured after the axon was hydrated in a stepwise manner. One experiment is presented in Fig. 5 and the other in Table 4. The increase in THO efflux is greater than expected from the decrease in diameter.

Discussion

In the text we intimated that the rate limiting structure for diffusional solvent flux may be the same as that for pressure driven solvent flow. This assumption may not be justified. For example, Hayes [5] found that in complex cellulose acetate membranes this assumption is not valid. He suggested that this may be the case in some biological systems (e.g., skin, bladder).

Our separation of surface structures into solvent and solute rate-limiting structures may be misleading. The solvent flux rate-limiting structure does offer some hindrance to solute flux. In normal axons this hindrance is not rate-limiting. In a recent study (to be presented elsewhere) we measured the efflux of radioactive glucose, sucrose and inulin in KF-stored and glutaraldehyde-fixed axons. In these axons the rate of efflux of these nonelectrolytes was an order of magnitude *lower* than that of THO, an order of magnitude *lower* than that expected from simple diffusion and 2–3 orders of magnitude *higher* than that seen in normal axons. In KF-stored axons whose diameter was $\sim 500\,\mu$ the $1/2\,t$ of efflux of glucose C-14, sucrose C-14 and inulin C-14 were around 4, 5 and 10 min, respectively. Apparently in these axons the relative efflux rate of these solutes and that of THO is a function of their diffusion constants in water.

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