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Cytoplasmic Gel and Water Relations of Axon

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Summary. A previous method of measuring the swelling pressure ($\Delta\Pi_g$) of the cytoplasmic gel of the giant axon of *Loligo vulgaris* was refined. The estimates of $\Delta\Pi_g$ made with the improved method were consistent with those made with the earlier method. In these methods the activity of the solvent in the gel is measured by increasing the activity of the solvent in the internal phase of the gel by application of hydrostatic pressure to the gel directly. Comparable values for the activity of the solvent in the gel were obtained also by an alternate method, in which the deswelling of the gel is measured upon decreasing the activity of the solvent in the external phase by addition of a nonpenetrating high mol wt polymer (i.e., Ficoll).

Additional support was obtained for the earlier suggestion that $\Delta\Pi_g$ contributes to the swelling and shrinkage pattern of the whole axon. In part, the new evidence involved two consecutive *direct* measurements of intraxonal pressure. The first measurement was that of a mixed pressure composed of $\Delta\Pi_g$ and $\Delta\Pi_m$ ($\Delta\Pi_m$ being the effective osmotic pressure due to the intra-extraxonal gradient in the activity of mobile solutes). The subsequent measurement was that of $\Delta\Pi_g$ alone. The latter measurement was made feasible by destroying the axolemma, thereby eliminating the contribution of $\Delta\Pi_m$. An estimate of $\Delta\Pi_m$ was obtained by subtracting $\Delta\Pi_g$ from the total pressure measured initially. The $\Delta\Pi_m$ determined by the above method was two orders of magnitude smaller than the theoretical osmotic pressure. This is consistent with the $\Delta\Pi_m$ determined previously, where osmotic intra-extraxonal filtration coefficients were compared to the hydrostatic. The mixed pressure experiments lend credence to the idea that the substantial contribution of $\Delta\Pi_g$ to the water relations of the whole axon is due to $\Delta\Pi_g$ being of the same order of magnitude as $\Delta\Pi_m$.

The degree of free swelling of axoplasmic gels was studied as a function of pH, salt concentration, and hydration radius of the anion of the salt used. The swelling increased with an increase in the reciprocal of the hydration radius, a decrease in salt concentration, and at pH below or above ~4.5.

The nature of the constraints to the free swelling of axoplasm in axons immersed in seawater was studied. With the seawater employed, these constraints appeared to be due more to the retractive forces of the sheath than to $\Delta\Pi_m$.

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Vargas (1968) and the author (Spyropoulos, 1977*a*) measured water filtration coefficients as a function of hydrostatic and osmotic pressure differences between the inside and the outside of internally perfused squid giant axons. In these experiments:

$$L_p \Delta P = (J_v)_{\Delta \Pi_{th}=0},$$
$$L_{PD} \Delta \Pi_{th} = (J_v)_{\Delta P=0}$$

and

$$\frac{L_p}{L_{PD}} = \frac{\Delta \Pi_{th}}{\Delta \Pi_m}$$

where L_p is the hydrostatic filtration coefficient, L_{PD} the osmotic filtration coefficient, J_v the rate of total water transport, ΔP, the hydrostatic pressure difference between the inside and the outside of the internally perfused axon, and $\Delta \Pi_{th}$ and $\Delta \Pi_m$, respectively, the theoretical and the effective intra-extraxonal osmotic pressure difference due to unequal distribution of mobile (diffusible) solutes. The results obtained by separate determinations of L_P and L_{PD} were consistent with the results obtained by measuring, as a function of $\Delta \Pi_{th}$, the applied steady-state hydrostatic pressure (ΔP) that sustains zero net water flow. In this method:

$$\frac{L_{PD}}{L_p} \Delta \Pi_{th} = -(\Delta P)_{J_v=0}$$

and

$$\frac{\Delta \Pi_{th}}{\Delta P} = \frac{\Delta \Pi_{th}}{\Delta \Pi_m}.$$

With both methods, $\Delta \Pi_m$ was found to be two orders of magnitude smaller than $\Delta \Pi_{th}$. Since the axolemma in the squid axon is almost completely impermeable to the solutes (e.g., sucrose, NaCl) involved in the determination of L_{PD}, Vargas (1968) interpreted this striking attenuation of $\Delta \Pi_{th}$ in terms of a leaky hole or holes; however, my experiments (Spyropoulos, 1977*a*) indicate that the attenuation of $\Delta \Pi_{th}$ is due to an in-series (to the axolemma) resistance to the flow of water. Since the attenuation of $\Delta \Pi_{th}$ was so great, I explored (Spyropoulos, 1977*b*) the consequences of $\Delta \Pi_m$ being of the order of $\Delta \Pi_g$, the swelling pressure of the gel (the cytoplasmic swollen polymer network) which is not attenuated, and of E, the elastic modulus of the sheath. I felt that if this was so, all three, $\Delta \Pi_m$, $\Delta \Pi_g$ and E, should be implicated in the volume relations of the axon.

To this end I measured E and $\Delta\Pi_g$ and found that they were of the same order of magnitude as $\Delta\Pi_m$ (Spyropoulos, 1977*b*). In these experiments E was estimated by measuring the volumetric elastic modulus of isolated sheaths, namely, sheaths that were emptied of axoplasm and filled with oil. The measurement of $\Delta\Pi_g$ was considerably more complex. $\Delta\Pi_g$ is viewed physically in the same manner and expressed in the same thermodynamic quantities as the osmotic pressure of solutions separated by a semi-permeable barrier. Both pressure differences represent the hydrostatic pressure difference required to elevate the vapor pressure of the solvent in the mixture so that it equals that of the pure solvent. The work done against either type of pressure difference is equal to the free energy decrease of the system. Although the conceptualization of the two types of pressures is similar, operationally, their respective measurement is quite different (*cf. Discussion*, for details). Since the methods for measuring $\Delta\Pi_g$ of synthetic gels were not directly applicable to axoplasmic gels, I developed a new method (Spyropoulos, 1977*b*). One of the aims of this paper is to present certain refinements and controls of my earlier $\Delta\Pi_g$ technique and also to present experiments where it was possible to estimate the activity of water in the gel by using a somewhat simpler approach. In the previous approach the activity of the solvent in the gel was measured by increasing it by application of hydrostatic pressure to the gel, while with the alternate approach the activity of the solvent in the gel is estimated by decreasing the activity of the solvent in the surrounding medium by addition of a polymer that does not penetrate the gel.

In my earlier work (Spyropoulos, 1977*b*) axonal volume was calculated as a function of $\Delta\Pi_{th}$ from experimentally obtained values for $\Delta\Pi_g$, $\Delta\Pi_m$ and E. These calculated volumes agreed with the experimentally observed volumes. It appeared that deviations in Boyle-vant Hoff's Law could be explained in most part by the contributions of $\Delta\Pi_g$ and E without necessarily invoking "osmotically inert materials." Ancillary experimental support for the validity of these estimations was obtained by circumventing the contribution of E and $\Delta\Pi_g$, where upon the axon appeared to obey Boyle-vant Hoff's Law, i.e., it behaved as a nearly perfect osmometer. This was accomplished by boring out the axoplasm in an intact axon and allowing the axoplasmic gel to swell inwardly in the lumen until it attained equilibrium. This effectively eliminated the contribution of $\Delta\Pi_g$ when the axon was immersed in hyperosmotic media (i.e., the network did not resist the decrease in volume due to $\Delta\Pi_m$). The contribution of E in the bored out axon was eliminated

by allowing increases in volume when the axon was immersed in hyposmotic media to occur not by a lateral expansion (thereby distending the sheath), but by a longitudinal expansion into a calibrated cannula open to atmospheric pressure. In this manner there was no stress on the sheath, no resistance to an increase in the intraxonal volume. In these previously reported experiments I measured axonal volume as a function of $\Delta\Pi_{th}$. In the experiments to be reported, in order to study the interplay of $\Delta\Pi_g$ and $\Delta\Pi_m$ in the water relations of the axon, I measured directly intraxonal pressure as a function of axonal volume and $\Delta\Pi_{th}$. At the onset of the experiment, I measured a mixed pressure (the sum of $\Delta\Pi_m$ and $\Delta\Pi_g$). This was followed by measurements of $\Delta\Pi_g$. By subtracting $\Delta\Pi_g$ from the mixed pressure, an estimate of $\Delta\Pi_m$ was obtained. These series of experiments constitute one of the main objectives of the work presented in this paper.

Finally, some experiments are included in this paper on the factors that influence the degree of unconstrained, free swelling of axoplasm and some experiments designed to elucidate the nature of the constraints to the free swelling of axoplasm in the normal axon immersed in seawater.

Materials and Methods

The giant axon of the stellate nerve of *Loligo vulgaris*, found in the coast of Italy and Greece, was used. The methods of axoplasmic extrusion of axonal cannulation, boring and perfusion, of measuring volume of extrudates or of the axoplasmic wall in bored out axons, of testing the electrical excitability of the axon, of injection of various substances, etc., are given in previous publications (Spyropoulos, 1977*a*, 1977*b*). Some modifications and pertinent additional details not given previously are presented below.

Axoplasmic Extrusion

The apparatus for cutting the axon (a guillotine type shutter composed of razor blades) and for rolling out the axoplasm (a miniatured version of the double rollers found in the wringers in old fashioned washing machines) used previously were employed again. Two main points of caution must be made. (i) The length of axon that one attempts to extrude must not be too long (not more than ~ 0.7 cm). This is so even though one is tempted to extrude longer portions since this appears feasible. (ii) One must make certain that the lips of the sheath in the cut region are not constricted. The cut end, if clogged, can be freed by flaring with a needle or by making a fresh cut. If the end is sealed or if the length of axon to be extruded was too long, frictional resistance was sufficiently high that rolling of the encapsulated axoplasm results in a longitudinal accordion-type compression that may lead to deswelling. I regarded an extrusion as being acceptable when the *instantaneous* volume of the extrudate was identical to its volume when encapsulated in the normal axon.

Measurement of Swelling of Axoplasmic Gels

The swelling ratio, q, is generally used to express the degree of swelling of a gel. It is equal to the ratio of the volumes of the swollen (wet) to unswollen (dry) network materials. q_m is the swelling ratio of a freely swollen (unconstrained) gel at equilibrium. I have chosen the expression q_1 to equal the swelling ratio of axoplasm in a normal axon immersed in seawater. Since the fraction of uncontaminated network materials is unknown, the absolute value of q is unknown also. The degree of swelling of the axoplasm will be expressed for this reason as a multiple of q_1. I have used the expression V_1 to equal the volume of the intraxonal compartment of a normal axon immersed in seawater.

The extrudate was enclosed in a transparent container whose dimensions were slightly larger than those anticipated for the extrudate. The pool was covered with a coverslip. When the swelling was pronounced (a large q), the axoplasm tended to sag due to its own weight, the network structure being too weak to sustain the cylindrical shape. A rough estimate of volume was made by very gently tilting the microscope (including the chamber containing the extrudate) by 90° so that the axoplasm came to rest on the side of the enclosing compartment. Extreme caution was necessary in measuring the volume of highly swollen axoplasm both extruded and bored out. It is difficult to make such ghostlike swollen gels visible under the microscope without careful manipulation of the optics. Slight water currents tend to sway the gel and either break it up, giving the appearance of dispersion in some instances, or reduce its degree of swelling irreversibly. Since the unstirred highly swollen gel can be made to deswell to the dimensions characteristic of a less swelling immersion medium, the meaning of the dispersion seen upon stirring may on occasion be limited. In previous methods of studying dispersion properties of axoplasm, the bathing medium was continuously stirred (e.g., Hodgkin & Katz, 1949, Gilbert, 1975*b*). Another rather important point not mentioned in the previous paper is the avoidance of surface denaturation. In my chamber the compartment enclosing the extrudate is covered and air is excluded. The extrudate can be transferred through air to different media; however, if it floats at an aqueous-air interface or if not completely covered with an aqueous solution, it may undergo surface denaturation. A miniature pH electrode (*cf.*, Spyropoulos, 1960) 100 μm in diameter and 1 mm long was placed in the vicinity of the extrudate. This was important, since many solutions were not buffered. Extreme care was necessary in measuring axoplasmic volume in KF-treated axons deswollen with Ficoll. At high concentrations of Ficoll the encapsulated axoplasm became noncylindrical and showed corrugations and longitudinal folds. The axon had to be rotated and examined carefully. Unless the estimate of the volume of axoplasm made through the sheath agreed with that made immediately after extrusion in the same Ficoll solutions, the experimental results were discarded.

The following procedure was used to ascertain the degree of swelling of extrudates as a function of pH, salt concentration, and anionic hydration radius. The terminal 3–6 mm segment of axon was extruded, the emptied sheath cut the now terminal 3–6 mm segment of axon extruded, and so on. The relative position of the extrudates while inside the axon was noted. For each stepwise change in pH, salt concentration, or anionic hydration radius a neighboring extrudate was used. At equilibrium swelling, the measurements were confirmed by switching the bathing media between neighboring extrudates. This exchange of immersion fluids was done only once with the pH and hydration radius experiments. With the salt concentration experiments it was repeated several times until each piece of axoplasm was exposed progressively to all the concentrations tested. Naturally, the aforementioned cross-checking procedure is limited to the cases where precipitation or dispersion did not take place. In the experiments where the swelling of extrudates was studied as a function of pH and salt concentration, the axons after excision and after determination of q_1 in sea water were kept 40 min to 2 hr in isosmotic KF. In these

experiments extrusion was carried out in isosmotic KF. Within 2 min of extrusion the extrudate was exposed to the test solution. In the experiments where the swelling of extrudates was studied as a function of anionic hydration radius, extrusion following excision and determination of q_1 was carried out in the test solution. In the experiments where the effects of "aging" were studied on the effects of anionic hydration radius, following excision and determination of q_1, the axons were stored in isosmotic KF or K gluconate (at 0–2 °C).

Swelling Pressure Measurements

A somewhat limited treatment of swelling pressure was given previously (Spyropoulos, 1977*b*); a more thorough one is given in the discussion section of the present paper. In the present series of experiments, I attempted to control three main shortcomings of my previous method (Spyropoulos, 1977*b*). (i) I had employed a method of destroying the axolemma that may have been too drastic. (ii) The liquid pressure mediator that I used in controlling q may have altered the properties of the gel. (iii) The three principal deformation (extension) ratios that characterize the strain of the gel network were not controlled, so they may have changed during a change in q.

1) Previously the axolemma was destroyed by moving the axoplasm back and forth in the axon using a rubber roller. Although axoplasm appeared to move longitudinally *en masse* and although this procedure did not entail a change of volume (within 3%), there may have been some network scrambling that may have reflected upon the pressures measured. In the experiments to be presented, the axolemma was made inoperative by immersing the axon for 8–15 hr in isosmotic KF. In *Results* I have presented some evidence, in addition to that given previously (Spyropoulos, 1977*a*), that KF treatment does destroy the solute flux rate-limiting properties of the axolemma.

In my earlier $\Delta\Pi_g$ experiments (Spyropoulos, 1977*b*) the axolemma was destroyed before the axoplasm was bored out. In the four $\Delta\Pi_g$ experiments presented in this paper, the axolemma was destroyed after the axoplasm had been bored out. In two of the four axons, before boring, the axon was perfused internally with isosmotic KF using a fine slitted capillary (*cf.* description of slitted capillary in section on mixed pressure measurements in *Methods*). In the remaining two axons (*cf.* section on Ficoll deswelling in *Results*) the axon had not been perfused internally before boring and introduction of the oil; thus, immediately following boring the mobile solute composition of the axoplasmic tube was that of the axoplasm in the intact axon.

2) In axonal swelling pressure measurements, the pressure mediating fluid makes direct contact with the gel. The fluid used should neither penetrate the gel under pressure, nor should it react chemically with the gel. In my method I used both mercury and oil. At first I wanted to avoid mercury (the mediator used by other investigators in the past) since it is known to unfold globular proteins, so I used water-saturated light (saybold viscosity 125/135) mineral oil; however, mineral oil seemed to "penetrate" axoplasm under pressure. I then resorted to using castor oil (H_2O-saturated). Castor oil did not appear to "penetrate" by more than 5–7 μm. This was ascertained by replacing the oil in the bore of the axon with an aqueous (isosmotic salt) solution, applying pressure, and noting the thickness of the oil layer between the aqueous gel and aqueous internal perfusate. Hoping that this thin layer of oil may protect the gel from adverse effects of mercury, I switched to the use of mercury at higher pressures. This procedure is tedious, and castor oil (whose composition is ill defined) and mercury, even with the presumably protective oil coating, may have affected the properties of the gel. In the present experiments mercury was avoided altogether at all pressures by using water-saturated Nujol oil (Plough, Inc., Memphis, Tenn.). It did not appear to penetrate axoplasm detectably even at pressures around 150–200 cm H_2O.

3) When swelling pressure studies are employed in the analysis of the structural and elasticity properties of polymer networks it is desirable that the network be amorphous and isotropically swollen at all values of q. This with one exception (von de Kraats, 1968) has not been attained even with synthetic gels. Intact axoplasm is not isotropically swollen. It is grossly nonuniform (e.g., its ectoplasmic regions are denser than the more endoplasmic), and there may be evidence (e.g., Gilbert, 1975*a*) for a helical structuring at several levels of organization. A truly isotropic amorphous state is impossible to attain and not germaine to the purpose of my experiments, which is to relegate a role to $\Delta\Pi_g$ in the water relations of the axon. To this end it would have been desirable to duplicate the state of strain existing in the axoplasmic gel of a normal axon subjected to anisosmotic external media; however, this would have been very tedious. So, I settled for maintaining the principal extension ratios constant at all values of q. The constancy of the extension ratios was accomplished by adjusting at each value of q the longitudinal tension on the axon by controlling the longitudinal separation of the cannulae. By this means it was possible to maintain the ratio $ID/OD/L$ constant where ID is the internal diameter of the axoplasmic tube (i.e., the diameter of the perfused bore) OD the outer diameter of the tube and L the length of the bored out axon. It must be pointed out though that the principle deformation ratios of the unstretched and unpressured but bored out oil-containing axon immersed in normal seawater need not be the same as those of the intact axon.[1]

After the axoplasm was bored (*cf.* Spyropoulos, 1977*b* for method of boring) and the borer emerged from the distal end of the axon, a 50–70 μm pipette was introduced into the lumen of the emerging borer so that its tip was located 2–3 mm from the distal end of the axon. This pipette was filled with H_2O-saturated Nujol oil and connected to a pressure head. As the borer was withdrawn the oil-filled pipette was commensurately advanced and the bore thus filled with oil. After both the borer and the oil-injecting pipette were withdrawn, the bored-out oil-filled axon was double cannulated and the oil in the bore connected to a pressure head (i.e., either a column of Hg or one of oil). The pressure head included a fine (~ 50 μm ID) calibrated capillary. The axoplasm was compressed by increasing the pressure head. q was ascertained by measuring the dimensions of the axoplasmic wall. These dimensions included (i) the outer diameter of the axoplasmic tube (exclusive of sheath), (ii) the thickness of the wall of the tube, and (iii) the inner diameter of the tube (i.e., the diameter of the bore). At first sight one may think that it would have been simpler to measure axoplasm volume from the meniscus of the fluid in the calibrated capillary in the pressure head. This actually is the method employed with $\Delta\Pi_g$ measurements on synthetic gels. Although this measurement proved to be feasible, it turned out to be more difficult than the measurement of the dimensions of the axoplasmic tube. The reason was that as the intraxonal pressure was increased the sheath stretched. This means that the position of the meniscus in the calibrated capillary reflected not only a decrease in axoplasmic volume but also an increase in total intraxonal volume. So it was necessary to measure the dimensions of the axoplasmic tube, which effectively gives axoplasmic volume. In two experiments I employed the meniscus method merely as a cross-check. The control of the thermal expansion of the fluid in the axon, the cannula, and the pressure head turned out to be a nuisance. My precautions to minimize the thermal changes in fluid volume were the following: The entire set-up, including the pressure head, the chamber containing the axon, manipulators, the vertical microscope for axonal observations, and the horizontal microscope used for observations of the pressure head meniscus, was enclosed (with the exception of the microscope oculars) in a double pane (spaced) plexiglass box. Most of the tube containing the pressure mediating fluid

[1] Monitoring the axoplasmic birefringence at different values of q was feasible and may have given some insight as to the state of strain of the system, but this was not pursued.

was doubly sleeved with oversized polyethylene tubing. The axon and the bathing medium were covered above and supported from below with a double pane (spaced) coverslip. Since the coefficient of thermal expansion of Hg was less than that of the oil, most of the pressure head and a part of the cannula, a safe distance from the axon, were filled with Hg. Two thermistors were attached to the surface of the outermost sleeve of the pressure head and one on the outside of the pool containing the axon. When the room temperature in the vicinity of the plexiglass box enclosing the entire set-up varied by a fraction of a degree centigrade, the meniscus method gave some added assurance that the technique of direct measurement of volume was reliable.

In $\Delta\Pi_g$ measurements volume determinations were more accurate with thicker axoplasmic walls (i.e., 1/3 or so the diameter of the axon). Unless q_m was very small and the wall thin with respect to the diameter of the axon, it is difficult to measure q_m in the bored out axon; the reason is that there is not enough room in the bore to accommodate free swelling. In one of my previous $\Delta\Pi_g$ experiments I allowed the gel to swell until it filled the bore and then I rebored the axoplasm and allowed the remaining gel to swell to equilibrium. The $\Delta\Pi_g$-q relation was determined by extrapolation. In all four of the $\Delta\Pi_g$ experiments presented in this paper and four of the five reported previously, q_m was determined by extruding the axoplasm from a segment of axon adjacent to the one used for $\Delta\Pi_g$ measurements. After boring, introduction of oil, and application of isosmotic KF to destroy the axolemma, a slight pressure (2–6 cm H_2O) was applied to the bore so that the swelling was arrested and bore-filling prevented while the axolemma was being destroyed. When the axolemma in the bored out oil-containing axon was intact this was not necessary, since the swelling observed is less than 5% (*cf. Results* for explanation).

In the earlier $\Delta\Pi_g$ measurements the swelling medium was an isosmotic (to seawater) solution that approximated the mobile solute composition of normal axoplasm. In this medium the q_m of axoplasm was greater than in isomotic KF. In addition, my earlier values for q_m in either medium were greater than the more recent ones (*cf. Results* section on factors influencing free-swelling of axoplasm). Despite the differences in q_m, in the composition of the swelling medium and differences due to refinement of the technique, namely, the method of destroying the axolemma, the use of oil instead of Hg as the pressure mediating fluid, and the maintenance of the deformation ratios of the gel, it was surprising that the $\Delta\Pi_g$-q relation did not vary more than it did. Since the variability in this relation using either technique, the older or more recent, was still appreciable, any differences due to the presumed refinements may be masked by some more important unknown variable that inadvertently I did not control.

Mixed Osmotic Pressure Measurements

The method for mixed pressure measurements is illustrated in Fig. 1. Only three axons were used. One was ruined before the experiment was completed. At the onset the intact axons were internally perfused (for 10–12 min) with KF solutions isosmotic to sea water. This perfusion was accomplished by inserting longitudinally all the way through the axon a 90–100 μm in diameter capillary[2] with a slit (25–30 μm in width) on one side (for details of technique *cf.* Spyropoulos & Teorell, 1968; Spyropoulos, 1972). The capillary initially contained in its lumen a platinum wire (50–60 μm in diameter). This served to prevent plugging or facilitated unplugging of the capillary while it was being advanced through the axon. When the slitted capillary emerged from the distal end of the axon the guard

[2] The tip of the capillary was beveled much like a syringe needle to minimize scrambling of axoplasm during insertion.

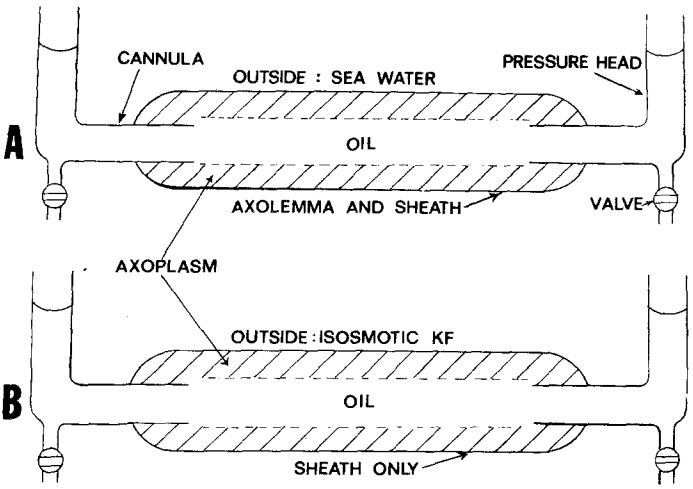

Fig. 1. Method for measuring the relation between pressure and volume of intraxonal aqueous compartment. (*A*): Axolemma is intact. (*B*): Axolemma is inoperative. (For more details of Method *B*, *cf.* Spyropoulos, 1977*b* and text)

platinum wire was pulled out and the axon perfused. It is questionable whether 10–12 min perfusion is sufficient for "full" equilibration of the mobile solute components of the axoplasmic and capillary compartments. Nevertheless, the purpose of the perfusion was to assure that $\Delta\Pi_{th}$ was closer to zero and that the axoplasmic mobile solutes more impermeable than they may be in the intact axon. At the end of perfusion the slitted capillary was withdrawn and the axon allowed to rest for 10–12 min so that the channel created by the slitted capillary was obliterated by the swelling of the axoplasm. At this point in most of the axon there was barely a trace of the tract. Measurements of axoplasmic volume before and after perfusion indicated that it did not vary by more than 0.3%. The remaining method for measurement of mixed pressures (i.e., $\Delta\Pi_g$ and $\Delta\Pi_m$) was the same as that for $\Delta\Pi_g$ measurements, except that initially the axolemma was intact and also except that a concerted effort was made to ensure that the oil filling process and the withdrawal of the borer and oil filling pipette were carried out as rapidly as possible (less than ~ 5 sec). Within 10–20 min, with $\Delta P=0$ (after correcting for capillarity) and the axon immersed in seawater, the axoplasmic wall swelled by 2.5–5.0%. The method of measuring axoplasmic volume was the same as for $\Delta\Pi_g$ except that only the dimensions of the axoplasmic tube were measured (i.e., the meniscus method was not used in conjunction). The pressure-axoplasmic volume relation was determined with the axon immersed in seawater. The intactness of the axolemma was tested by checking the excitability (i.e., a conducted action potential) of the axon by using a micropipette electrode introduced through the surface and two external platinum stimulating electrodes. For $\Delta\Pi_g$ measurements the external seawater was merely replaced with isosmotic KF. $\Delta\Pi_g$ measurements were carried out 8–9 hr after immersions in KF.

Measurement of Intra-Extraxonal Difference in pH

These experiments were undertaken in order to evaluate the properties of the surface of KF-treated axons. The methods were the same as used previously (Spyropoulos, 1960),

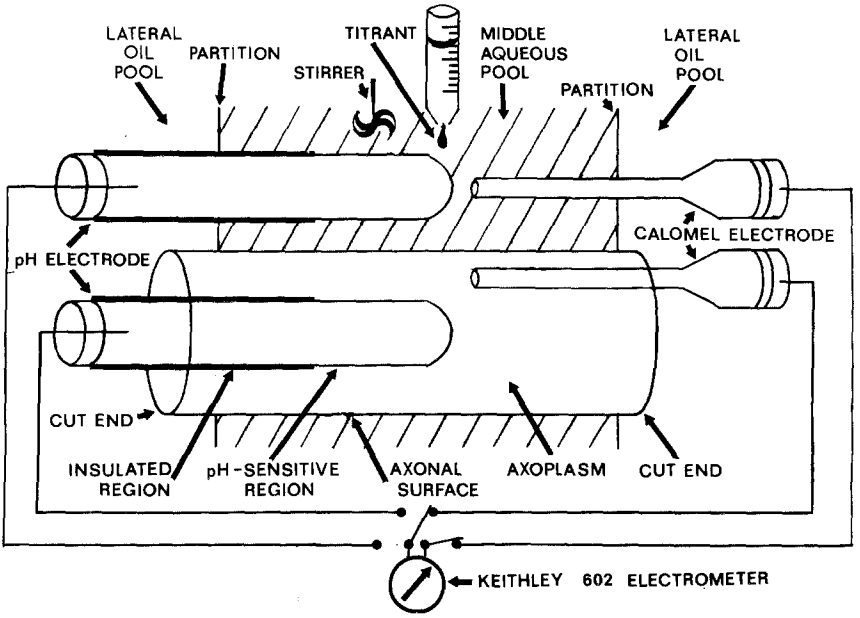

Fig. 2. Method of measuring intraxonal and extraxonal pH (for details, *cf.* text)

except that in addition to the fine internal pH glass electrode, a gross pH glass electrode was immersed in the solution bathing the axon. The method is illustrated in Fig. 2. The potential difference was measured between (i) the internal pH and the external pH electrode, (ii) the internal pH and the external or internal calomel electrode, (iii) the external pH and the internal or external calomel electrode, and finally, (iv) the external and the internal calomel electrodes. At 21 °C the pH of the interior of the normal axon immersed in fresh seawater was found to be the same (i.e., $\sim 7.35 \pm 0.5$) as that found previously (Spyropoulos, 1960) at the same temperature.

Osmotic Pressure of Ficoll Solutions

Ficoll 400 was obtained from Pharmacia. Ficoll was dialyzed with a Union Carbide dialysis tubing against deionized and quartz double distilled water and lyophilized. Ficoll was dissolved in isosmotic KF in concentrations of 2, 4, 6, 8 and 10% (wt/wt). These solutions were used in the axoplasmic deswelling experiments after their osmotic pressure was measured. Previous (Williams, Kraft & Shortman, 1972; Munthe-Kass & Seglen, 1974) osmotic pressure measurements on Ficoll did not reveal values in the region of concentrations in which I was interested. In my method I employed the same type of membranes (Union Carbide dialysis) that were used in the Ficoll purification procedure. They were backed with a nylon mesh to prevent ballooning. These Union Carbide membranes are regenerated cellulose membranes with a molecular weight cut off at 12,000–14,000. Both a zero-flow and a static equilibrium-type, 2-cell type osmometric method were used. The temperature was kept at 21 °C. The osmotic pressure of the Ficoll solutions was measured against the supporting electrolyte (isosmotic KF). The results of the experiments are given in Fig. 3.

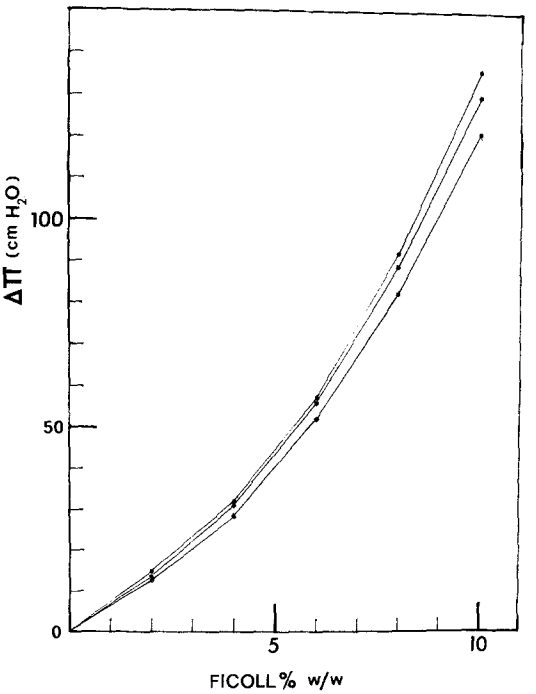

Fig. 3. Determination of osmotic pressure of Ficoll solutions. Temperature was 21.0 °C. The zero-flow method with a 2-cell type osmometer was used. The membrane was a Union Carbide dialysis membrane. The Ficoll-400 was obtained from Pharmacia. It was purified by dialysis (again a Union Carbide dialysis membrane was used) and lyophilized. Ficoll was dissolved in isosmotic (to seawater) KF. The osmotic pressure of the Ficoll solution was measured against isosmotic KF

Measurement of Osmotic Pressure due to Diffusible Components of Axoplasmic Extrudates

Only two determinations were undertaken. One pooled batch of axoplasm was derived from 28 and the other from 32 axons ~ 500–800 μm in diameter. The axons after excision were carefully blotted with lens paper. The double roller extrusion apparatus described previously was mounted vertically over a vessel containing 1 cc deionized and quartz distilled (initially pH 5.6) H_2O and a removable platinum wire mesh at the bottom. The vessel rested on the pan of a Mettler balance so that the weight of the vessel alone, the platinum mesh, the fluid, and the axoplasm could be measured progressively. The extrudate, as it emerged from the axon, dropped directly into the fluid of the vessel and upon sinking came to rest on the platinum mesh platform. The extrudates were allowed to stand for 8–9 hr to swell and lose their diffusible components. At the end of this period the fluid was stirred very gently for 10 min and the solid material removed by lifting the platinum mesh out of the vessel. The osmotic pressure was measured with a Fiske osmometer.

Tracer Efflux Measurements

Glucose C-14, sucrose C-14, inulin C-14, and tritiated water (THO) were Amersham products. The radioactivity was measured with a Packard Tricarb Liquid Scintillation

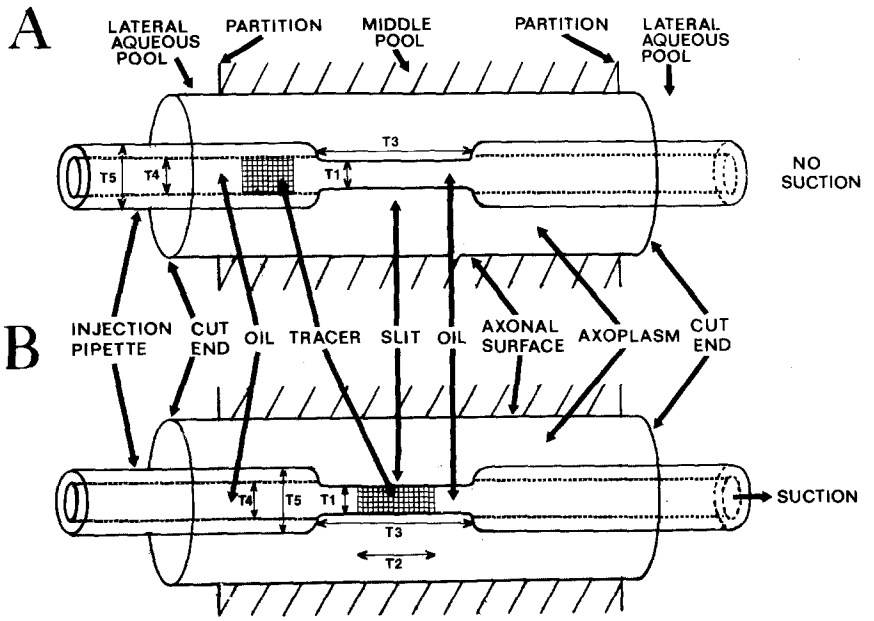

Fig. 4. Method of introduction of tritiated water (THO) into axon without increasing the internal pressure. The slit width was ~ 50–55 μm and the slit length 11 mm. T_1 was ~ 60 μm, T_2, ~ 9 mm; T_3, ~ 11 mm; T_4, ~ 95 μm; and T_5, ~ 120 μm

spectrometer 574. For efflux measurements, glucose C-14, sucrose C-14, and inulin C-14 were dissolved in isosmotic KF and injected into the axon as described previously (Spyropoulos, 1977*a*). The method of determining THO efflux was different. This is presented in Fig. 4. A glass capillary with two lateral slits was inserted all the way through the axon so that the slits were located in the middle of the axon. Initially, (condition *A* in the figure) the lumen of the capillary in the region of the slits contained H_2O-saturated light mineral oil. Since the capillary was fragile, a tungsten wire was slipped into its lumen. When the tip of the capillary emerged from the opposite end of the axon, the wire was removed. A short distance (~ 3–4 mm) away in the unslitted region the capillary contained isosmotic KF and THO.[3] The external fluid ran past the axon and was collected in a fractionator. The lack of any appreciable radioactivity in the effluent indicated that the oil interposed between the slits and the THO containing aqueous plug in the capillary acted as an efficient sealant. In condition *B* in the figure a slight suction was applied from one end of the capillary so that the aqueous contents of the capillary were moved to the slitted region and made contact with the axoplasm. In this manner, THO was introduced into the axon with a transient, slightly negative pressure. This variation in the THO efflux technique was prompted by an objection that the time course of the initial efflux may be affected by the pressure inherent in the injection procedure used previously (Spyropoulos, 1977*a*). The fluid flowing by the axon was seawater or 0.54 M KF. The resolution of the fractionator that collected the effluent was 0.2–0.3 sec.

[3] It would have been desirable that the length of the THO plug be more than 9 mm (the length that was used) to minimize end effects, namely, the contribution of longitudinal diffusion. However, this proved technically difficult.

Temperature

The temperature, unless indicated otherwise, was 21–22 °C.

Water and pH

The water used was deionized and quartz distilled. Unless indicated otherwise, the pH of the solutions was 7.35 ± 0.5.

Results

Direct Measurements of $\Delta\Pi_m$ and $\Delta\Pi_g$ in the Same Axon

Previously (Spyropoulos, 1977*b*) I measured $\Delta\Pi_g$ in a set of axons, $\Delta\Pi_m$ in another set, and E in the sheaths of still another set. From the average values of $\Delta\Pi_m$, $\Delta\Pi_g$ and E, I calculated axonal volume as a function of $\Delta\Pi_{th}$. The calculated volumes were in agreement with the experimentally observed volumes. The deviations in Boyle-vant Hoff's Law could be accounted for by the most part by the contribution of $\Delta\Pi_g$ and E. By circumventing the contribution of $\Delta\Pi_g$ and E the axon obeyed Boyle-vant Hoff's Law; i.e., it behaved as a near perfect osmometer. In the experiments to be presented the interplay of $\Delta\Pi_g$ and $\Delta\Pi_m$ (not of E)[4] was approached from a different angle, namely, by measuring in the same axon $\Delta\Pi_g$ and $\Delta\Pi_m$ at different $\Delta\Pi_{th}$ and axonal volumes. This approach involved two consecutive direct measurements of pressure,

[4] At first sight, since I measured (*cf. Methods*) total intraxonal volume as a function of internal hydrostatic pressure, one may think that E could have also been measured. However, the experiments were done with the double cannulation technique, and longitudinal tension was always applied to the axon by adjusting the intercannular separation distance. Since the longitudinal load on the sheath is unknown, E could not be determined. Previously (Spyropoulos, 1977*b*) I employed both the single and the double cannulation technique in determining E of the isolated sheath. With the double cannulation technique at no time was longitudinal tension applied. The increase in length at higher internal pressures was ascertained by just barely straightening out the bow in the sheath that was the result of the two ends being fixed. The distance between two reference points on the sheath that had been marked in the intact axon by ejection of a dye through a micropipette gave a measure of length referred to that in the intact axon. Still, from the experiments mentioned in this section, one can conclude that since the sheath can withstand the combined pressures of $\Delta\Pi_g$ and $\Delta\Pi_m$ (i.e., since it acts as a back-up resistive well against which axoplasm is compressed) that at high internal pressures E is very large. As a matter of observation the sheath can withstand more than 6 atm of hydrostatic pressure without rupturing; however, its yield point is exceeded at these high pressures. Between 1 and 6 atm the distension is slight. What the breaking point is I do not know, since it was around 6 atm that the cannulae leaked.

one of a mixed pressure (the sum of $\Delta\Pi_g$ and $\Delta\Pi_m$), and one of $\Delta\Pi_g$ alone. The method is illustrated in Fig. 1 and described in *Methods*. The experimental set-up was the same as that used in $\Delta\Pi_g$ measurements. Namely, the axoplasm in an axon was bored out, the lumen filled with oil, the oil through a canulla and tubing was connected to a pressure head that incorporated a calibrated fine capillary. The method differed from the $\Delta\Pi_g$ method only in that the axolemma was intact initially; consequently, the pressure measured initially was the sum of $\Delta\Pi_g$ and $\Delta\Pi_m$.

As mentioned in *Methods* at the beginning of the mixed pressure experiment, with the axon immersed in seawater and before the axoplasm was bored out, an attempt was made to assure that $\Delta\Pi_{th}=0$. This was done by perfusing the axon with isomotic KF, (without axoplasmic boring), using a slitted fine capillary. Under these conditions at zero applied pressure upon boring and introduction of the oil, I expected that the gel should not be in a steady state. Since $\Delta\Pi_{th} \sim 0$, I expected the gel to be hypertonic to the extracellular phase by an amount equal to $\Delta\Pi_g$. The steady state, I felt, would be attained when water flowed across the axolemma and swelled the gel until $\Delta\Pi_g$ was balanced by the resulting $\Delta\Pi_m$. This turned out to be the case. Within 10–20 min following the introduction of oil into the bore, the axoplasmic wall swelled by 2.5% (axon *3*, Fig. 5), 3% (axon *1*, Fig. 5) and 5% (axon *2*, Fig. 5)[5]. As the internal pressure was increased from zero by raising the pressure head (thereby compressing the axoplasm against the sheath), I expected that $\Delta\Pi_g$ should increase nonlinearly with the reciprocal of q, while $\Delta\Pi_m$ should go through zero reverse sign and increase linearly with the reciprocal of axoplasmic volume. Finally, I had also anticipated that upon destroying the axolemma with KF-treatment[6] that the contri-

[5] The swelling of the bored out axon immersed in seawater was slight only when the axolemma was intact and the bore filled with oil. When the axolemma was destroyed and the bore filled with oil at $\Delta P=0$, the swelling of the axoplasmic wall was far more pronounced; it tended to approach swelling equilibrium, opposed only by the resistance of the oil being displaced out of the cannulae. When the bore was not large enough the gel swelled, filling the lumen. Since the rate of swelling along the bore was not the same, the oil was not simply ejected through the axonal ends. Invariably some oil was trapped and usually broken up into beads. With the axolemma intact or destroyed and the bore filled with aqueous isosmotic solutions, the gel swelled freely, provided there was enough room in the lumen (Spyropoulos, 1977*b*).

[6] Previously (Spyropoulos, 1977*a*) I demonstrated that when axons were immersed in isosmotic KF (our presently employed method for destroying the axolemma) $L_{PD}=0$ which means $\Delta\Pi_m=0$. Further controls in support of the idea that KF treatment destroys the axolemma are given in a later section.

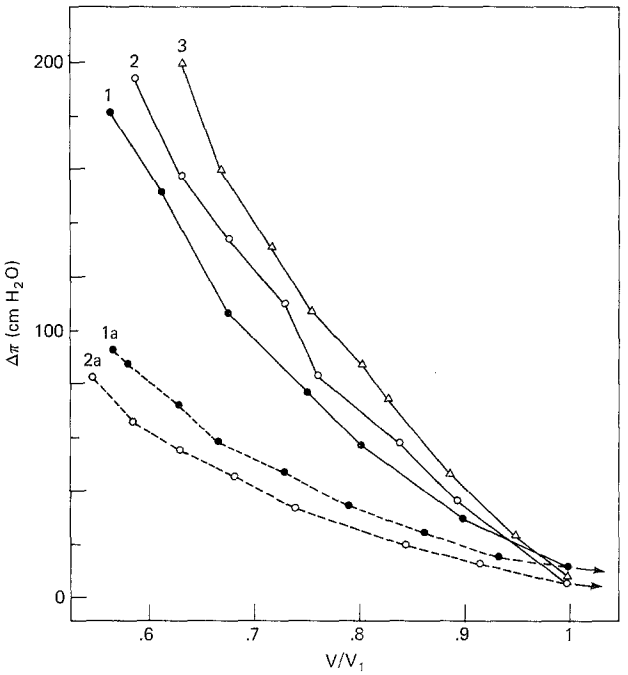

Fig. 5. The relation between pressure ($\Delta\pi$) and relative volume (V/V_1) of intraxonal aqueous compartment with axolemma intact and axolemma inoperative. The method of Fig. 2 was employed. The experiments with the axolemma intact are indicated by the continuous line and those with the axolemma inoperative (by KF treatment) by the interrupted line. With the axolemma intact the pressure-volume relation was carried out with the axon immersed in seawater, and with the axolemma inoperative, in isosmotic KF. V_1 is the volume of the intraxonal compartment of a normal axon immersed in seawater *minus* the volume of the bored-out axoplasm. When the axolemma was made inoperative it would have been more orthodox to plot $\Delta\pi$ *vs.* q/q_1 (*cf. Methods* for definitions); however, in this particular experiment $V/V_1 = q/q_1$. Axon number 3 was ruined before the hydrostatic pressure-deswelling measurements could be carried out. A comparison of the hydrostatic pressure-deswelling curves in this figure and in Fig. 7 of this paper with those of Fig. 4 in my previous paper (Spyropoulos, 1977*b*) show that refinement in the technique did not yield markedly different results. The diameter of axon number 1 was 710–722 μm, axon 2, 736–752 μm, and axon 3, 645–658 μm. The temperature was 20–21 °C. The q_m/q_1 in isosmotic KF of axon No. 1 was 2.3; of axon No. 2, 1.9; and of axon No. 3, 2.0

bution of $\Delta\Pi_m$ to the $\Delta\Pi$ *vs.* V/V_1 relation would be obviated and the relation would be reduced to $\Delta\Pi_g$ *vs.* q. It can be seen from Fig. 5 that these expectations appear to have been realized. The mixed pressure measurements are given in curves 1–3. It can be seen that although the pressures measured are greater than $\Delta\Pi_g$ given previously (Spyropoulos, 1977*b*), they are two orders of magnitude smaller than expected had $\Delta\Pi_{th}$ not been attenuated. It can also be seen (curves 1*a* and 1*b*) that KF treatment results in a reduction of the pressures measured and

that these are of the order of the $\Delta\Pi_g$ seen previously by the author. One of the axons (i.e., No. *3*) was ruined after the mixed pressure measurement. Although $\Delta\Pi_g$ data are not available on this axon, it was included in the figure to show that the mixed pressure was of the order of magnitude seen in the other two axons.

Since, with the axolemma intact the compressed axoplasmic volumes in the experiments of Fig. 5 were steady state, one may assume that the internal mobile solutes (in large part KF) are impermeable. Initially at $\Delta P = 0$ the osmolality due to diffusible solutes in the axoplasm should have been close to that of the immersion medium (seawater). This osmolality is close to 1.0. From this assumption I made an estimate of the osmolality of the interior of the axon at different clamped axonal volumes. From these I obtained $\Delta\Pi_{th}$. $\Delta\Pi_m$ is an expression of the attenuation of $\Delta\Pi_{th}$. In the two axons shown in the figure the attenuation ratio $\Delta\Pi_{th}/\Delta\Pi_m$ was around 120 and 160. These values compare fairly well with those obtained previously (Vargas, 1968; Spyropoulos, 1977*a*) by comparing osmotic to hydrostatic filtration coefficients. In contrast to the previous methods, in the present one the problem of leakage is not easily invokable as long as the volume of the internal aqueous compartment is constant at elevated internal hydrostatic pressures. One of the main conclusions of the experiments of Fig. 5 is that $\Delta\Pi_g$ and $\Delta\Pi_m$ are of the same order of magnitude and that they both can contribute substantially in determining the volume of the intraxonal aqueous compartment.

Evaluation of the experiments presented in this section, as well as those presented in the following section, is predicated to some extent on understanding the properties of the surface of KF-treated fibers. These experiments are presented in still a later section.

There is a feature of my experimental technique that may complicate my picture of what is going on upon compressing the axoplasmic wall with the axolemma intact. The oil in the bore was H_2O-saturated by shaking for a day or two Nujol oil with isosmotic solutions of KF. Two to three days were allowed for phase separation after the shaking. When, in the mixed pressure experiments, the axoplasmic wall is compressed and the concentration of solutes in the wall is increased, there may well be a flux of solutes and water between the two phases (i.e., the now hyperosmotic axoplasmic wall phase and the oil phase previously equilibrated with isosmotic KF). The solubility of electrolytes and water in oil is quite small; however, so is the volume of the axoplasmic wall; in contrast, the volume of the oil in the bore may be regarded

as being considerably greater since it mixes with the oil in the cannula during the experiment. The other possibility, that pressure *per se* ($\sim$ 100–200 cm H_2O) can affect salt and H_2O distribution between the oil and aqueous phases, also cannot be excluded entirely.

Estimates of Activity of Solvent in Axoplasmic Gels by Measurements of Deswelling with Nonpenetrating Polymers

The two swelling pressure measurements given in the previous section were consistent with the five reported previously (Spyropoulos, 1977*b*). The swelling pressure technique was refined and some disturbing features controlled. Still both methods involve drastic operations (e.g., boring out of axoplasm contact with oil, etc.) that I felt may be controlled by using an entirely different approach. This is the purpose of the experiments presented in this section.

Since equilibrium swelling of a gel is attained when the thermodynamic potentials of the solvent outside and inside the gel are equal, it follows that lowering the solvent activity outside the gel without changing that of the solvent activity in the gel should result in a deswelling of the gel. One method whereby the activity of the solvent outside the gel can be lowered is by dissolving a polymer in it which, because of its size, does not penetrate the gel phase (*cf. Discussion* for further treatment of this problem). These experiments in which the external activity of H_2O is lowered by the inclusion of a high molecular weight polymer yield essentially the same type of information as the experiments where the internal activity is raised by application of hydrostatic pressure to the gel (i.e., the hydrostatic pressure-deswelling technique). Operationally, in both types of experiments the activity is determined osmometrically. The polymer selected to reduce the activity of the salt solution surrounding the gel was Ficoll 400. Ficoll is a synthetic copolymer of epichlorohydrin and sucrose. Its average mol wt is 400,000, it has a high solubility in water ($< 50\%$), a Stoke's radius $\sim$ 2000 nM, and its osmotic pressure at concentrations where the solutions were not too viscous were sufficiently large for the type of experiments envisioned.

Surprisingly, in Ficoll solutions the deswelling of extruded axoplasm was transient. Transient deswelling was observed (i) when the extrudates that had been allowed to swell maximally in isosmotic KF were exposed to Ficoll and (ii) when axoplasm was extruded directly in isosmotic KF solutions containing Ficoll. This implies either that axoplasm is

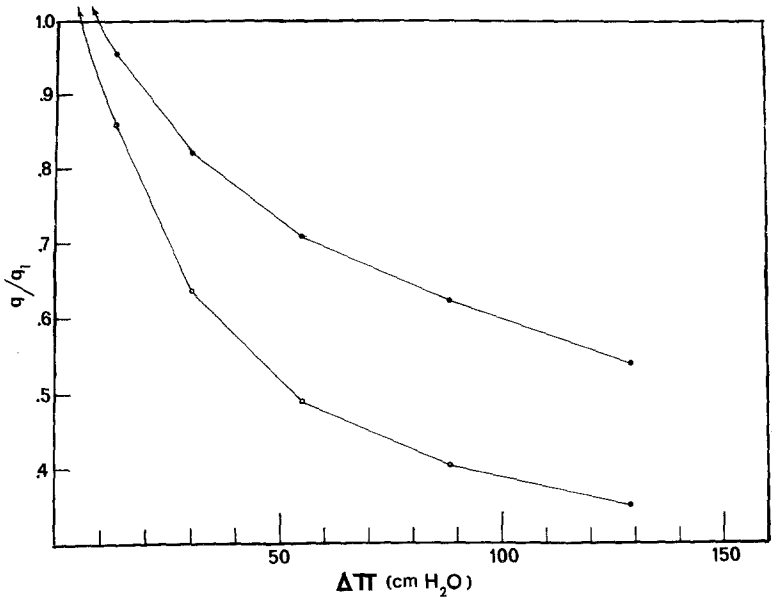

Fig. 6. Relative swelling ratios of axoplasmic gels as a function of the osmotic pressure of Ficoll solutions. Ficoll concentrations were translated into $\Delta\pi$ from the experiments of Fig. 3. The osmotic pressure of the Ficoll solutions bathing the sheathed axoplasm is plotted against q/q_1, the relative swelling ratios of axoplasm. The swelling ratio, q, is equal to the ratio of the volumes of wet (swollen) to dry (unswollen) polymer network components. q_1 is the swelling ratio of axoplasm in the normal axon immersed in seawater (*cf. Methods* for definitions). The two experiments presented were obtained from axoplasmic gels showing the highest and lowest $\Delta\pi$ in 34 preparations. The axons had been immersed in isosmotic KF for 10 hr before the experiments started. Comparison of the results in this figure with the results of Figs. 5 and 7 this paper and Fig. 4 in a previous (Spyropoulos, 1977*b*) reveals that the values obtained for the osmotic pressure of the axoplasmic gel using the Ficoll-deswelling are similar to those obtained using the hydrostatic pressure-deswelling technique. The axon diameter in the experiment represented by the upper curve was 517-522 μm and that by the lower curve 583–605 μm. The q_m/q_1 of the continuous line axon was 1.8, and of the interrupted, 2.1

permeable to Ficoll inherently, or that the process of extrusion *per se* makes it permeable. Steady-state extrudate deswelling was obtained with Blue Dextran T-2000 (obtained from Pharmacia). This has a mol wt of around 2,000,000; however, its high viscosity (at 20°/η 0.7) and low osmotic pressure made it unsuitable. I then resorted to examining the effects of Ficoll on the axoplasm of KF-treated whole axons. As mentioned previously, in these axons the axolemma is inoperative. The deswelling obtained was not transient. I felt that it did not matter whether this is because the investitures of axoplasm or axoplasm itself is impermeable to Ficoll.

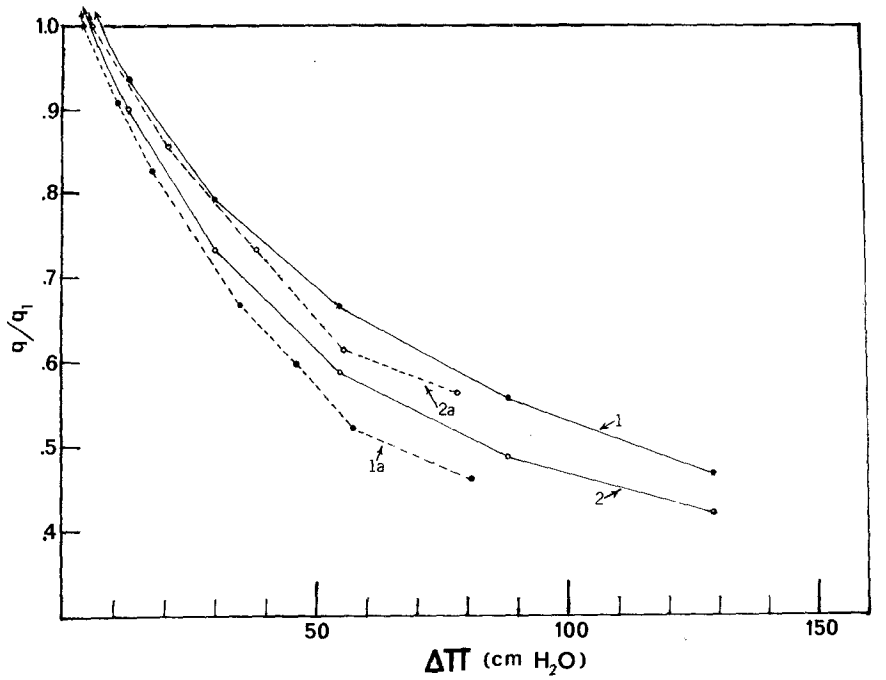

Fig. 7. Comparative measurements of osmotic pressure of axoplasmic gels using both the hydrostatic pressure- and the impermeable polymer-deswelling method on the same axon. $\Delta\pi$ (interrupted lines) represents, with the hydrostatic pressure-deswelling method, the hydrostatic pressure measured. With the impermeable polymer-deswelling method $\Delta\pi$ (continuous lines) represents the osmotic pressure of the Ficoll solution as seen in Fig. 3. Both the polymer-deswelling and hydrostatic pressure-deswelling experiments were carried out with the axon immersed in isosmotic KF. Before the experiments started the axons had been immersed in KF for 8–10 hr. Axon number 1 was 673–688 μm in diameter and 12 cm long. Axon number 2 was 715–735 μm in diameter and 15 cm long. 4.5–5.0 cm from each axon were used for the swelling pressure experiments. The q_m/q_1 in isosmotic KF of the continuous line axon was 1.8 and of the interrupted line axon, 2.0

The results of experiments on 34 axons are presented in Fig. 6. A comparison of this figure with Fig. 4 in my previous paper (Spyropoulos, 1977*b*) and with the two swelling pressure experiments in Fig. 5 of the present paper indicate that the estimates of the pressures developed by the axoplasmic polymer network made by the two different methods (i.e., Ficoll-deswelling and hydrostatic pressure-deswelling) are similar. Some further assurance that the measurements were reliable was obtained by using both methods on adjacent portions of the same axon. The q_m of the axoplasm was determined by extrusion of adjacent portions of the same axon. These results are presented in Fig. 7. An observation of some consequence (*cf.* later section and *Discussion*) was made while the axon was still immersed in seawater and its axolemma as yet had

not been destroyed. In preparation for the swelling pressure experiments the axoplasm was bored out and the bore filled with oil. At $\Delta P=0$, within 10–20 min the wall swelled by 2% (axon of curve *1*) and 4.5% (axon of curve *2*). After the swelling of the wall reached a steady state, the axolemma was destroyed by KF-treatment and the $\Delta\Pi_g$ experiments were initiated.

That the sheath and/or the axoplasmic surface of KF-treated axons is impermeable to Ficoll is indicated by the experiments presented in Fig. 8. In these experiments the axons are bored out, filled with isosmotic KF, and immersed in isosmotic KF containing various concentrations of Ficoll. The external solution was stirred. In order to prevent a change in the osmotic pressure of the intraxonal compartment, the fluid in the bore was continuously renewed. This was accomplished simply by keeping one of the two pressure head capillaries higher by 1–2 cm in an alternating, see-saw fashion. The capillaries through a reservoir containing isosmotic KF were connected to the bore of the axon. The reservoir fluid was stirred with an externally operated magnet. The stirred reservoir fluid in conjunction with the see-sawing action gave some assurance that the fluid in the bore was renewed. The hydrostatic pressure required for zero flow was determined at different Ficoll concentrations. The osmotic pressure measured with the bored out KF-treated axon agrees fairly well with the data of Fig. 3, where the pressure was measured with a classical osmometer employing a dialysis membrane. Actually, two of the axons (Nos. *1* and *3*) of Fig. 8 had been used in the swelling pressure experiments of Fig. 7. Simply, at the end of the swelling pressure experiment, the oil in the bore of the axon was replaced with isosmotic KF. In the experiment in Fig. 8 the axoplasm does not contribute to the "colloid" osmotic pressure of the intraxonal compartment. The reason is that the gel is bored out and in swelling equilibrium with the aqueous fluid in the bore so that $\Delta\Pi_g=0$.

Permeability of KF-treated Axons

A serious source of concern in the evaluation of both the hydrostatic pressure- and the Ficoll-deswelling experiments is the validity of using KF treatment to eliminate the mobile solute-barrier characteristics of the axolemma. To alleviate this concern, I attempted to further characterize the permeability properties of the surface structures of KF-treated

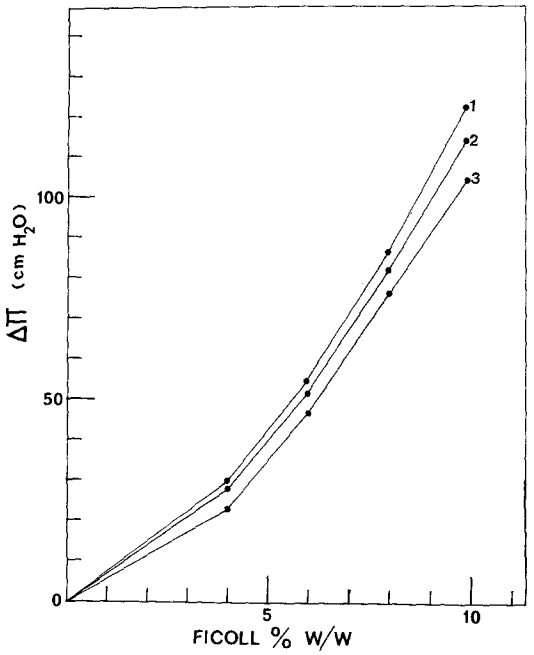

Fig. 8. Relation of concentration of Ficoll to the hydrostatic pressure required to sustain zero net volume flow across the surface of perfused KF-treated axon. The bore of the axon contained isosmotic KF at pH 7.4, and the axon was immersed in isosmotic KF, pH 7.4, containing various concentrations of Ficoll. Pressure was applied to the interior of the axon. Axons 1 and 3 are the same as those used in Fig. 7 except that the oil in the bore was replaced with isosmotic KF. Axons 1 and 3 had been exposed to isosmotic KF for 29 hr. Axon 2 was exposed for 11 hr. Comparison of the results in this figure with those of Fig. 3 show that the system (bored-out KF-treated axon) behaves as an osmometer; i.e., the values obtained with a dialysis membrane were similar. The temperature was 21 °C. The diameter of axon 1 was 673–688 μm, of axon 2, 613–632 μm; and of axon 3, 715–730 μm

axons. Previous experiments (Spyropoulos, 1977*a*, 1977*b*) reveal that such axons show (i) an L_{pt} that is close to zero, (ii) a L_p and efflux rate of THO that are indistinguishable from those of the normal axon, (iii) a $\sim$ 100-fold increase in the efflux rate of injected Na_{22}, and (iv) no significant membrane electrical resistance or capacitance. To these properties should be added the observation presented in the previous section indicating that KF-treated bored out axons are impermeable to Ficoll. In the present work I studied the intra-extraxonal exchange of H^+ and OH^-, the efflux rate of injected glucose C-14, sucrose C-14 and inulin C-14, and, using a new method again, of THO. The results of these experiments are presented in Figs. 9–11. Only three axons (curves

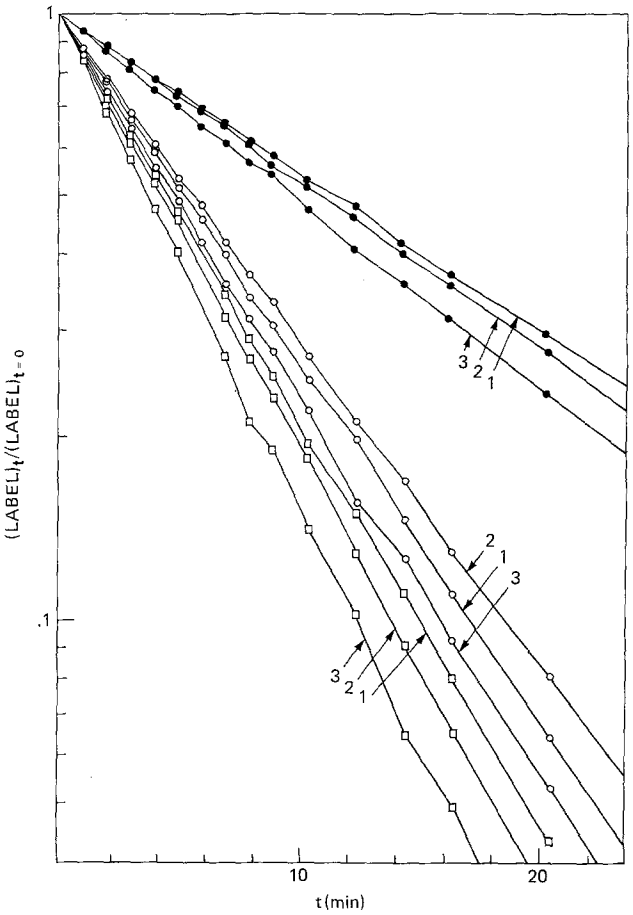

Fig. 9. Efflux rates of injected labeled nonelectrolytes in KF-treated axons. The logarithm of the fraction of initial labeled injected nonelectrolyte remaining in the axon and sheath $[(\text{Label})_t/(\text{Label})_{t=0}]$ is plotted against efflux time. The KF treatment lasted 12–13 hr. Note that curves are straight lines (with an intercept at $t=0$ close to unity). Three (coded *1, 2, 3* in figure) axons were used. All three labeled nonelectrolytes and THO were injected in each axon. The filled circle depicts inulin, the open circle, sucrose and the open squares, glucose. The rate of efflux of the nonelectrolytes in the normal axon was too slow and the rate of THO efflux was too rapid to be included in the figure. The rate of nonelectrolyte efflux is given in the text. The rate of THO efflux in the KF-treated axon was 42, 44 and 47 seconds, respectively, for axons *1, 2* and *3*. Two hr were allowed between consecutive injections so that the previously injected label was washed out almost completely. The sequence of injections was in the order of decreasing efflux rates. The diameter of axon *1* was 508–521 μm, axon *2*, 513–525 μm, and axon *3*, 503–518 μm. The temperature was 20–21 °C

1–3 in Fig. 9) were used in studying the effects of KF-treatment on labeled glucose, sucrose, and inulin efflux. *After* KF-treatment the efflux of all three labeled compounds were measured on each axon. The effluxes

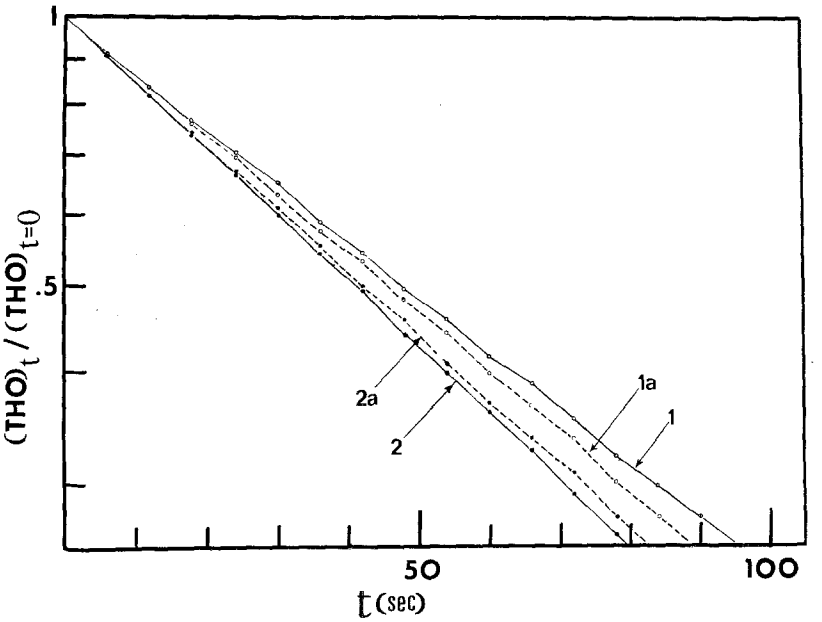

Fig. 10. Efflux rates of THO from normal and KF-treated axons. This is a semilogarithmic plot of fraction of initial labeled water remaining in axon and sheath $[(THO)_t/(THO)_{t=0}]$ against efflux (washout) time (t). The axon was loaded with THO, using the technique of Fig. 4. Continuous lines refer to normal axons immersed in seawater. Interrupted lines refer to axons that had been kept in isosmotic KF for 15–16 hr. *1* and *1a* are from the same axon; *2* and *2a* from another axon. Note that the efflux curve is a straight line (i.e., with an intercept at $t=0$ close to unity). These efflux curves obtained with a new technique are similar to those previously obtained (Spyropoulos, 1977*a*), using the simple injection method. The diameter of axon *1* was 512–515 μm and of axon 2, 504–517 μm

in the normal axon (i.e., before KF-treatment) were more limited.[7] Glucose C-14 was injected in the normal axon No. 1, inulin in the normal axon No. 2, and sucrose in the normal axon No. 3. In the normal axons immersed in seawater the 1/2 time of efflux of glucose C-14 was 32 hr, that of sucrose C-14, 135 hr, and that of inulin C-14 more than 200 hr. Inulin measurements were close to the resolution of my technique. After the normal effluxes were measured the axons were immersed in several changes of isosmotic KF. KF treatment lasted for 12–13 hr. At this time sampling for radioactivity in the bathing medium of the axons revealed that all of the injected label had been washed out. The efflux

[7] The altered permeability properties are due to KF treatment, not a consequence of repeated injections in the same axon. Multiple injections of inocuous agents can easily be carried out in the squid axon without any apparent effects on its permeability properties (e.g., Brady, Spyropoulos & Tasaki, 1958).

of injected sucrose C-14 has been studied previously in the normal squid axon (Tasaki & Spyropoulos, 1961). My present data agree with the previous ones. To my knowledge the efflux rates of injected glucose C-14 and inulin C-14 has not been studied previously. Particularly in the case of glucose C-14, the normal efflux measured in the present study may reflect not only the efflux of injected glucose C-14 but also the efflux of its metabolic breakdown products. In addition, an "active" process cannot be excluded.

In KF-treated axons the relative efflux rates of the injected nonelectrolytes and THO is a function of their diffusion constants in water, although an order of magnitude lower. When the logarithm of the amount of intraxonal label remaining in the axon at time t is expressed as a fraction of the initial amount and plotted against t, a straight line is obtained whose intercept is unity. This characteristic is indicative of a surface-limited diffusion process. That the crucial initial time course is reliable and not affected by the pressure inherent in the injection method is indicated by the experiment on THO efflux. In previous experiments (Spyropoulos, 1977*a*) THO was introduced into the axon by positive pressure-injection, in the current experiments, by suction (i.e., negative pressure). The characteristics of THO efflux with the two methods are the same (*compare* Fig. 10 to Figs. 4 and 5*b* of Spyropoulos, 1977*a*).

It is known (Caldwell, 1958; Spyropoulos, 1960) that in the normal axon the internal pH, within limits, does not vary as the extracellular pH is changed by addition of NaOH or HCl. In contrast, in KF-treated axons the surface of the axon appears to offer no hindrance to hydrogen or hydroxyl ion exchange. These results are presented in Fig. 11. That the internal pH is almost exactly the same as the external is not surprising. The theoretically expected Donnan contribution at 0.54 M KF and 4% protein of the axoplasmic type very likely is below the resolution of our pH measurements (which is ~ 0.03 pH units).

The mechanism of how KF treatment results in an inoperative axolemma is not certain. The electrophysiological survival of axons perfused internally with fluoride is well known to be greater than that of axons perfused with anions of a larger hydration radius (e.g., chloride; *cf.*, Tasaki, Singer & Takenaka, 1965). In contrast, externally applied fluoride destroys the solute barrier characteristics of the axolemma. This may be related to the Ca-sequestering properties of fluoride. During KF-treatment calcium is not added and traces left may, in the presence of half molar KF, be expected to be minimal. Prolonged exposure of the axon to divalent ion-free media is known to impair the axolemmal

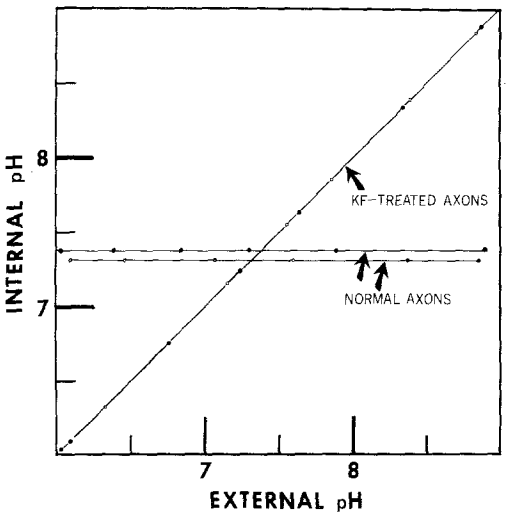

Fig. 11. The relation of external to internal pH in normal and KF-treated axons. The normal axon was initially immersed in seawater, pH 7.95–8.0. The dependence of internal to external pH was ascertained by addition of NaOH or HCl to an "infinitely" large external seawater medium. After this dependence was made, the axon was immersed for 20 hr in isosmotic KF. At the end of this period the external pH was varied by addition of KF and KOH. The method of Fig. 2 was used. The temperature was 21 °C. Experiments were done only on 2 axons. The axon diameter of the open circle axon was 694–708 μm and of the filled circle, 705–720 μm

properties. Internally or externally applied fluoride preserves axoplasmic structure more than similarly applied anions of a larger hydration radius (e.g., Spyropoulos, 1977*b*, and later section of this paper). In some instances Ca sequestering may contribute to this anionic hydration radius effect on structure. Hodgkin and Katz (1949) noted that dispersion of axoplasm in chloride media is potentiated by small amounts of calcium. Gilbert (1975*b*) has obtained evidence that this potentiation of dispersal is due to Ca-activation of axoplasmic proteases.

The Constraints to the Free-Swelling of the Axoplasmic Gel in the Normal Axon Immersed in Seawater

Previously (Spyropoulos, 1977*b*) I had pointed out that when $\Delta\Pi_{th}$, the intra-extraxonal difference in the theoretical osmotic pressure due to diffusible solutes, is zero the swelling out of the axoplasmic gel may be constrained by the retractive forces of the encapsulating elastic

sheath.[8] The clearest example of an axon in such a state (i.e., where $\Delta\Pi_{th}=0$) is one whose axolemma has been destroyed (e.g., by KF-treatment). I had assumed in my earlier work that in the intact axon immersed in seawater $\Delta\Pi_{th}$ is close enough to zero[9] so that $\Delta\Pi_m$ (the intra-extraxonal difference in effective osmotic pressure due to diffusible solutes) does not contribute substantially to the constraints suppressing axoplasmic free-swelling. In other words, I had assumed that the contribution of $\Delta\Pi_m$ to σ (the stress on the sheath) is insignificant, so that the condition $\Delta\Pi_g+\Delta\Pi_m=-\sigma$ reduces to $\Delta\Pi_g=-\sigma$. It means that in the normal axon immersed in seawater there is a slight turgor pressure due to $\Delta\Pi_g$, that $\Delta\Pi_g$ distends the sheath. What prompted the aforementioned assumption was mainly the finding that $\Delta\Pi_g$ at q_1 (the swelling ratio of the axoplasmic gel in the normal axon immersed in seawater) was of the same order as the hydrostatic pressure required to inflate the isolated sheath so that its enclosed volume was equal to V_1 (the volume of the intraxonal compartment in a normal axon immersed in seawater). This finding made the alternative possibility that $\Delta\Pi_m=-\sigma$ rather unlikely. In the experiments presented in this paper, I attempted to evaluate the assumption that in seawater the sheath constrains axoplasmic network swelling (or, conversely, that the swelling pressure of the gel inflates the sheath) primarily by showing that with the axon immersed in seawater $\Delta\Pi_m$ is small in comparison to σ or $\Delta\Pi_g$. To this end I employed three methods: (i) I tried to accurately measure the osmotic pressure of the mobile (diffusible) solutes in the axoplasm and that of the surrounding seawater. (ii) I tried to destroy the axolemma selectively (thereby eliminating the contribution of $\Delta\Pi_m$) and note the change in axonal volume. (iii) I bored out the axoplasm in normal axons immersed in seawater, filled the bore with oil and noted the volume of the axoplasmic wall with respect to time. In approach *iii* if the axoplasm did not swell appreciably it would mean that in the intact axon $\Delta\Pi_g$ was balanced by $\Delta\Pi_m$, primarily.

The technique for measuring the osmotic pressure of the diffusible components of the axoplasm and of the seawater is described in the

[8] That the degree of free swelling of axoplasm is far greater than the swelling in the axon, I had noted previously (Spyropoulos, 1977*b*) and is studied more extensively in the following section of this paper.

[9] In accordance with this assumption, isosmotic and anisosmotic artificial internal and external solutions were such in reference to seawater and to what I assumed to be the osmotic pressure of the *diffusible* (exclusive of network polymers and bound solutes) solutes of the internal compartment.

Methods section. The osmotic pressure of the seawater I used was 1.005 to 1.006. I was able to make only two estimates of the osmotic pressure of pooled axoplasm; the reason was that the technique was somewhat tedious and dozens of axons were needed for each determination. The osmotic pressure of the diffusible solutes in the one pool of axoplasm was $1.007 \pm .005$ osmolal and in the second $1.015 \pm .005$ osmolal. The data are limited but I feel they may indicate that $\Delta\Pi_{th}$ was less than ~ 15 mosmol. Assuming a conservative[10] $\Delta\Pi_{th}/\Delta\Pi_m \sim 150$, then $\Delta\Pi_m$ should be 2 or less cm H_2O. In contrast, from the hydrostatic pressure and Ficoll deswelling experiments $\Delta\Pi_g$ at q_1 is 2–12 cm H_2O and σ at V_1 is 1.5 to 6.0 cm H_2O (*cf.* Spyropoulos, 1977*a*).

My experiments on the osmotic pressure of the diffusible components of the axoplasm of *Loligo vulgaris* appear to be consistent with similar previous experiments on *myxicola* axoplasm by Gilbert (1975*b*). Gilbert employed both the Fiske osmometer method and the freezing point method. With the latter method, he reported a difference in osmotic pressure between seawater and axoplasm of less than 10 mosmol and with the former method (one determination) 3 ± 26 mosmol.

In the second approach to elucidating the constraints to free-swelling, I attempted to notice axonal volume changes upon eliminating the contribution of $\Delta\Pi_m$. As noted in my previous report (Spyropoulos, 1977*a*) and in the previous section of this paper, KF treatment destroys the solute barrier properties of the axolemma. In 8 axons I noticed a 0.3 to 0.9% increase in intraxonal volume after 45 min to 2 hr exposure to isosmotic (to seawater) KF solutions. Assuming for the moment that the increase in intraxonal volume was due to abolition of $\Delta\Pi_{th}$ and assuming also that the axon acted as a perfect osmometer, a change in volume of 0.3 to 0.9% would indicate a $\Delta\Pi_{th}$ of ~ 3 to 9 mosmol. Actually the axon is known not to act as a perfect osmometer (Hill, 1950; Villegas & Villegas, 1960; Freeman *et al.*, 1966; Spyropoulos, 1977*b*). The deviation from perfect osmometric behavior varies from 25 to more than 45%. I arbitrarily corrected for 30% so that a change in intraxonal volume of 0.3 to 0.9% would indicate a $\Delta\Pi_{th} \sim 4$–12 mosmol. Assuming a conservative $\Delta\Pi_{th}/\Delta\Pi_m \sim 150$, then $\Delta\Pi_m$ in the axon immersed in seawater from this approach should be less than 1.5 cm H_2O. Previously (Spyropoulos, 1977*b*) I destroyed the axolemma very crudely, by rolling the axoplasmic cylinder back and forth within the axon. I mentioned that if there was a volume increase it would have

[10] *cf.* footnote 14 in *Discussion* for justification.

had to have been less than 3%. The reason for giving this poor resolution is the following. Even after the axolemma was destroyed (by rolling or KF treatment) volume readings made following successive rollings differ by ± 0.2 to 1.8%.[11] The increase in intraxonal volume observed following KF treatment need not be due to the abolition of $\Delta\Pi_{th}$ alone. Potassium in the presence of calcium is known to swell the axon even though the osmotic properties of the axolemma are not impaired. Calcium depletion also is known to swell the axon. KF may act in part by sequestering calcium. Finally, following KF-treatment the diffusible solutes in the axon may be expected to be washed out and replaced with KF. This altered internal milieu may affect the swelling pressure of the sheathed gel (*cf.* next section).

The results of the third and final approach to the nature of the constraints to free swelling of the axoplasm in the axon immersed in seawater are somewhat more convincing than those from the previous two approaches. The axoplasm of a normal axon immersed in seawater was bored out, and the bore was filled with H_2O-saturated oil. In two such bored-out axons at zero applied internal pressure the axoplasmic wall was found to swell by 2.0 and 4.5%. Had the swelling of axoplasm been constrained primarily by $\Delta\Pi_m$ (i.e., had $\Delta\Pi_g$ been balanced by $\Delta\Pi_m$) there should have been no swelling. I feel the swelling observed was simply an expression of allowing the gel to swell into the bore instead of straining the sheath. In this experiment if $\Delta\Pi_g$ at q_1 is 2–12 cm H_2O – let's say 5 cm H_2O – then this may imply that $\Delta\Pi_{th}$ is attenuated by a factor ~ 80–180. This follows since an increase in volume of 2 to 4.5% would imply a $\Delta\Pi_{th}$ of 20 to 45 mosmol.[12] The conditions of the two experiments on boring of axons immersed in seawater were mentioned in greater detail in *Results* in the section on Ficoll deswelling (*cf.*, the two axons that were used for swelling pressure experiments). In three other axons (also used previously and mentioned in *Results* in the section on mixed pressure measurements) just before boring out, the axoplasm had been perfused with isosmotic (to seawater) KF. A slitted fine capillary was used so there was no loss of axoplasm. In

[11] This does not imply that axonal volume in fact changed. More likely, it means that the accuracy of my volume measurements was attenuated due to the necessity of having to make multiple diameter measurements along the axon, not merely one measurement at a reference region. Axonal diameter is not uniform along the axon, and this nonuniformity is altered by the rolling procedure.

[12] In this crude estimation no correction for imperfect osmometric behavior of the axon is necessary.

these axons before boring, there was some assurance that $\Delta\Pi_{th}$ was zero. Upon boring and filling the bore with oil, the axoplasm swelled in the three axons by 2.5, 3.0, and 5%. These values obtained with $\Delta\Pi_{th}$ presumably equal to zero compare fairly well with the values of 2 and 4.5% (given above) that were obtained on normal, unexposed to isosmotic KF axoplasm. This implies that the osmotic pressure of the diffusible components of normal axoplasm may be close to that of isosmotic *to seawater* KF and that $\Delta\Pi_m$ in the normal axon immersed in seawater may be smaller than $\Delta\Pi_g$ or σ.

The estimates of the value of $\Delta\Pi_m$ relative to that of $\Delta\Pi_g$ and σ in the normal axon immersed in seawater are not as convincing as I would have desired. One reason is that there is too much scatter in practically all the measurements (e.g., osmotic pressure of axoplasm due to diffusible components, L_P/L_{PD}, $\Delta\Pi_g$, σ).

There are obvious approaches to resolve this problem more unequivocally; however, I do not feel this issue is so consequential as to warrant further pursuit. The reasons are the following: Despite the routine use in this field of seawater as the standard external medium, the choice of the osmotic pressure of seawater as the reference is arbitrary. The osmotic pressure of different seawaters vary, and the osmotic pressure of axoplasms from different excised axons may vary. Neither the diffusible solute osmotic pressure of the seawater nor that of the excised axon need be the same as that in the intraxonal or extraxonal compartments in the animal. Finally, the elastic modulus of an isolated, cleaned, oil-filled axonal sheath may be different than that of the axoplasmic coverings in the animal or in isolated uncleaned intact axons.

Unconstrained, Free-Swelling of Axoplasmic Gels

From a previous paper (Spyropoulos, 1977*b*) and previous sections of this paper it is apparent that in the axon the cytoplasmic gel network is not free to swell out completely. When the axon was immersed in media hyposmotic to seawater and maybe when immersed in normal seawater, these constraints appeared to be due mostly to the mechanical retractive forces of the encapsulating sheath. In hyperosmotic (to seawater) external media the constraints appeared to be mainly osmotic (i.e., due to $\Delta\Pi_m$). The constraints to gel free-swelling existing in the axon can be eliminated or circumvented, respectively, by extruding or boring out the axoplasm. By boring out the axoplasm, space (the lumen)

is allocated to accommodate the inward swelling of the axoplasmic wall (i.e., the swelling of the annular axoplasm juxtaposed to the axonal investitures). Following extrusion, axoplasm increases in volume.[13] This swelling, upon freeing cytoplasm from its covering, is not unique to the axon; it has also been observed, for example, with the cytoplasm of muscle fibers [April, Brandt & Elliott, 1971, 1972; Ford & Podolsky, 1972 (*cf.* p. 1); Matsubara & Elliott, 1972; April, 1975; Godt & Maughan, 1977]. In contrast to constrained axoplasm the degree of swelling of extruded axoplasm depends markedly upon pH, salt concentration, and the hydration radius of the anion of the salt used. Although a concerted effort was made to maintain conditions constant, q_m, in a given medium, varied tremendously. Most, not all, of our recent values for q_m are considerably lower than our older values. The effects of pH, hydration radius of anions, and ionic strength can be compared only on a given extrudate or in adjacent segments from the same axon.

The Effects of the Hydration Radius of the Anion. Hofmeister first suggested that anions can be arranged in a particular order (Lyotropic order of Hofmeister Series) depending upon their swelling action on gels. He found that swelling was enhanced with an increase in the reciprocal of the ionic hydration radius. The swelling or dispersing action of anions on axoplasmic gels was found to obey the lyotropic relation. An example of these experiments is given in Fig. 12 and additional data in Table 1. All the salt solutions were isosmotic to seawater. The cation in all cases was potassium. In isosmotic chloride salts or in salts whose anion had a smaller ionic hydration radius than chloride, the *fresh* axoplasm dispersed. In the case of chloride the dispersing action is limited to a range of concentration close to the isosmotic one. Actually Hodgkin and Katz (1949) had first reported the dispersion of extrudates in isosmotic chlorides. In anions whose hydration radius was larger than that of chloride, the *fresh* axoplasm did not disperse but invariably swelled, the q_m being a function of the Hofmeister rule. The *rate* of dispersion of axoplasm also varied in accordance with the Hofmeister series. Dispersion in chloride took 20–40 min and in thiocyanate it occurred instantaneously. In the majority of the cases maximal swelling occurred within

[13] This swelling is not related to the condition of suddenly exposing the gel to an environment whose composition, pH, etc., is alien to that existing inside the axon (*cf.* Spyropoulos, 1977*b*). For example, after immersing the axon for hours in isosmotic KF, the axoplasmic volume does not change markedly but the intraxonal composition of the diffusible solutes is almost the same as that of the immersion medium. Once released from the confines of the sheath, the axoplasm of these KF-treated axons still swells markedly in isosmotic media.

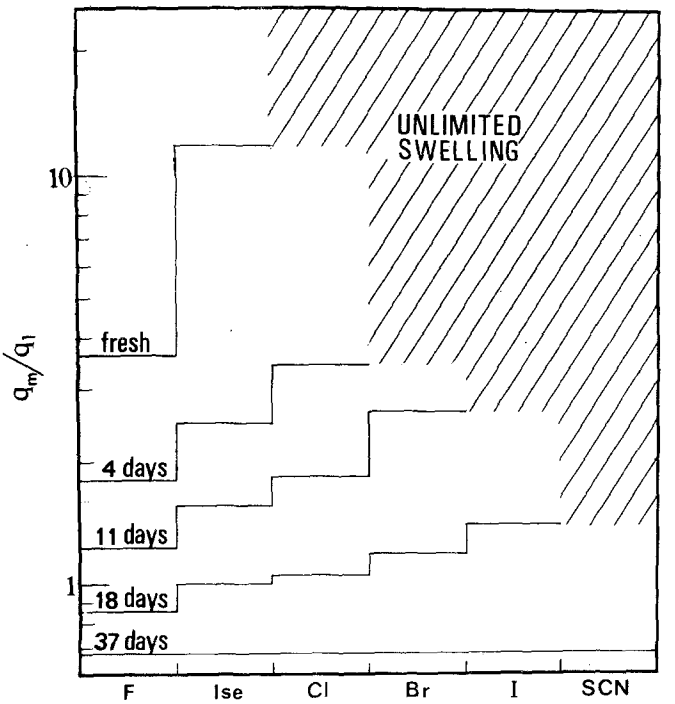

Fig. 12. Relative free-swelling volume of axoplasmic gels as function of hydration radius of anion. The swelling ratio of the freely swollen axoplasm (q_m) is expressed in the figure as a multiple of the swelling ratio of the axoplasm in the normal axon (q_l). Unlimited swelling means dispersion. All salts were isosmotic to seawater. The cation in all cases was potassium. The pH was 7.35–7.4. The axon diameter was 715–745 μm and its length 16.5 cm. Half of the axon was stored and half used for the fresh extrudate experiment

Table 1. Variability in q_m/q_l (Relative free-swelling ratio of extruded axoplasm)[a]

Swelling agent	q_m/q_l	Number of preparations
K gluconate	1.9–9.5	23
KF	1.7–11	45
K glutamate	2.0–17	9
Medium approximating mobile ion composition of axoplasm	2.7–30	22
K-isethionate	3.2–40	13
Distilled water[b]	6.0–48	41

[a] The pH of the salt solutions was 7.35–7.4; that of distilled water, 7.0. All salt solutions were isosmotic to seawater.

[b] In eight extrudates, an unlimited swelling (i.e., dispersion) was observed. Two extrudates dispersed, even at pH 6.0–6.2.

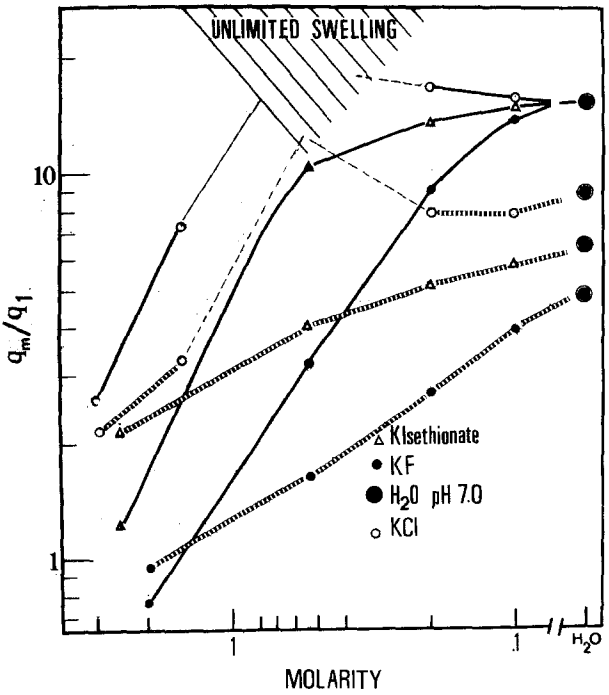

Fig. 13. Relative free-swelling volume of axoplasmic gels as function of ionic strength. The swelling ratio of the freely swollen axoplasm, q_m, is expressed in the figure as a multiple of the swelling ratio of the axoplasm in the normal axon, q_1. The pH was 7.0; the cation in all cases was potassium. The curves in the figure were obtained from two axons. One (continuous line) showed the highest q_m and had a diameter of 705–742 μm and a length of 16 cm, and the other (dotted line) showed the lowest q_m and had a diameter of 572–578 μm and a length of 12 cm. The results of the experiments on 18 other axons fell within the limits defined by the continuous and dotted curves. The interrupted line in the figure means that there were no intermediate experimental points, so the exact concentration at which dispersion occurred in this particular fiber is not known. However, dispersion did occur at 0.54 M KCl. The expression *unlimited swelling* in the figure means dispersion

15–80 min. In approximately 10% of the cases 2–5 hr were required. The time (following excision) that the axon was stored in isosmotic nondispersing media (e.g., KF, K gluconate) markedly affected q_m and dispersion. In the experiment illustrated in the figure the axon was stored in isosmotic K gluconate. The time of storage is indicated. Progressively axoplasm is not dispersed in Cl^-, Br^-, I^-, and thiocyanate and the lyotropic series is extended but the degree of swelling becomes smaller. At 37 days storage syneresis had set in to the extent that $q_m/q_1 < 1$.

The Effects of Salt Concentration. These experiments are summarized in Fig. 13. In the case of chloride, dispersion (unlimited swelling in figure)

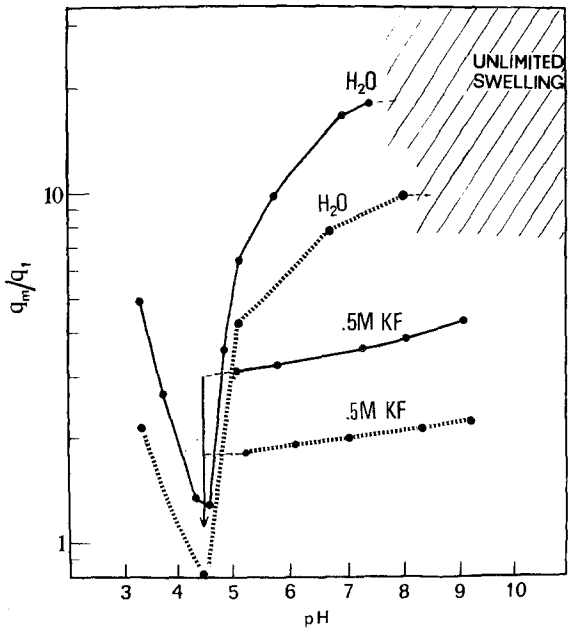

Fig. 14. Relative free-swelling volume of axoplasmic gels as function of pH. The swelling ratio of the freely swollen axoplasm, q_m, is expressed in the figure as a multiple of the swelling ratio of the axoplasm in the normal axon, q_1. One axon (continuous line) was 695–730 μm in diameter and 17 cm in length and showed the highest q_m of the 13 axons where this particular experiment was performed. The other axon (620–632 μm in diameter, grated line in figure, and 13.5 cm in length) showed the lowest q_m. The curves obtained with the remaining axons fell within the limits of the two curves shown in the figure. *Unlimited swelling* means dispersion. The interrupted line in the figure means that I did not have any intermediate points; i.e., I did not know the exact pH at which precipitation or dispersion occurred. The vertical arrow in the curves, obtained at high salt concentration, means that the gel was precipitated

occurred over a limited range of concentrations. The old idea that some gels swell more in chloride than in distilled H_2O and less in anions of a smaller hydration radius than chloride appears applicable to axoplasmic gels. At pH 7.0, around 3/4 of the extrudates did not disperse in distilled water (*cf.* Table 1). The concentration dependence of the volume of extrudates was marked. The more dilute the medium, the greater the degree of swelling. With equimolar solutions of different salts the degree of swelling varied according to the Hofmeister series (with respect to the anion).

The Effects of pH. If axoplasmic gels behave as amphoteric polyelectrolytes, there should be a pH of the immersion medium (isoelectric point) where the ionizable salt groups are minimal and consequently in accordance with the Donnan equilibrium the concentration of mobile coions, the osmotic pressure difference, and the volume of the gel also

minimal. Addition of either acid or base to the isoelectric gel should result in swelling. The data on the effects of pH are summarized in Fig. 14. Axoplasm dispersed at high pH. The pH at which this happened varied from axon to axon. At higher salt concentrations the pH required for dispersion was higher. The dependence of the volume of axoplasm on pH was marked and dependent on the salt concentration. The higher the salt concentration the lower the dependence of q upon pH. At high ionic strengths and around pH 4.5 axoplasm precipitated. This precipitation was only slightly reversible, namely, upon raising the pH of the immersion fluid neither the shape or the degree of swelling was completely restored. It appears that the isoelectric point of the network protein is around pH 4.5. This was the pH region of minimal swelling.

From the results presented in this section there is some indication that changes in Donnan distribution are involved in the effects of salts and pH on the q of axoplasmic gels. However, the effects of salt, pH, and anions on other properties of the network may be even more important. pH, salt, and anion dependent alteration in organized protein structures are considered a result of changes in electrostatic repulsive and attractive forces (e.g., by salt screening and pH charge neutralization), changes in water lattice structure, changes in hydrogen bonds, and van der Waal's forces, disruption of α-helix, etc. (*cf.* Record, Anderson & Lohman, 1978, for recent review). There appears to be some evidence that a multilevel helical type of architecture pervades the entire axoplasmic network, including the denser ectoplasm (e.g., Metuzals & Izzard, 1969; Metuzals, 1969; Wuerker & Kirkpatrick, 1972; Gilbert, 1975*a*). At the lowest level, that of the α-helix, there may be in some instances at high values of q, helix-coil transitions. At higher levels of structural organization there may be untwisting, changes in pitch, etc. The very marked dependence of q_m on anionic hydration radius, pH, and salt concentration, the high values of q_m attained, and the extreme irreproducibility in the observed values of q_m in a given medium cannot be expected by applying Donnan theory to a simple amorphous polyelectrolyte gel system; it is more consistent with complex effects on a structurally complex network.

Discussion

The experiments presented in the present paper and previous papers (Spyropoulos, 1977*a* and *b*) strongly indicate that in the giant axon of the squid $\Delta\Pi_{th}$ is sufficiently attenuated for $\Delta\Pi_g$ to become significant in the volume relations of the axon.[14] Still, the situation in the giant axon is regarded by most as being paradoxical, the general view being

that in cells with nearly ideal semipermeable membranes $\Delta\Pi_{th}=\Delta\Pi_m$. This is an assumption, since the type of crucial experiments required to establish an attenuation of $\Delta\Pi_{th}$ (e.g., a comparison of L_P to L_{PD} under internal perfusion conditions or an introduction into the cell of a pressure indicator) have only been done on the squid axon and recently (*see* below) on some plant cells. The observation that a cell behaves as a perfect osmometer (i.e., that it obeys Boyle-vant Hoff's Law) is regarded by some as indicating that $\Delta\Pi_{th}=\Delta\Pi_m$. This does not follow either from theory or from experimental observations. For example, the squid axon can be made to act as a perfect osmometer (Spyropoulos, 1977*b*) but still $\Delta\Pi_{th}\gg\Delta\Pi_m$.[15] Recently (C.S. Spyropoulos, *unpublished*) in an attempt to point out that the situation $\Delta\Pi_{th}>\Delta\Pi_m$ is not unique to the axon, I measured $\Delta\Pi_{th}/\Delta\Pi_m$ in *nitella*. In large part I chose *nitella* since it is a classical type of preparation for the study of osmotic phenomena in cells and it behaves as a perfect osmometer. As was done in the squid axon, I measured $L_P/L_{\Delta\Pi}$ ratios under internal perfusion conditions; in addition I measured the internal pressure as a function of $\Delta\Pi_{th}$ using Green's (1968) micromanometer. At *near isosmotic* conditions $\Delta\Pi_{th}/\Delta\Pi_m$ was around 3 to 12. In more turgored internodes this ratio was closer to, but not quite, unity. My observations on *nitella* can be interpreted as being consistent with previous work

[14] The attenuation of $\Delta\Pi_{th}$ has been effectively shown in the axon by four somewhat different methods: (i) a measurement of L_P and L_{PD} under perfusion conditions, (ii) a measurement of the ΔP required to sustain zero net water flow under perfusion conditions, (iii) in the mixed pressure experiments a measurement of the internal hydrostatic pressure required to maintain with the bore filled with oil a particular intraxonal aqueous volume at a particular $\Delta\Pi_{th}$, and (iv) a measurement at zero internal pressure of the increase in the volume of the axoplasmic wall following boring and oil filling of an axon immersed in seawater (*cf.* appropriate section in *Results* on constraints to free swelling). (i) Using one perfusion method, Vargas (1968) found an average L_P/L_{PD} of 220; using another method, 66. My (Spyropoulos, 1977*a*) average L_P/L_{PD} ratio was 210. (ii) My average $\Delta\Pi_{th}/\Delta P$ ratio (i.e., in the aqueous perfusion experiments, the ratio of $\Delta\Pi_{th}$ to applied ΔP at which zero net water flow is sustained) was 240. (iii) My average (from only two experiments) $\Delta\Pi_{th}/\Delta P$ from the mixed-pressure experiments was 150. (iv) My average (from two experiments only and assuming arbitrarily that $\Delta\Pi_g$ at $q=5$ cm of H_2O) $\Delta\Pi_{th}/\Delta\Pi_m$ from the experiments where the degree of swelling is measured at $\Delta P=0$ in a bored out oil-filled axon immersed in seawater was 135.

[15] Hill (1950), Villegas and Villegas (1960) and Freeman *et al.* (1966) felt, consistent with general practice, that the deviations in the normal squid axon from Boyle-vant Hoff's Law were due to the presence in the axoplasm of osmotically inert materials. In accordance with my view (Spyropoulos, 1977*b*) this deviation from perfect osmometer behavior when the axon is immersed in hyperosmotic media is due primarily to the swelling pressure of the gel opposing axonal shrinkage, while when the axon is immersed in hyposmotic media it is due primarily to the retractive forces of the elastically distended sheath opposing axonal swelling.

of Steudle and Zimmerman (1974) who used a different method on *nitella* that was not perfused internally.

A discussion of some work on inanimate gels may be of pertinence in evaluating the role of cytoplasmic gels in the water relations of the axon and perhaps other cells. The degree of swelling of nonionic gels at equilibrium is determined by the equality of the chemical potential of the solvent inside with that of the excess solvent outside the swollen gel (Flory & Rehner, 1943; Flory, 1950, 1953; Dusek & Prins, 1969). When the outside solvent is at activity a_1 then $\Delta\mu_1 = (\Delta\mu_1)_{el} + (\Delta\mu_1)_{dil} = KTLna_1$ where K is the Boltzmann constant, T, the absolute temperature and $(\Delta\mu_1)_{dil}$ and $(\Delta\mu_1)_{el}$, the change of the chemical potential of the solvent in the swollen network, respectively, due to dilution of the polymer with the solvent and due to the elastic deformation of the network.

With ionic (polyelectrolyte) swollen polymer networks, the reduction in the chemical potential of the solvent inside the network includes in addition the term $(\Delta\mu_1)_i$ due to mixing of the solvent with all the mobile ionic constituents (the Donnan effect) (e.g., Flory, 1953; Katchalsky, 1951; Katchalsky, Lifson & Eisenberg, 1951; Smith, 1974), and unless the network charges are salt-screened in some systems the term $(\Delta\mu_1)_{rep}$, which stems from repulsive electrostatic forces exerted between similarly charged groups on the network. At swelling equilibrium $(\Delta\mu_1^*)_i - (\Delta\mu_1)_i = (\Delta\mu_1)_{dil} + (\Delta\mu_1)_{el} + (\Delta\mu_1)_{rep}$ where the asterisk refers to the external solution. At high ionic strengths in the solvent compared to the fixed charge density of the network, Donnan contributions and electrostatic repulsive forces are reduced and the swelling relations of some ionic networks may tend to revert to those of nonionic networks.

A change of swelling and a retractive force can be induced in a polymer network by imposing a uni-directional strain (Flory, 1953; Hoeve & Flory, 1962; Rijke & Taylor, 1967; Bashaw and Smith, 1968; Knibbe, 1968; Shen & Croucher, 1975). Here, only the term $(\Delta\mu_1)_{el}$ of the equilibrium equation is altered. A deswelling can also be achieved by lowering the solvent activity around the swollen nonionic network by reducing the partial pressure of the external solvent in the vapor phase (Gee & Orr, 1946; Gee, Herbert & Roberts, 1965). Here, for nonionic gels $\Delta\mu_1 = (\Delta\mu_1)_{el} + (\Delta\mu_1)_{dil} = KTlna_1 = KTlnp/p_0$. A lowering of the external solvent activity and a deswelling can be obtained by inclusion of nonpenetrating high mol wt polymers in the external solvent (Blow & Stamberger, 1929; Powers & Robinson, 1942; Boyer, 1945; Rijke & Prins, 1962; Mukherji & Prins, 1964). Here, for nonionic gels $\Delta\mu_1 = (\Delta\mu_1)_{el} + (\Delta\mu_1)_{dil} = KTlna_1 = KTln(1-v_2) + (1-1/\chi)v_2 + \chi v_2^2$ where v_2 is the

volume fraction of polymer in solution, χ, the ratio of the partial molecular volumes of the solvent and the polymer, respectively, and χ, the corresponding Flory-Huggins polymer-solvent interaction parameter. Finally, a deswelling can be attained by raising the solvent activity inside the gel by application of a hydrostatic pressure to the gel [Reinke, 1879; von Schroder, 1903 (note footnote p. 117); Posnjak, 1912; Pennings & Prins, 1961; von de Kraats, 1968; von de Kraats *et al.*, 1969; Borchard, 1966, 1975]. The same condition obtains if the gel is somehow mechanically constrained so that it cannot swell out completely. The equilibrium conditions given above for nonionic and ionic gels are then replaced respectively with $\Delta\mu_1=(\Delta\mu_1)_{\text{dil}}+(\Delta\mu_1)_{\text{el}}+\bar{V}_1\Delta\Pi_g=0$ and $(\Delta\mu_1^*)_i-(\Delta\mu_1)_i=(\Delta\mu_1)_{\text{dil}}+(\Delta\mu_1)_{\text{el}}+(\Delta\mu_1)_{\text{rep}}+\bar{V}_1\Delta\Pi_g$, where $\bar{V}_1$ is the partial molal volume of the solvent and $\Delta\Pi_g$ is commonly referred to as the swelling pressure of the gel (Flory, 1953; Dusek & Prins, 1969).

The very general description given above should apply qualitatively with some limitations to axoplasmic and other biological protein gels devoid of semipermeable (to mobile solutes) coverings; however, the quantitative statistical thermodynamic approaches (e.g., Flory, 1953; Dusek & Prins, 1969) used with synthetic gels where theories of rubber elasticity are combined with theories of polymers in solution are inapplicable. In large part the reason is that swollen protein networks cannot be treated from the view of a Gaussian coil. In considering what determines the degree of swelling or the swelling pressure of axoplasmic gels (devoid of a plasmalemma), the network polymer dilution and the Donnan distribution contributions may be less significant than they are in synthetic gels treated so far. In axoplasmic gels the repulsive and cohesive forces exist between segments of a polypeptide chain, between polypeptide chains in a filament and between filaments. The cohesive forces involve not only covalent linkages (as in the synthetic gels treated this far) but also other more tenuous forces such as electrostatic-hydrogen bonds and van der Waals forces. The axoplasmic gel is grossly nonuniform (e.g., the ectoplasm is denser than the endoplasm). In addition, there is evidence (e.g., Metuzals, 1969; Metuzals & Izzard, 1969; Wuerker & Kirkpatrick, 1972; Gilbert, 1975*a*) that at various levels of organization there is a helical type of architecture. Under certain extreme conditions helix-coil transitions of the α-helix may take place; under certain conditions a change in pitch, or untwisting of more organized structures are also conceivable. Some of the conditions that I had imposed on the axoplasm were sufficiently drastic that some such changes are likely.

Probably the most important difference for our purposes between biological and polyelectrolytic synthetic gels is that the distribution of mobile solutes is determined usually by a semipermeable covering not by a Donnan effect. Cellular gels may be covered not only by a semipermeable barrier but also by other extraneous coats that may constrain gel swelling. Very few cells are naked, devoid of material external to the semipermeable barrier. For a cytoplasmic gel coated only with an ideally semipermeable membrane offering no mechanical resistance to swelling, one may expect at swelling equilibrium that $(\Delta\mu_1^*)_m-(\Delta\mu_1)_m=(\Delta\mu_1)_{el}+(\Delta\mu_1)_{dil}+(\Delta\mu_1)_{rep}$ and for a gel covered in addition with a coat offering a mechanical resistance to the gel swelling out: $(\Delta\mu_1{}^*)_m-(\Delta\mu_1)_m=(\Delta\mu_1)_{el}+(\Delta\mu_1)_{dil}+(\Delta\mu_1)_{rep}+\bar{V}\Delta\Pi$, where the asterisk identifies the external solution and where m refers to all the mobile solutes exclusive of network polymers. Under the former condition $\Delta\Pi_g=-\Delta\Pi_m$ [16] and under the latter the total osmotic pressure, $\Delta\Pi=\Delta\Pi_g+\Delta\Pi_m$ where $\Delta\Pi_m$ is the effective osmotic pressure difference due to mobile (i.e., exclusive of network polymers) solute-activity gradients across the semipermeable coating of the gel. *Unless $\Delta\Pi_m$ is much smaller than $\Delta\Pi_{th}$ (as appears to be the case in the axon) the contribution of $\Delta\Pi_g$ to $\Delta\Pi$ can be ignored since it is too small in comparison.*

In Fig. 15 I have illustrated the various methods that have been used since before the turn of the century in measuring the swelling pressure of swollen polymer networks (gels). My method is presented in *f*. I have not included in the figure one very recent method by Borchard (1975) that utilizes the analytical ultracentrifuge. All the methods of Fig. 15 have the following in common: the gel is mechanically constrained so that it cannot swell out completely. Part of the constraining enclosure is porous so that the confined gel can make contact with an external liquid swelling agent. The pressure developed in the gel is measured at various swelling ratios, q. q is varied either (a) by making the enclosure more or less confining and thereby allowing the gel to deswell or swell, respectively, or (b) by using a series of gels of identical dimensions but of different values of q. Method *b* has the advantage that (i) gel volume measurements are not necessary and more importantly (ii) that all gels can be prepared so that they are *isotropically* swollen. *i* and *ii* are actually the major difficulties implicit in methods of type *a*. In

[16] In such cells, destruction of the semipermeability properties of the membrane should allow the gel to swell to equilibrium so that $\Delta\Pi_g=0$. One may point out in this connection the well-known observation that a variety of cells, upon being subjected to various types of injury, swell.

previous type *a* methods the gel is hidden so that volume measurements are indirect, and, if they are to be accurate, difficult. Since one face of the gel of necessity is fixed at the porous region of the enclosure (so that it makes contact with the swelling agent) attempts at mechanically changing q must be accomplished by application of pressure anisotropically through the remaining surface of the gel. Attempts at minimizing this deformation, for example, as was done in *d* by shaping the gel in the form of a cap or momentarily lifting the gel from the porous region, have met with questionable success. Although my method (method *f*) is of type *a*, I had less difficulty in measuring gel volume and keeping the three principal extension ratios that characterize the strain of the gel constant.

In previous methods a porous metal or earthenware membrane was used as the porous region of the enclosure. The requirements are that the number and diameter of the pores be large enough and their length short enough so that the membrane does not unnecessarily delay the equilibration of the gel with the swelling agent. At the same time the pores must be appropriately small in diameter so that the swelling of the gel is restrained (so that the gel does not swell through the pore). In my method on the axon, by good fortune, I had a built-in porous membrane, namely, the axonal sheath (the Schwann layer and adhering connective tissue). To satisfy the requirements of the porous membrane, I destroyed the plasmalemma. Had I not, the pressure measured would be mixed, namely, the sum of $\Delta\Pi_g$ and $\Delta\Pi_m$.

The properties of the axonal sheath, in principle, appear suitable for it to act as the porous region of the enclosure. Electron microscopy (Villegas & Villegas, 1960) reveals that it is traversed by numerous continuous zig-zag slits ~ 60 Å in width and 43,000 Å in length. That solvent and solute traffic across the sheath occurs exclusively through the slits (between Schwann cells) and not across the Schwann cells is not yet quite proven. Measurements of pressure conjugated water flow and diffusional flow of THO, Na_{22}, glucose C-14, sucrose C-14, and inulin C-14 on KF-treated axons (Spyropoulos, 1977*a*, and present paper) would indicate that the enclosure of the gel does not offer a hindrance to the flow of the external swelling medium sufficient to delay swelling pressure equilibrium.[17]

The concentrically layered cylindrical arrangement consisting of the

[17] It appears immaterial, as regards the steady state $\Delta\Pi_g$-q relation, whether the axonal sheath or the gel surface is the rate-limiting structure for solvent flow (*cf.* Spyropoulos, 1977*a*, for discussion).

circumferential sheath acting as the porous enclosure against which the gel is compressed on the one side, the axial nonpenetrating fluid used to mediate the pressure on the other side, and the gel sandwiched in between, coupled with the fact (Spyropoulos, 1977*b*) that the sheath is elastic, may have certain advantages over most previous arrangements. The deformation ratios of the gel could be kept constant by adjusting the longitudinal tension on the axon.

In my method, since the sheath is transparent, axoplasmic volume could be measured directly (visually). This is not the case with the methods used previously.

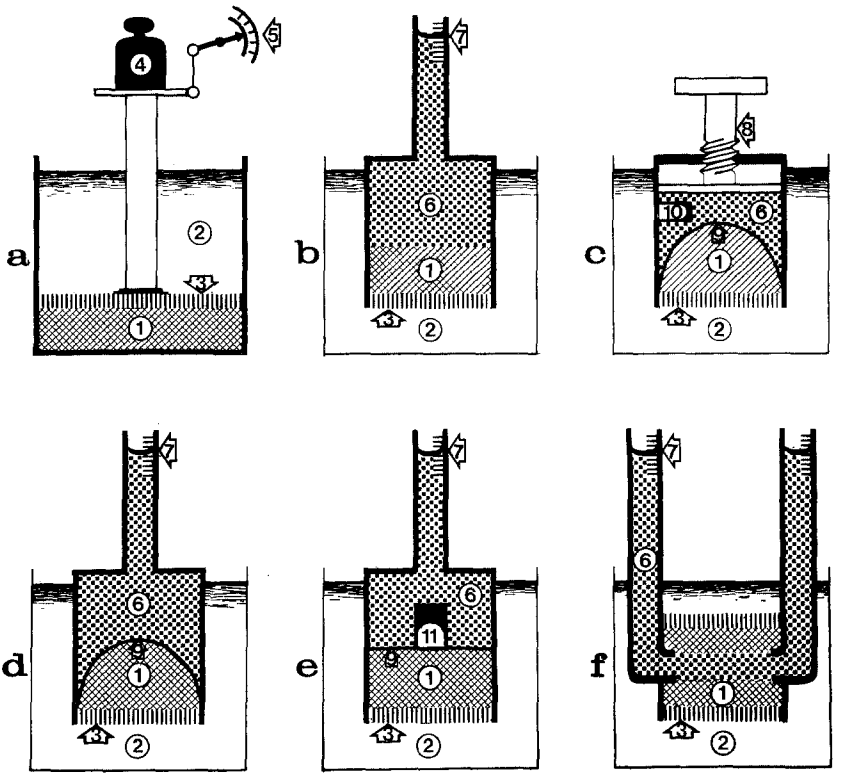

Fig. 15. Comparison of methods for measurement of swelling pressure of gels. In all methods the gel (*1* in figures) is tightly enclosed in a vessel with part of the wall (*3* in figures) permeable to the swelling medium (*2* in figures): The dilation tendency of the gel gives rise to a pressure. (*a*): In the original method of Reinke (1879) the swelling substance (*1*) was placed in the bottom of a hollow metal cylinder. Upon it rested a porous metal piston (*3*) that permitted the swelling agent to pass. The piston was equipped on top with a platform upon which could be placed weights (*4*) that prevented the piston from rising. Such weights expressed per unit surface area gave the swelling pressure. The volume of the swelling substance was registered with a pointer (*5*) mounted on the piston. (*b*) In Posnjak's (1912) method the earthenware porous region (*3*) of the gel enclosure is

Except in the earliest method used a century ago by Reinke (method *a*, in figure), in order to vary q the pressure applied to the gel is mediated through a fluid (usually mercury). With methods *c* and *e*, a flexible platinum foil, and with method *b*, polyethylene film is interposed between the pressure mediating fluid and the gel. With method *b* and *f*, the pressure mediating fluid makes direct contact with the gel. Obviously in the latter instance the fluid used (i) should not penetrate the gel under pressure, and (ii) should not react chemically with the gel. In my more recent method I used oil (Nujol) in direct contact with the gel. It seemed to meet both requirements.

There is a limitation in both the Ficoll-deswelling and the hydrostatic pressure-deswelling experiments. If the volume of the denser ectoplasm

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fixed by cementing it to the bottom of a glass tube. The gel was placed against the porous region and the vessel was filled with mercury (*6*). Mercury extended into a connecting calibrated capillary sealed to the pressure vessel. Mercury made direct contact with the gel. The capillary was connected to a gas bomb and a manometer that indicated the pressure. The volume of the gel was measured from the level of mercury in the capillary. (*c*) In Pennings' and Prins' (1961) method the previous methods *a* and *b* are combined and improved. The pressure was mediated with mercury (*6*). The volume was controlled by advancing or withdrawing a piston using a calibrated micrometer screw (*8*). The pressure developed was followed with a pressure transducer (*10*) that was in contact with the mercury. The gel that rested on the fixed porous metallic region (*3*) of the pressure vessel was wrapped with a polyethylene film (*9*). The interposition of the film prevented direct contact between the gel and mercury. It also served as a gasket. The shape of the gel, although given in the form of a cap in my illustration, is not clear in Pennings' and Prins' description. They claim to have ensured isotropicity of swelling by maintaining the larger diameter of the gel appreciably smaller than the diameter of the porous region of the enclosure; this is somewhat questionable. The device in their work was mounted horizontally. The upright position in *c* is to facilitate comparison of the methods. (*d*) Essentially Borchard's (1966) method in principle is a reversion to Posnjaks' method. To ensure isotropicity of swelling, he shaped the gel in the form of a cap and periodically applied a pressure to the solvent compartment (*2*) of the pressure chamber so that the gel was lifted off the porous metallic membrane. The level of the mercury in the capillary gave a measure of the pressure exerted on the gel and of gel volume. A flexible foil (*9*) was placed between the gel and the mercury. In my illustration, for conformity, Borchard's method is turned upside down. (*e*) The uniqueness of van de Kraat's (1968) method resides in the feature whereby, in order to avoid anisotropic effects, the volume of the gel was kept constant. For every q value in the q *vs.* $\Delta\pi_g$ plot a new gel was prepared of a different swelling ratio. van de Kraats employed an inflexible porous nickel membrane (*3*) that allowed the diffusion of the solvent in the gel. A flexible thin (100 μm) platinum nonporous membrane (*9*) separated the gel from the pressure mediating fluid (silicon and, in series, mercury). The latter in large part was contained in a flexible plastic tube. The output of displacement transducer (*11*) attached to the platinum separator (*9*) hoisted through a servo-system the mercury column until the pressure of this column matched the swelling pressure of the gel; i.e., until the displacement of (*9*) was retracted and the gel volume kept constant. For illustrative purposes, I have turned van de Kraats' set-up upside down. For explanation of method (*f*), *cf.* text

constitutes an appreciable fraction of the total axoplasmic volume or if there is a gradient in network density from the axial center of the axoplasmic cylinder to the curved surface, then it would have been somewhat more appropriate for me to plot something less committing instead of the conventional q. At the lower pressures the more central regions would be compressed more than the more superficial regions. If this were the case, in the hydrostatic pressure-deswelling experiments, the initial ratio of the diameter of the axon over the diameter of the bore should affect the characteristics of the $\Delta\Pi_g - q$ relation. In my experiments, this ratio varied from 2.6 to 3.4. A partially redeeming finding, however, is that both methods for determining the activity of the water in the gel yield similar relations, even though in one (the Ficoll-deswelling method) all of the axoplasm is present.

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