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Osmotic Relations of Nerve Fiber

C.S. Spyropoulos

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

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Summary. The volumetric elastic modulus of the sheath and the osmotic swelling pressure of the axoplasmic polymer network of the giant axon of *Loligo vulgaris* were measured. Evidence was obtained that (1) the elastic modulus of the sheath, (2) the swelling pressure of axoplasm, and (3) the *effective* osmotic pressure difference due to mobile solutes determine axonal volume. The contributions of the sheath and the axoplasm were significant because the *effective* osmotic pressure due to mobile solutes was a small fraction of the *theoretical* bulk osmotic pressure due to these solutes. The giant axon was converted from an imperfect to a near perfect osmometer by minimizing the contribution of the sheath and the axoplasmic gel.

Equilibrium swelling of nonionic swollen polymer networks (gels) is ascribed to the balance of the free energy of mixing (ΔF_{mix}) of the polymer with the solvent and the elastic free energy (ΔF_{el}) incident to the expansion of the network. At equilibrium the solvent activity a_l outside and inside the gel are equal: $RT \ln a_l = \partial \Delta F_{\text{mix}}/\partial n_l + \partial \Delta F_{\text{el}}/\partial n_l$. If the gel is somehow constrained so that it cannot attain its maximum degree of swelling (q_m), an osmotic swelling pressure difference ($\Delta \Pi_g$) will develop between the solvent and the gel [7–10]. The above equilibrium condition becomes: $0 = \partial \Delta F_{\text{mix}}/\partial n_l + \partial \Delta F_{\text{el}}/\partial n_l + \bar{V} \Delta \Pi_g$, where $\bar{V}_l$ is the partial molal volume of the solvent. With ionic networks, the Donnan effect and the interaction between charged groups on the network also must be taken into account.

It is well known that the cytoplasm of the *Loligo* axon is a gel. We will present experiments indicating that the gel is constrained by the axonal sheath, so that it cannot swell out completely, and that $\Delta \Pi_g$ and E (the elastic modulus of the sheath) in conjunction with $\Delta \Pi_m$ (the *effective* transmembranal osmotic pressure difference due to mobile solutes) determine axonal volume. This conclusion conflicts with the generally held

* 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.

belief that cytoplasmic polymer networks are incapable of sufficient mixing with the solvent to elevate appreciably the total entropy of the system, thereby decreasing the chemical potential of the solvent and creating an osmotic pressure comparable to that due to mobile solutes.

Our approach to evaluating the role of $\Delta\Pi_g$ is the following: at the mobile solute and network polymer concentrations encountered in the axon $\Pi_g \ll \Pi_{th}$, where Π_{th} is the theoretical bulk Π due to *mobile* (i.e., exclusive of network polymers) solutes. It follows that $\Delta\Pi_g$ can constitute a substantial fraction of the total effective $\Delta\Pi$ only if $\Delta\Pi_m/\Delta\Pi_{th} \ll 1$. We ascertained that $\Delta\Pi_m/\Delta\Pi_{th} \ll 1$ by (1) a determination of ΔP, the steady state hydrostatic pressure that balances $\Delta\Pi_{th}$ to sustain zero net flow and (2) a determination of the ratio: osmotic filtration coefficient/hydrostatic filtration coefficient, L_{PD}/L_p. These results are presented in the foregoing paper [13].

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 [12].

Axoplasmic Extrusion with Excised Axons

Axoplasm was extruded using either a single or a double rubber roller. The extrusion apparatus used in the double roller technique essentially was a miniaturized version of the double roller wringer on old-fashioned washing machines. The pressure between the rollers was adjusted with a micrometer screw.

Axoplasmic Extrusion with In Situ Axons

In situ extrusion was successful with lateral giant axons radiating from the stellate ganglion. A squid was pinned to a board without circulating sea water. An incision was made on the ventral mantle. The axon was cut in the immediate vicinity of the stellate ganglion. In this region the axon was flared. Within a few mm the axon enters a tunnel in the mantle. The wall of the tunnel was cut open lengthwise with a single incision and the axon exposed. The axoplasm was extruded with a metal probe whose end was enlarged and spherical. The terminal sphere of the probe was coated with rubber. The diameter of the sphere was slightly larger than the diameter of the tunnel. The tunnel acted as a guide for the extruding probe and simultaneously held the axon in a fixed position. The whole procedure took about 1–2 min from the time the squid was pinned.

Measurement of Volume of Extrudates

The extrudate was immobilized in a groove just wide, deep and long enough to accommodate the anticipated increase in the volume. The groove was covered with a piece of nylon mesh to prevent the tendency of the extrudate to float. The volume (i.e., swelling)

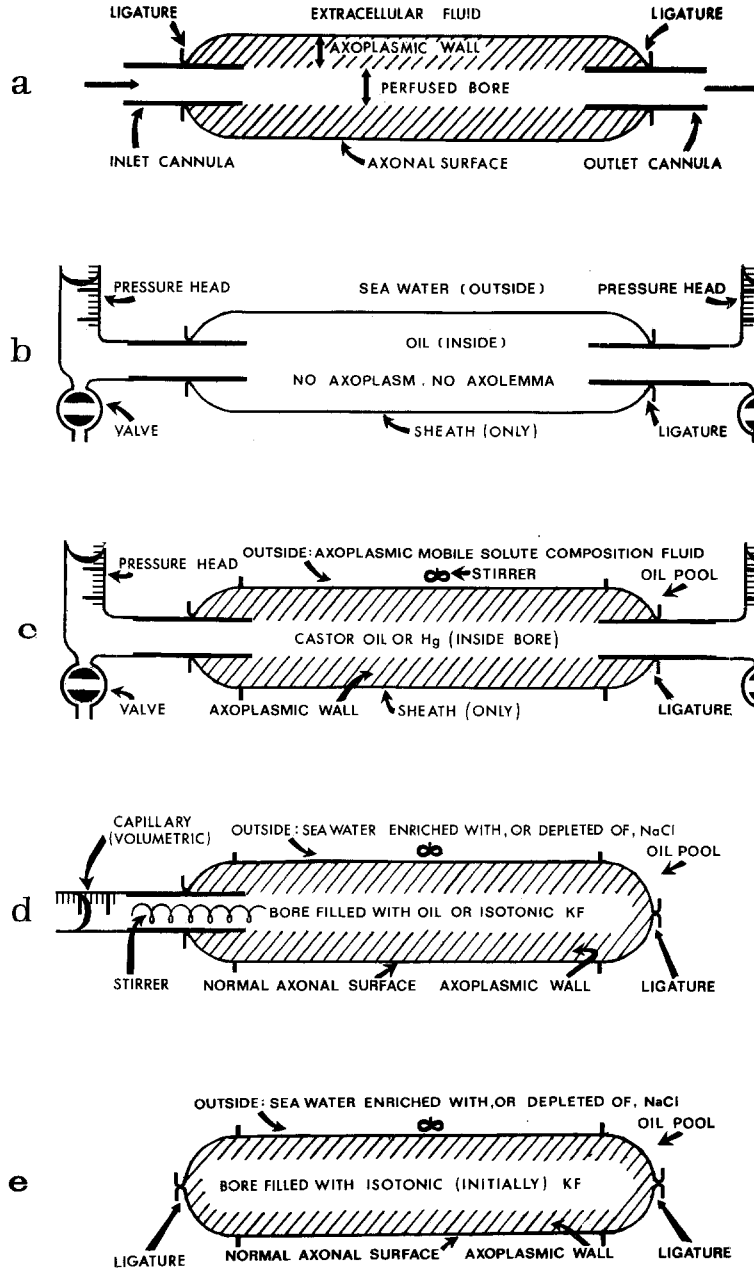

Fig. 1. (*a*) Method of measurement of volume of axoplasm in perfused axon. (*b*) Method of measurement of volumetric elastic modulus of sheath. (*c*) Method of measurement of osmotic swelling pressure of axoplasmic gel. (*d*) Method of conversion of axon into near perfect osmometer, both in hypotonic and hypertonic external media. (*e*) Method of conversion of axon into a near perfect osmometer in hypertonic, but not in hypotonic external media

of the extrudates was measured by measuring their length with an ocular micrometer and their diameter at several points with a Watson Image Shearing device. When swelling was marked, the extrudates were fragile, and sagged. Apparently, the gel framework was too weak to sustain the cylindrical shape.

Measurement of Axoplasmic Volume in Perfused Axons

The method is illustrated in Fig. 1*a*. The axons were thoroughly cleaned except at the region of the cannulae. The author's double cannulation method of internal perfusion was used. The borer pipette was made of thin wall glass and the drawn portion was of uniform diameter for a length approximating the length of the axon. The diameter varied with the diameter of the axon. The outside surface of the terminal portion of the borer was coated with a thin layer of parafin and the tip was repeatedly dipped and withdrawn in HF. This resulted in a circular serated knife edge at the tip and a further thinning of the wall of the borer. Before insertion the "borer pipette" was either empty or filled with light mineral oil. The cleaned axon was immersed in mineral oil. While the borer was advanced into the axon (from the cut end) gentle suction was applied. The inner wall of the sheathed axoplasmic cylinder was smooth. The axoplasmic volume in perfused axons was ascertained by measuring the outside and inside diameter of the axoplasmic tube. In these measurements the sheath was excluded. The "normal" volume of the axoplasmic wall was determined with mineral oil both in the bore and in the extracellular space. This volume usually was close to that estimated by knowing the internal diameter of the borer. After swelling completely filled the channel and could no longer be accommodated, the channel was rebored. As maximal swelling was approached, perfusion was discontinued to avoid sloughing of the delicate wall. The measurement of the volume of highly swollen axoplasm was not accurate. The oils were H_2O-saturated.

Measurement of Elastic Modulus of Sheath (Schwann Layer and Connective Tissue)

The method is illustrated in Fig. 1*b*. Both the length and the internal diameter of the fluid-filled tube composed of sheath alone were measured. The axoplasm was extruded using the rubber roller method. Either the axon rested horizontally or was suspended vertically. When suspended vertically the axon was enclosed in a chamber composed of 2 large coverslips in apposition separated by a spacer 1.5 mm thick. The axon was filled with castor or mineral oil and immersed in oil or sea water. Under pressure oil did not cross the sheath. Either pressure was applied from both ends or one end was ligated and pressure applied through the cannula at the other end. The volume of the intraxonal compartment in the collapsed state of the axon could not be ascertained by measuring the internal diameter and length of the ghost axon because the surface was corrugated. At zero or near zero internal pressures the vertical approach was more reliable. In the collapsed state, the internal volume was determined by extrusion (using a roller) of the contents of the axon into the cannula and noting the change in the meniscus. This technique was not accurate and necessitated that during extrusion the horizontal orientation be assumed. The pressure source was a column of oil, water or mercury or a set-up described previously [12]. Care was taken that the region of axon used was branchless. Leakages were controlled by staining the contents of the ghost axon.

Measurement of Swelling Pressure of the Axoplasmic Gel

For previous methods of measuring swelling pressures of gels, *cf.* references [7–10]. Our method is illustrated in Fig. 1*c*. The axon was bored and cannulated as described above. Mercury was used at higher pressures since at lower pressures its weight distorted

the axoplasmic wall and volume measurements were difficult. Castor oil instead of mineral oil was used at the lower pressure ranges since under pressure it penetrates axoplasm less readily and distorts the wall less (its density being closer to that of water). Obviously nonpenetration of the fluid mediating the pressure is a prerequisite for meaningful swelling pressure measurements. That the sheath is appropriately porous for restraint of swelling and solvent equilibration is evidenced from curve 3*B*, Fig. 3, left frame, and Fig. 4 in the foregoing paper [13] and from previous electronmicroscopic studies [15]. The axolemma had to be destroyed (by the rolling method); if it were intact the pressure measured reflected not only $\Delta\Pi_g$ but also $\Delta\Pi_m$. Such measurements are in process but have proven to be very difficult. The external medium was a solution approximating the mobile solute composition of axoplasm (*cf.* [6] for composition). The oils were H_2O-saturated.

Method for Conversion of Axon to Near Perfect Osmometer

The methods are illustrated in Fig. 1*d* and *e*. The axon was bored and cannulated as described above. The bore was filled initially with mineral oil or isotonic KF. In 1*d* the volume of the intraxonal aqueous compartment was determined by noting both the axonal dimensions and the meniscus in the capillary. In Fig. 1*d* the flowmeter capillary was horizontal to avoid an increase in the internal pressure due to osmotic flow. An insulated iron coil was inserted in the capillary and functioned as a stirrer; it was moved back and forth with an external magnet and served to accelerate equilibration of the aqueous medium in the capillary. When the bore was filled with oil the stirrer was not used. The external osmotic pressure was varied by NaCl enrichment or depletion of sea water (*cf.* [1] for calculation). The oils were H_2O-saturated.

Perforation of Axonal Surface

Holes in the axonal sheath were made (1) by a transverse (to the long axis) cut leaving the cut end open, (2) by a parallel (to the long axis) cut at the origin of a branch, and (3) by a tangential shaving cut in a branchless region.

Whether or not the holes functioned efficiently presumably as vents depended in large part upon the manner in which the orifices were made. Using scissors or a single razor blade as has been used in the past was not always satisfactory. The cutter we used was essentially a guillotine shutter. The blades of the shutter were thin razor blades. The cutting edges of the razor blades outlined the aperture of the shutter. Three razor blades were used. The blades alternately overlapped so that the departure from isoplanicity of the cutting edges was determined by the thinness of the blades only.

pH of Solutions

With the exception of sea water or when otherwise indicated the pH was 7.35. Apparently this is the pH of axoplasm of an axon immersed in sea water [11].

Control of Osmotic Pressure of Solutions

The osmotic pressure of sea water was varied by varying the NaCl component. [1] should be consulted for this method. Isotonic solutions of other substances were matched against sea water using a Fiske osmometer. Solutions were also spot checked using the freezing point depression method.

Temperature

Except when indicated otherwise the temperature was 20–23 °C.

Results

The Role of the Sheath and Axoplasm when $\Delta\Pi_{th}=0$

We determined that free swelling (q_m) was suppressed by the sheath as follows:

1. We released the axoplasm from the confines of the sheath by extrusion and noted that it swelled markedly when bathed in isotonic media (Table 1 and Fig. 2*a* and *b*). These media included one (*Ax*) whose mobile solute composition approximated that of normal axoplasm. The composition of medium *Ax* is based on data given in [6]. q_m varied with the anion in the medium. In chloride or in halides of a smaller

Table 1. q_m/q_t (Relative free swelling volume of axoplasm)[a]

Swelling agent	Extruded axoplasm	Bored out axoplasm	
		Axolemma intact[b]	Axolemma destroyed[c]
Isotonic			
K gluconate	5.2–9.5 (18)	4.2–8.0 (6)	4.3–7.1 (4)
NaF, KF	6.1–11 (16)	5.0–8.4 (9)	4.7–7.9 (5)
K glutamate	8.3–17 (6)	7.2–13 (3)	–
Ax pH 7.35	14–30 (12)	12–27 (5)	13–29 (9)
Ax pH 6.4	12–22 (5)	11–24 (3)	10–25 (3)
Ax pH 8.4	21–31 (5)	18–30 (3)	17–27 (3)
Ax+20 mM NaCN	16–28 (5)	15–26 (3)	–
Ax+20 mM NaN_3	15–26 (5)	16–23 (3)	19 (1)
K Isethionate	25–40 (9)	22–34 (5)	19–30 (4)
NaCl, KCl	*D* (21)	*D* (8)	*D* (7)
KBr	*D* (6)	*D* (3)	*D* (2)
KI	*D* (8)	*D* (2)	*D* (1)
KSCN	*D* (6)	*D* (2)	*D* (1)
1.5 M			
KF	1.5–2.3 (6)	–	1.4–2.1 (4)
K gluconate	1.4–2.1 (7)	–	1.3–1.8 (4)
K Isethionate	4.2–9.1 (5)	–	3.8–8.3 (4)

[a] *D*, means dispersed. The number of experiments performed is enclosed in parentheses. *Isotonic* means isotonic in reference to sea water. Unless otherwise indicated the pH was 7.35. The composition of *Ax* is given in [6]. It contained K, Na, Cl, isethionate, aspartate and glutamate in the ratios given in [6], the concentrations adjusted so that the solution was isosmotic to sea water.

[b] The external solution was sea water and the perfusate was the swelling agent indicated. The axolemma was intact at the beginning of the experiment, not necessarily after exposure to a dispersing anion.

[c] The external solution was the same as the perfusate (i.e., the swelling agent indicated).

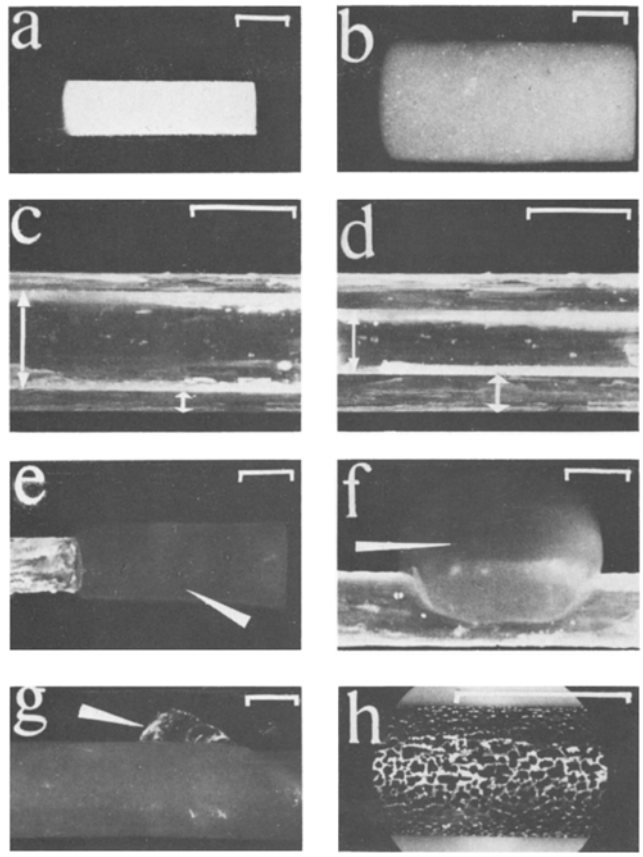

Fig. 2. In all frames the calibration is 0.5 mm. The darkfield background, but not the preparation is retouched. (*a*) The extrudate is immersed in 2.2 M KF. In this medium the extrudate has the same volume as it had while it was encapsulated in the axon (i.e., $q_m = q_l$). (*b*) This is the same extrudate as depicted in frame *a*. It was immersed in isotonic KF and swollen maximally (i.e., $q_m = q$). At this q_m the extrudate is still cylindrical not flattened due to its own weight. (*c*) One double ended arrow subtends the bore in the axoplasm and the other the remaining concentric structure that includes the axoplasmic wall and the sheath. A higher magnification is required to differentiate between the two concentric structures and determine axoplasmic volume. The bore is filled with mineral oil. The degree of swelling of the axoplasmic wall is the same as that of the axoplasm in the normal axon before it was bored (i.e., $q = q_l$). (*d*) This is the same bored out axon as depicted in *c*. The mineral oil in the bore was replaced with isotonic KF. After the axoplasm swelled and filled the bore it was bored out again with the same borer. The new bore was filled with isotonic KF, the axoplasmic wall was allowed to swell maximally and the photograph taken. In this frame $q = q_m$. (*e*) The pointer is on the axoplasmic protrusion from a hole obtained by complete transection of the axon. The length of the protrusion varied in other axons from 0.2–2.5 mm. The shape varied from cylindrical and spherical to amorphous. Usually the diameter of the protrusion next to the cut is larger than the axonal diameter. The preparation was immersed in isotonic KF. (*f*) The pointer is on the axoplasmic protrusion from a hole on the surface of the axon. The hole was made by tangential shaving off of sheath. The preparation was immersed in isotonic KF. (*g*) The pointer is on a cortical flap that was peeled off of the extrudate. A needle was used for this localized skinning. The extrudate was immersed in isotonic KF. (*h*) Perforated perfusion pipette used in water flux measurements presented in the preceding paper [13]. The increase in length seen in *b* did not always occur upon swelling

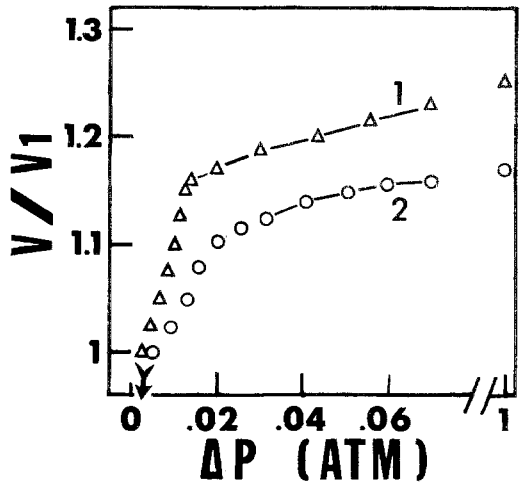

Fig. 3. The volumetric elastic modulus of the axonal sheath. the relative intraxonal volume is plotted against the internal hydrostatic pressure of oil-filled sac comprised of the axonal sheath alone. V_l is the volume of the intraxonal compartment of the normal parent (i.e., before axoplasmic extrusion) axon immersed in sea water. The near vertical arrow near zero ΔP indicates that the volume enclosed by the sheath fell to $^1/_3$–$^1/_7$ of V_l. A ΔP of two to six cm of water was required to attain V_l. The sheaths studied in curves 1 and 2 showed the highest and lowest elastic modulus encountered in 9 sheaths studied. The diameter of the parent axon in curve 1 was 572–579 μ and in curve 2, 592–603 μ

hydration radius the extrudate dispersed. When not dispersed $q_m/q_l = 5$–40. The swelling ratio q is equal to the ratio of the volumes of the swollen (wet) to unswollen (dry) network materials. q_l is the swelling ratio of axoplasm in a normal axon immersed in sea water. The value of q_l is uncertain; it is more than 30 and less than 200 [5].

2. We bored out the axon and noted that the resulting axoplasmic tube upon being perfused with isotonic media swelled only by a decrease in internal diameter, the outer diameter remaining constant (Table 1 and Fig. 2*c* and *d* using method of Fig. 1*a*). The "clogging" of the perfusion channel encountered routinely by investigators working on the perfused squid axon clearly is due to $q_m \gg q_l$.

3. We made an opening in the sheath and observed that axoplasm swelled through it, the opening assuming the appearance of a vent (Fig. 2*e* and *f*). In all nondispersing media of Table 1, the protrusion remained intact indefinitely. In this connection the common allusion to a "flow" of axoplasm from the cut ends of axons immersed in sea water is misleading since it is a dispersion followed by a flow.

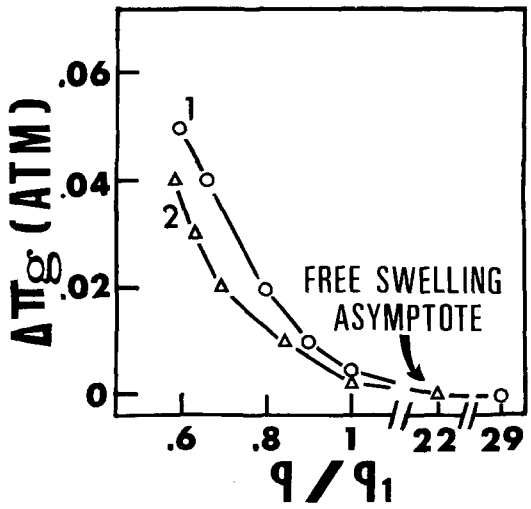

Fig. 4. The osmotic swelling pressure of axoplasmic gels. The hydrostatic pressure is plotted against q/q_l, the relative swelling ratio of the volumes of wet to dry network materials. q_l is the swelling ratio of the axoplasm in a normal axon immersed in sea water. The more orthodox plot of $\Delta\Pi_g$, against q is not possible with the axoplasmic gel since the volume fraction of the uncontaminated network materials is unknown. Curves 1 and 2 were obtained from axoplasmic gels showing the highest and lowest $\Delta\Pi_g$ seen in 5 axons. In curve 1 the $\Delta\Pi_g$ at $q/q_l=1$ was 6.5 cm of H_2O; in curve 2, 1.5 cm of H_2O. The values of 22 and 29 on the q/q_l axis are the q_m/q_l values of the axoplasmic gel of curve 2 and curve 1, respectively. q_m is the swelling ratio of the mechanically unconstrained (free swelling) gel. The diameter of the axon of curve 1 was 782–802 μ and that of curve 2, 805–816 μ

4. We measured the volumetric elastic modulus of the sheath. At zero pressure the sheath collapsed and the volume enclosed by the sheath was $^1/_3$ to $^1/_7$ of V_l (the volume of the intraxonal compartment of the normal axon immersed in sea water). These data are presented in Fig. 3. The localized constriction, or collapse of the sheath seen in cut or otherwise damaged regions of axons immersed in sea water, very likely is related to the absence of the swelling pressure since the axoplasm is liquefied and exuded.

The obvious question is: what pressure keeps the sheath inflated in the "normal" axon? Is the stress on the sheath, $\sigma=-\Delta\Pi_g$, or is $\sigma=-\Delta\Pi_m$? It is more likely that $\sigma=-\Delta\Pi_g$, since the ΔP at V_l (in Fig. 3*a*) coincided with $\Delta\Pi_g$ at q_l (the swelling ratio at V_l) seen in Fig. 4. It is unlikely that $\sigma=-\Delta\Pi_m$ since this may imply a $\Delta\Pi_{th}\sim 20$ to 90 mosmoles and such a difference in chemical composition between the axoplasm and sea water has not been reported in the literature. This calculation is based on measurements given in the adjoining paper [13] where it was found that $\Delta\Pi_m/\Delta\Pi_{th}=0.003$–$0.005$. It is unlikely that $\sigma=-\Delta\Pi_m$ since upon

destroying the solute barrier the volume of the intraxonal compartment did not change appreciably (within 3%). The solute barrier was destroyed by rolling the axoplasm back and forth in the axon. This procedure of destroying the axolemma is effective in the axon of *Loligo vulgaris* and *pealii*, but apparently not always in the axon of *Loligo forbesii*. Destruction was evidenced by (1) an immeasurably low (i.e., less than $^1/_{50}$ the normal) steady state osmotic flow and (2) the absence of any appreciable membrane resistance or potential (in NaCl media). From Table 1 it is seen that other factors (aside from E) need not be considered in explaining q_m/q_l. Namely, since $q_m \gg q_l$ in media differing in composition, concentration and pH, Donnan effects[1] or exposure of the gel to a fluid not quite *identical* to the normal are not contributing factors. Since q_m is the same in the presence of KF or other inhibitors as it is bored out but otherwise intact axons, metabolism is not a consequential factor. Since the q_m of extrudates obtained from *in situ* axons (*cf., Materials and Methods*) is the same as the q_m of extrudates from excised axons, excision *per se* is not responsible for $q_m \gg q_l$.

The Role of the Sheath and Axoplasm when $\Delta\Pi_{th} \neq 0$

In the foregoing paper [13] and in [14] it was shown that $\Delta\Pi_m \ll \Delta\Pi_{th}$. This makes it feasible, when $\Delta\Pi_{th} \neq 0$, that both $\Delta\Pi_g$ and E may play a role in the volume relations of the axon. In other words, if the intra-extraxonal difference in bulk osmotic pressure due to mobile solutes were not attenuated, the resulting swelling or shrinkage of the axon could not be expected to be opposed significantly by the retractive forces of the sheath or the expansive forces of the gel, respectively. The reason for this is that E and more so $\Delta\Pi_g$ are small in comparison to $\Delta\Pi_{th}$. In order to test this idea and attempt to calculate the volume of the axon we had to first measure $\Delta\Pi_m$ (or $\Delta\Pi_m/\Delta\Pi_{th}$), E and $\Delta\Pi_g$. When the axon is immersed in hyperosmotic media the curve is calculated from $-\Delta\Pi_g = \sigma + \Delta\Pi_m$; in hyposmotic media it is calculated from $\Delta\Pi_m + \Delta\Pi_g = -\sigma$. In this calculation we made the serious assumption that the $\Delta\Pi_m$ of unperfused axons is the same as that determined in perfused axons. This may not be justified if certain types of mechanisms are operating to attain the negative anomalous osmosis (*cf.* [13]). The

[1] With the axolemma intact Donnan contributions to q may be ignored since axolemma related mechanisms, not the ionizable groups of gel, control ion distributions. In the absence of the axolemma, Donnan contributions are reduced in isotonic media since salt concentrations are high (~ 0.5 M).

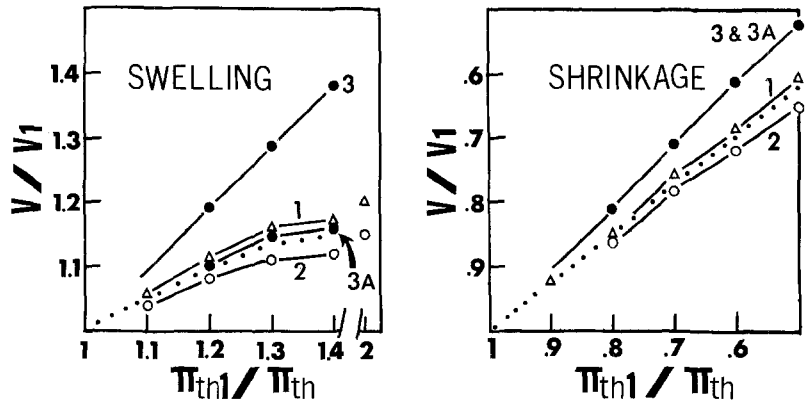

Fig. 5. Osmotic relations of normal axons and of an axon that was converted into a near perfect osmometer. In our unorthodox presentation the experiments in one frame are continued in the adjacent one. In both figures the relative volume of the intraxonal compartment (V/V_l) is plotted against the relative osmotic pressure of the external medium ($\Pi_{th\,l}/\Pi_{th}$). V_l is the volume of the intraxonal compartment of the normal axon bathed in sea water. $\Pi_{th\,l}$ is the osmotic pressure of sea water (~ 1000 milliosmolal). Curves 1 and 2 were obtained from normal axons that were the most and least osmotically responsive of the 18 axons studied. Curves 3 and 3*A* were obtained from the same axon. In both 3 and 3*A* the axoplasm was bored out. Curve 3 was obtained using the method *d* of Fig. 1 and curve 3*A* was obtained after curve 3 by using method *e* of Fig. 1 (i.e., by applying a ligature to the axon in the vicinity of the volumetric cannula). It can be seen that when method *d* of Fig. 1 was used the osmometric behavior both in hypotonic and hypertonic media was near that predicted by Boyle's law. With method *e* of Fig. 1 near perfect osmometric behavior was seen only in hypertonic media. The axon diameter in curve 1 was 526–537 μ; in curve 2, 547–555 μ; in curves 3 and 3*A*, 695–708 μ

values for $\Delta\Pi_m/\Delta\Pi_{th}$ are given in the adjoining paper [13], Figs. 2, 3 and 5 (top) and Tables 1–3. The value for E, the volumetric elastic modulus of the sheath, is given in Fig. 3 of this paper and was mentioned in the previous section. The values for $\Delta\Pi_g$ are given in Fig. 4. At $q/q_l = 1$ (the degree of swelling of axoplasm in the normal axon) $\Delta\Pi_g$ was 1.5 to 6.5 cm of H_2O. As mentioned previously, this pressure is close to the pressure required to inflate the sheath to the point where the volume enclosed by it is the same as that of the intraxonal volume of the normal axon. This indicates that it may be the swelling pressure of the gel that keeps the sheath inflated in the "normal" axon.

Having roughly estimated $\Delta\Pi_m$, E and $\Delta\Pi_g$ we attempted to calculate the volume relations of the axon. The dotted curve in Fig. 5 is the calculated $\Pi_{th\,l}/\Pi_{th}$ versus V/V_l relation of the axon. Curves 1, 2, 3 and 3*A* in the left frame of Fig. 5 are continued in the right frame. We assumed that the membrane is ideally semipermeable and that there was no osmotically

"dead speace" or osmotically "inert material". In this calculation we used the mean values for $\Delta\Pi_m/\Delta\Pi_{th}$, E and $\Delta\Pi_g$. The calculated dotted curve agrees with the experimental curves (curves 1 and 2, Fig. 5) obtained from normal axons. They were the most and least osmotically responsive axons out of 18. Parts of curves 1 and 2 are in agreement with previous work by other investigators [3, 4, 15].

That this agreement in curve fitting is not entirely fortuitous is suggested by an experiment where we contrived an axon that behaved as a near perfect osmometer (Fig. 5, curves 3 and 3*A* using methods of Fig. 1*d* and *c*). Simply, in order to test the idea that $\Delta\Pi_g$ and E affect the volume relations, both $\Delta\Pi_g$ and the stress on the sheath, σ, were minimized. We accomplished this by boring out most of the axoplasm and by allowing the fluid in the bore to extend into a cannula open to atmospheric pressure. By boring out most of the axoplasm we allocated sufficient room for the remaining gel to swell maximally into the bore, i.e., to come in swelling equilibrium with the fluid in the bore. Under these conditions $q=q_m$ and $\Delta\Pi_g \sim 0$, the contribution of $\Delta\Pi_g$ to the total $\Delta\Pi$ is minimized and the resistance to axonal shrinkage by the gel thereby commensurately reduced. Since with method *d* of Fig. 1 the intraxonal compartment is an open system, increases in the volume of this compartment can occur free of the constraints imposed by the sheath. Curves 3 and 3*A* were obtained from the same axon. Curve 3 was obtained by first using method *d* of Fig. 1, then ligating the axon near the cannula so that it became a closed system, and curve 3*A* was obtained. Four other axons showed near perfect osmometric behavior.

Discussion

The entire cytoplasm of many cells and many inclusions and maybe the ectoplasm of all cells are complex swollen polymer networks. Such gels (e.g., "skinned" muscle fibers, nuclei) when released from the confines of the cell surface swell or disperse in isotonic media as does axoplasm. Nevertheless, accepted theories of osmotic behavior dismiss intracellular polymer networks, the cell being viewed as a fluid filled sac. That is to say it is generally thought that the chemical potential of the solvent is not lowered appreciably by the cytoplasmic polymer network. Conformity to Boyle's law is arrived at by invoking that part of the cellular volume (b) is inert so that $P(V-b)=k$. In our treatment (in order to abide by Boyle's law) the pressure, not the volume term, is adjusted.

If our interpretation of the experiments on the axon can be extended to other systems some speculations may be feasible. In the squid axon immersed in sea water we think the stress on the sheath, $\sigma = -\Delta\Pi_g$, and the condition is not truly isosmotic; the interior is hypertonic by an amount equal to the osmotic swelling pressure. In other words in the normal axon there is a turgor pressure as in plant cells though much smaller and due to $\Delta\Pi_g$ not to $\Delta\Pi_m$. In cells with higher protein concentrations (e.g., muscles) the contribution of $\Delta\Pi_g$ to volume relations may be greater than it is in the squid axon.

At q_l the concentration in the axoplasm of the neurofilament protein filarin (presumably the skeletal moiety of the network) is $\sim 0.5\%$ and its $MW \sim 75{,}000$, the concentration of protein adsorbed strongly to filarin, $\sim 1.5\%$, and the concentration of protein in particulates entrapped in the network, $\sim 2\%$ [5]. While primarily the crosslinked filarin may be expected to determine ΔF_{el} other proteins strongly bound to the network may also contribute to ΔF_{mix}. If only filarin were involved a q_m of over 10,000 and a $\Delta\Pi_g$ of over 50 cm H_2O at a q of ~ 120 (i.e., when $V/V_l = 0.6$) would obtain. To our knowledge both these values would be far greater than encountered in any known polymer network. However, assuming 2–3% protein (a fraction of this of MW appreciably lower than 75,000) were involved at q_l, the q_m and $\Delta\Pi_g$ observed would be more realistic. For example this q_m would be of the order seen in hagfish mitin and vitreous body gels. Such attempts at estimating axoplasmic gel properties are speculative. For that matter any attempt at a rigorous treatment of an *anisotropically* swollen, grossly inhomogeneous protein network such as the axoplasmic at present is hopeless. Although nonuniformities are recently being investigated in inanimate swollen networks [e.g., 2], there still is no treatment combining theories of elasticity with theories of polymers in solution that describe the behavior of networks composed of polymers with strong intersegmental forces such as proteins. Also, the elasticity of proteins may be determined by conformational transitions.

As yet certain experiments presented in this paper are not sufficiently well controlled: (1) In the experiment of Fig. 4 we assumed that the scrambling of the axoplasm when the axolemma was being destroyed did not alter appreciably the value of $\Delta\Pi_g$. (2) In calculating the dotted curve of Fig. 5 we assumed that with the axolemma *intact* the change in intraxonal solute concentration as V/V_l was varied did not alter $\Delta\Pi_g$ appreciably. Undoubtedly it did change; the correction factor must be ascertained by a determination of the swelling pressure as a function of the solute concentration in the external fluid with which the gel is equilibrated.

(3) In the experiment of curves 3 and 3*A* of Fig. 5, right frame, we assumed that the boring of the axoplasm, not the replacement of the normal axoplasmic solutes with isotonic *KF*, was responsible for the near perfect osmometric properties observed. (4) In the experiment of curves 3 and 3*A* of Fig. 4 we assumed that boring did not remove "osmotically" inert material. (5) Crucial direct measurements of internal pressure *vs.* volume of aqueous compartment *with the axolemma intact* are not available as yet. (6) In calculating the dotted curve of Fig. 5, we used mean values from different fibers. This curve would have been more convincing if all measurements were made on the same fiber.

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Note in Proof

In very recent experiments the q_m/q_l of extruded axoplasm in isosmotic K isethionate was 10–16 and in solution *Ax* pH 7.35, 6–11.

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