

Osmometrically Determined Characteristics of the Cell Membrane of Squid and Lobster Giant Axons

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ABSTRACT Lobster and squid giant nerve fibers respond differently when subjected to osmotic challenges. The axons proper, as distinct from the total (fiber) complex formed by the axon and connective sheath, both behave as "fast" osmometers for changes in the concentration of NaCl, but the maximum degree of swelling in hyposmotic media is by about 60% in lobster and only by 20% in squid. The relative volume intercepts of the van't Hoff relation are about 0.2 for lobster and 0.4 for squid. The sheaths of both axons undergo only small, apparently passive changes in volume. Lobster axons are permeable to Cl, but squid axons are impermeable to this anion. Lobster axons are also permeable to glycerol. The implications of the data as to the nature of volume regulation of cells are discussed.

INTRODUCTION

The experiments to be reported here were initiated in conjunction with osmometric studies on muscle fibers of crayfish (Reuben et al., 1964) and frog (Reuben et al., 1963). Muscle fibers have a complexly structured cell membrane which invaginates deep into the fibers through the transverse tubular system (Girardier et al., 1963; Brandt et al., 1965 *b*; Huxley, 1964; Endo, 1964). The axonal membrane (axolemma), on the contrary, seems to be structurally a relatively simple envelope (Fig. 1). Axons, however, possess a layer of Schwann cells which are closely apposed to the axolemma, separated from the latter by a space of about 100 Å. There is also a dense, multi-layered sheath.

Frog and crayfish muscle fibers are permeable to Cl (Boyle and Conway, 1941; Hodgkin and Horowicz, 1959; Adrian, 1960; Reuben et al., 1964). Squid giant axons were at one time also thought to have a high Cl permea-

bility (Hodgkin, 1951), and a value of 0.35 to 0.4 was assigned (Hodgkin and Katz, 1949) to the "permeability coefficient" for Cl (P_{Cl}) relative to K in the constant field equation (Goldman, 1943). However, microinjection experiments (Grundfest et al., 1954) indicated that Cl must be relatively impermeant, since the membrane potential was not altered when a substantial amount of K was introduced either as the Cl salt or as salt of other anions. These findings have been confirmed by recent studies on perfused giant axons and the value for P_{Cl} currently estimated from such work is about 0.02, or of the same order as P_{Na} in the resting axon (Baker et al., 1964).

Osmometric studies on axons thus could also serve to test the degree of correspondence of such data with electrophysiological measurements. Because of the year round availability of the material, these studies were first done on lobster axons. The data demonstrated that the membrane of lobster axons is quite permeable to Cl. Accordingly, similar studies were then also carried out on squid giant axons. These confirmed the electrophysiological finding that the squid axon is effectively impermeable to Cl.

This paper therefore presents comparative data on the osmotic properties of two axons which differ in their permeability characteristics. Electrophysiological and morphological correlates of these differences were studied, particularly on the lobster axons, for which published data are less extensive than they are for squid axons. The electrophysiological studies (Freeman et al., 1964 *b*) on lobster axons will be reported fully at another time and only a few of the data will be presented here. Preliminary accounts of the present work have appeared (Freeman et al., 1964 *a*, 1965; Brandt et al., 1965 *a*).

METHODS

Studies on lobster axons were done mainly in New York, but experiments were also carried out at the Marine Biological Laboratory at Woods Hole in order to check the possibility of seasonal variations such as have been observed in frog muscle fibers (Reuben et al., 1963, and unpublished data). None was found. Experiments on squid axons were done at Woods Hole.

Motor axons of the lobster walking legs, which are known functionally as the " opener" and the "fast closer" (Wiersma, 1961) were dissected from the meropodite. Isolated axons were also prepared from the circumesophageal connectives (Wright and Reuben, 1958; Julian et al., 1962). The fibers of the walking legs ranged in diameter between 50 and 60 μ , and those of the connectives were up to 120 μ in diameter. Squid axons were all close to 500 μ in diameter and were cleaned of the smaller fibers in cold sea water. The connective tissue sheath which surrounds the squid axon has been described previously (cf. Villegas and Villegas, 1960; Baker et al., 1962). Our own data are in general accord with the earlier findings and will not be presented. Lobster axons also have a connective tissue sheath (Fig. 1) which differs somewhat in structure from that of squid axons. In addition to the Schwann cell layer it consists of a number of concentric layers of what is probably collagenous material with rather thin coverings of interposed connective tissue. As will be de-

scribed below, the responses of the axon proper and of its sheath to a given osmotic challenge could differ considerably.

Both dimensional changes and electrophysiological characteristics could be examined at the same time while making various changes in the bathing medium. The isolated axon was mounted in a Lucite chamber which was placed on the stage of an inverted microscope, equipped for photomicrography. The ends of the fiber were held in clamps. Electrodes were fixed in the chamber for stimulation and for external recording and a microelectrode was often inserted for intracellular recording. The electrophysiological equipment was standard for the laboratory. The experiments were done at room temperature (18° to 23° C). As far as possible, experiments were carried out in pairs, on axons from the same animal, one being exposed to a medium which caused swelling, the other to a medium which caused shrinkage. Squid and lobster axons which were representative of each experimental condition that was employed were fixed for electron microscopy.

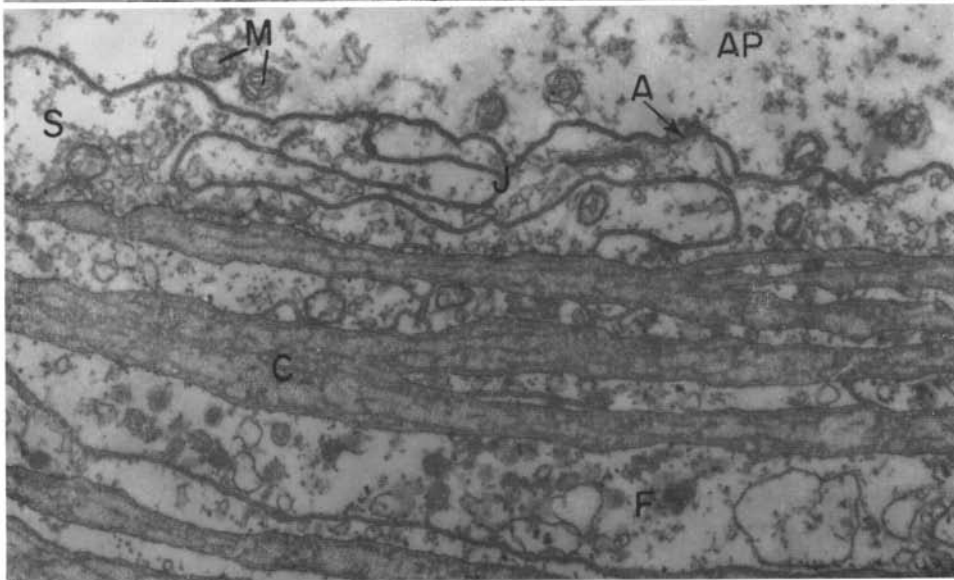
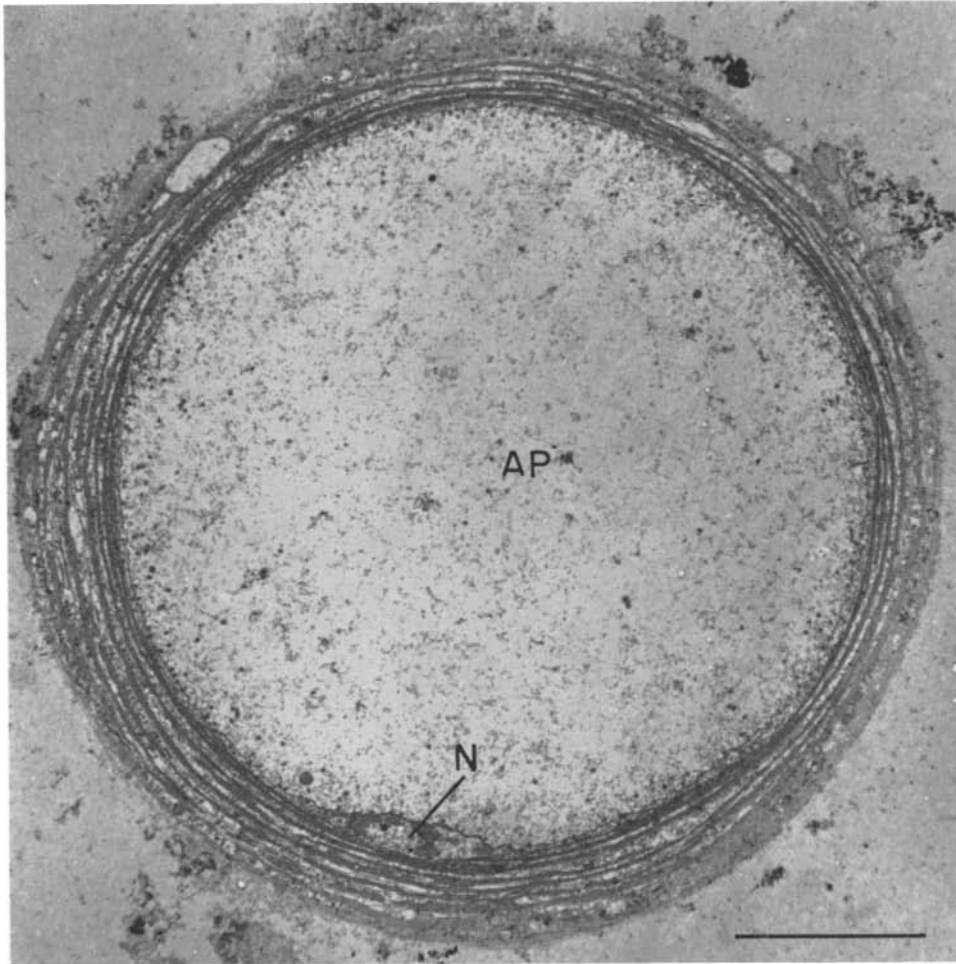
For the osmometric measurements the diameters of the fibers were estimated from enlarged photomicrographs (Fig. 2), with an error of less than $\pm 2\%$. The major source of error in the measurements lies in the estimation of the boundary between the axon proper and the connective tissue sheath which forms the remainder of the nerve fiber (Figs. 1 and 2). The accuracy of these estimations varied somewhat depending upon the experimental conditions, since under some conditions clear spaces could be observed between the axoplasm and connective tissue. The measurements on living lobster axons were compared with measurements on the same fibers after they had been fixed for electron microscopy. These paired measurements, expressed in terms of the ratios of the axonal and fiber volumes $(r_i/r_o)^2$, agreed remarkably closely, as will be described below (Table I).

In one series of experiments a lobster fiber was mounted in a holder which permitted full rotation of the axon about its longitudinal axis. The measurements showed that the axon responded as a uniform cylinder to changes in the NaCl concentration of the medium. In several experiments squid axons were injected with chlorphenol red (Grundfest et al., 1954), in order to permit better visualization of the axon as distinct from the connective tissue sheath. These fibers were then also exposed to various changes in the medium and were photographed either under ordinary light or in a polarization microscope.

The control bathing medium for the experiments on both types of lobster axons was that described by Dalton but with SO_4 omitted (1958).¹ The axon was first equilibrated in this medium, measurements being made frequently to ascertain that the volume was not undergoing changes. Changes in osmotic pressure were produced by variation of the sodium and/or the potassium salt concentration of the medium and specific experimental conditions will be given in connection with the particular experiments. The relative osmotic pressure of a given solution in which the Na salt was increased or decreased was calculated according to the expression

$$\frac{2k (\text{Na salt concentration})_{\text{initial}} + R}{2k (\text{Na salt concentration})_{\text{final}} + R} \quad (1)$$

¹ The composition was (in milliequivalents per liter): Na 465, K 10, Ca 50, Mg 16, Cl 533.



k is the osmotic coefficient and R represents the osmotic contribution of the other, invariant constituents of the medium.

Changes in the relative volume of the axoplasm and of the connective tissue were calculated separately since it was found that the two compartments exhibited different osmotic characteristics (Fig. 2). The volumes were calculated on the basis of the finding that the axons showed radial symmetry. For a radially expanding or contracting cylinder of constant length,

$$\frac{(r_{\text{final}}^2)}{(r_{\text{initial}}^2)} = \frac{V_{\text{final}}}{V_{\text{initial}}} = V_{\text{relative}}; \text{ where } r = \text{radius} \quad (2)$$

Thus, employing the nomenclature given in Fig. 2

$$\frac{A_{\text{final}}^2}{A_{\text{initial}}^2} = \text{relative volume change of axoplasm} \quad (3)$$

$$\frac{B_{\text{final}}^2}{B_{\text{initial}}^2} = \text{relative total volume change of both compartments} \quad (4)$$

$$\frac{B_{\text{final}}^2 - A_{\text{final}}^2}{B_{\text{initial}}^2 - A_{\text{initial}}^2} = \text{relative volume change of connective tissue} \quad (5)$$

RESULTS

A. Lobster Axons

BEHAVIOR OF THE AXON AS AN OSMOMETER FOR NaCl Whether isolated from the nerve cord or from the nerve trunks of the walking legs, lobster axons gave essentially similar osmometric data. They exhibited a clear cut compartmentalization (Fig. 2). The inner, axoplasmic compartment, which is bounded by the axolemma, shrank markedly in hyperosmotic NaCl media while there was little apparent change in the over-all diameter of the fiber (Figs. 2 and 3). In hyposmotic media the entire fiber swelled. However, examination of the boundaries of the different compartments of the nerve fiber (Fig. 2) showed that the axon proper swelled markedly at the expense

FIGURE 1. Electron micrographs of axon from lobster walking leg. Above, at low magnification the whole axon and its sheaths are seen. Below, at higher magnification. In the axoplasm (AP) adjacent to the axolemma (A) are numerous mitochondria (M) oriented with their long axes perpendicular to the plane of the cross-section. The axolemma is closely invested by a layer of Schwann cells (S). The junction (J) between two interdigitating Schwann cells is separated by a space of about 100 Å which is continuous with the periaxolemmal space of about the same dimension. There appears to be no basement membrane (as there is in squid) to separate the Schwann cells from the overlying connective tissue. The latter is formed by layers of fibrous collagen-like material (C) and of connective tissue cells (F). In the low power micrograph the nucleus of a Schwann cell (N) is visible. Line represents 12.5 μ for the upper micrograph and 1 μ for the lower.

of the space occupied by the connective tissue sheath, the latter undergoing a relative diminution in volume (Fig. 3).

The combined data of experiments on lobster motor axons obtained by changing the NaCl concentration are shown in terms of the π - V relation in

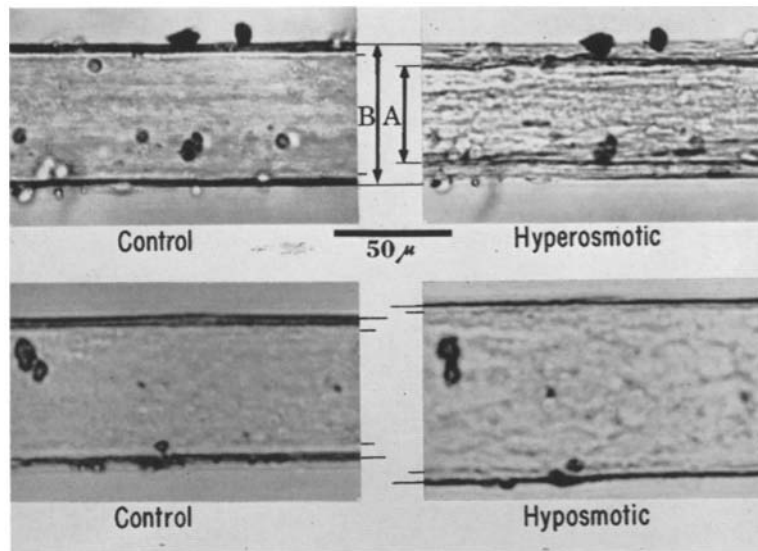


FIGURE 2. Photomicrographs of two lobster axons from the same animal, one exposed to a hyposmotic NaCl medium, the other to a hyperosmotic medium. Lines are drawn in to show the edges of the inner compartment (*A*) and the outer (*B*) which are respectively the axon proper and the sheath of connective tissue surrounding the axon. Note the different responses of the two compartments to the osmotic challenges.

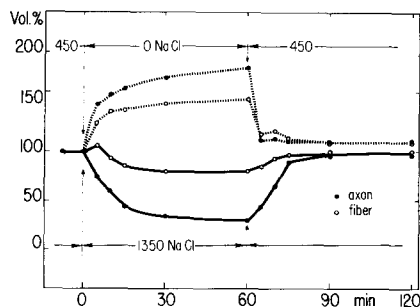


FIGURE 3. Time course of the relative volume changes in lobster axons during and after challenges by hyposmotic (0 NaCl) and hyperosmotic (three times NaCl) media. The volume of the axon (filled circles) changed much more than did the volume of the whole fiber.

Fig. 4. The relative volume changes of the axoplasmic compartment and of the whole fiber are plotted separately. The axoplasmic compartment responds linearly to changes in relative osmotic pressure from three times the normal level of osmotic pressure to about 75% decrease in the pressure. The maximum shrinkage of the axon proper as extrapolated from the data of Fig. 4 is to about 20% of the original volume and the maximum swelling is to about 170% of

that volume. The maximum degree of swelling is about the same as in single frog muscle fibers (Reuben et al., 1963), but is less than that of crayfish fibers (Reuben et al., 1964). The extrapolated maximum degree of shrinkage is greater than is that for either type of muscle fiber.

The volumes of the nerve fibers as measured from their outer diameters changed relatively less. This was particularly so in hyperosmotic media, when

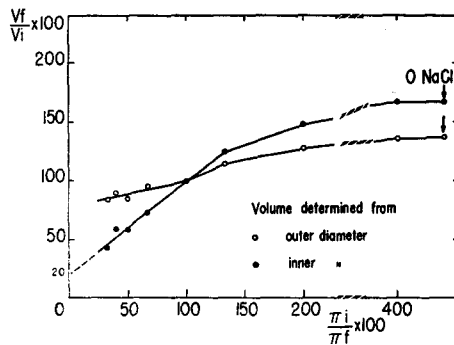


FIGURE 4. Pressure-volume relation for lobster nerve fibers exposed to different osmotic challenges. Abscissa, relative change in osmotic pressure. Ordinate, relative change in volume. The changes in the volume of the axon proper (filled circles) and of the whole fiber (open circles) are plotted separately. Further description in text.

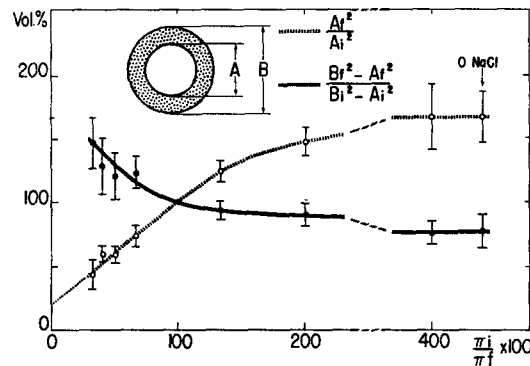


FIGURE 5. Pressure-volume relation of Fig. 4 for the axon proper shown as the open circles, the bars representing the standard error of the measurements. Filled circles and solid line show the relative volume changes of the connective tissue compartment alone. Inset shows the method of calculating the two volumes. Further description in text.

the axon shrank markedly while the change in relative volume calculated from the measurements of the outer diameter of the fiber was very small. In fact, if the intercept on the ordinate were used for an estimate of the osmotically inactive fraction, a value of 75% of the whole fiber would be obtained.

The reason for this anomaly is given by the data of Fig. 5 which describe the changes in relative volume of the inner compartment (A, as in Fig. 4), and of the annular element, or the outer compartment. The relative volume of the latter decreased somewhat as the inner compartment swelled in hypotonic media, indicating that the swelling of A was causing some compres-

sion of the annular space. As the axon proper diminished in volume in hyperosmotic media, the annular compartment increased. It did so, however, by occupying part of the space given up by the inner compartment, since the over-all volume of the fiber declined slightly (Fig. 4).

That the outer compartment does not behave as an osmometer for Na chloride, whereas the inner compartment does, is shown in Fig. 6 in which a pair of fibers were exposed to the same final level of hyperosmotic solution. One fiber was transferred at once to this solution while the other was exposed to stepwise increases in the osmotic pressure, each lasting 10 min. Compartment *A*, the axon proper (filled circles), shrank to the same degree in both experiments. The diameter of the fiber which was exposed to the doubled concentration of NaCl in one step diminished, but to a lesser degree than did

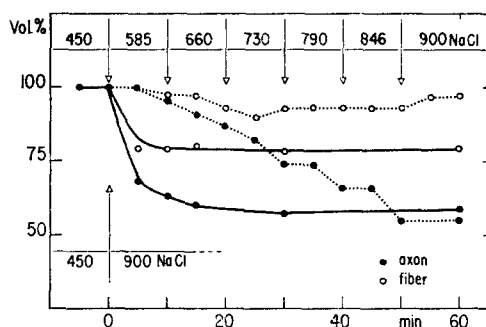


FIGURE 6. Different responses of axon and connective tissue compartments to differently presented, but eventually identical, osmotic challenges. Solid lines show the time course of the shrinkage in the axon (filled circles) and of the whole fiber (*open circles*) when the fiber was exposed to 900 mm/liter NaCl. The broken lines represent corresponding measurements on raising the concentration of NaCl gradually, as indicated in the upper portion of the figure. Further description in text.

the inner diameter, as in Figs. 2 and 3. The final volume of the fiber which was exposed to the stepwise changes in osmotic pressure showed almost no change from the control level. The foregoing data indicate that the boundary of the inner compartment has a reflection coefficient (Staverman, 1951) of unity for NaCl, while that of the outer compartment must be small.

The micrographs of Fig. 2 and the measurements shown in Figs. 3 to 6 were made on living axons, capable of conducting spikes with an average velocity of 6 m/sec. Dimensional measurements were also made on axons after they had been fixed as preparations for electron microscopy. Table I shows comparisons of the measurements of the two compartments before and after fixation in a representative sample of lobster axons. The values are expressed in terms of the ratios of the relative volumes calculated in the living and fixed material for the different experimental conditions. It is clear that the inner

and outer compartments, as determined with the light microscope, represent the axon proper and connective tissue space, respectively. From this point on relative volume changes of the inner compartment only will be dealt with.

Exposure of the lobster axons to media made hyperosmotic by addition of NaCl, or the reversal from that condition, did not alter their ability to generate and conduct spikes. Conduction of impulses invariably failed when the

TABLE I
COMPARISON OF THE RATIO OF CROSS-SECTIONAL
AREAS OF LOBSTER AXONS AND OF THE WHOLE FIBER IN
LIVING AND FIXED PREPARATIONS EXPOSED
TO DIFFERENT EXPERIMENTAL CONDITIONS
 r_o = radius of whole fiber, r_i = radius of axon

Experimental condition	$(r_i/r_o)^2$	
	Living	Fixed
Control	0.68	0.65
Hypotonic NaCl medium	0.81	0.84
Isotonic KCl medium	0.80	0.86
Hyperosmotic NaCl medium	0.41	0.45

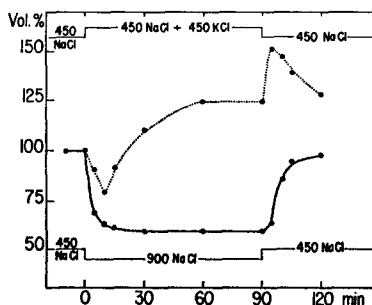


FIGURE 7. Responses of a pair of axons to media made hyperosmotic by adding 450 mM/liter NaCl (solid line) and by adding 450 mM/liter KCl (broken line). Decreased initial shrinkage and subsequent swelling of the axon exposed to high KCl indicate entry of this salt. Note the further swelling on returning the fiber to the control medium and the slow rate of the subsequent decrease in volume.

osmotic pressure was reduced to approximately 25% of the normal level. This failure was not due solely to the diminution in the Na level, however, for it was not reversible when the fibers were restored to the control medium. The electron micrographs of the fibers which had been exposed to the very hypotonic media and which had become unresponsive invariably showed obvious and numerous discontinuities of the axolemma. Such breaks were never seen in control preparations or in those which had been fixed while

exposed to strongly hyperosmotic media, or which were swollen in media made hyposmotic by removing only 50 to 60% of the NaCl.

OSMOTIC RESPONSES TO INCREASING KCl When the bathing medium was made hyperosmotic with the addition of 450 mM/liter KCl to the standard medium (Figs. 7 to 9), the axon at first shrank and then swelled to a new steady level, which ranged from 15 to 30% above the initial volume. On returning to the control medium the axon first swelled further and then gradually diminished in volume. However, the axons did not regain their initial volume even as long as 1.5 hr later (Fig. 9, broken line curve).

The changes in volume which result when the lobster axon is exposed to a medium made hyperosmotic by increasing the KCl of the solution are similar

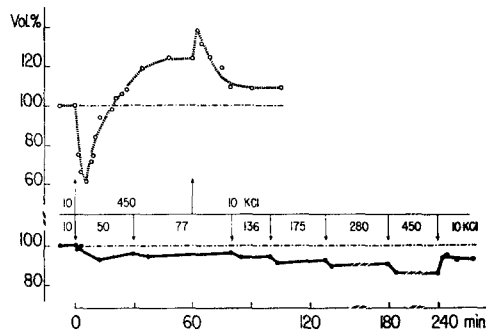


FIGURE 8. Different responses of lobster axons to sudden and gradual enrichment of the medium with KCl. Upper graph, time course of relative volume changes during transfer of an axon to a medium made hyperosmotic by addition of 450 mM/liter KCl and after return to the control medium. Lower graph, this axon was exposed to a small stepwise increase in KCl, but after 3 hr the final solution also contained 450 mM/liter KCl. Further description in text.

in form to those observed in muscle fibers of frog (Boyle and Conway, 1941, Reuben et al., 1963) and crayfish (Reuben et al., 1964). They are regarded as denoting the initial efflux of water and its reentry as KCl enters the axon. Failure of the volume to return to the original level when the axon is returned to the control medium is apparently related to changes in cellular properties which occur when the axon is suddenly subjected to a high concentration of KCl. These interpretations, however, must be viewed with caution as is shown by experiments like those of Fig. 8.

One of the two axons illustrated in Fig. 8 (open circles and broken line) was subjected to a medium made hyperosmotic by adding 450 mM/liter KCl, as in Fig. 7, with essentially similar results. The other axon (solid line and filled circles) was subjected to gradual additions of KCl, starting with an increase to 50 mM/liter. While it had been expected that this second axon would show less initial shrinkage, the final steady-state volume of the two

axons should have been the same if the ionic distribution across the cell boundary can be expressed merely as a function of the external concentration of KCl (Boyle and Conway, 1941). In fact, the second axon remained at about 15% below its initial volume in the 450 mM/liter K solution. Furthermore, upon returning to the control saline, the axon swelled but still remained approximately 5% below the initial volume. This small swelling of 10% suggests that either the axon had gained less KCl during the prolonged gradual increase in the KCl concentration of the medium or that there was a coincidental loss of some intracellular salt, perhaps NaCl.

When the medium bathing the nerve fiber was maintained isosmotic, but with substitution of KCl for all the Na, the axon swelled markedly to a maximum volume about 70% above the original level (Fig. 9, solid line). Thus, KCl and water must have entered the cell until a limit of swelling was reached,

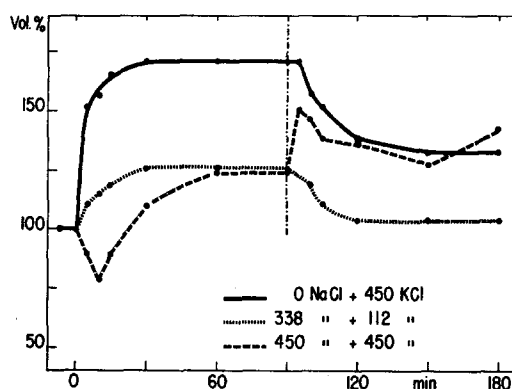


FIGURE 9. Changes in volume produced by hyperosmotic and isosmotic KCl-enriched media. Three different axons. Broken line, axon had been exposed for 90 min to a medium made hyperosmotic by addition of 450 mM/liter KCl. Dotted line, this axon was exposed to an isosmotic medium but with one-fourth the NaCl replaced with KCl. Note that the swelling reversed monotonically when the axon was returned to the control medium. Solid line, this axon was placed in an isosmotic medium in which KCl had replaced all the NaCl. The larger degree of swelling was less readily reversible. The decrease of the volume paralleled that of the fiber which had been exposed to the hyperosmotic medium. The vertical line indicates the time when the axons were returned to the control saline.

similar to that observed in hyposmotic NaCl media. When only 25% of the NaCl was replaced with KCl (dotted line) the fiber volume increased by 25%, further evidence that the membrane is highly permeable to KCl.

In the fiber which was exposed only to a 25% isosmotic substitution of KCl for NaCl, the volume of the axon regained its initial level on restoring the normal bathing medium, whereas the axon which was exposed to the 450 mM/liter (isosmotic) KCl solution remained considerably swollen even 1.5 hr after it had been returned to the control medium.

OSMOTIC RESPONSE TO K PROPIONATE The conclusion that lobster axons are permeable to Cl as well as to K was confirmed by experiments like that of Fig. 10 which presents parallel measurements of the axon volume when a fiber was exposed to an isosmotic KCl medium or to isosmotic K propionate. In the chloride saline, isosmotic substitution of KCl for NaCl gave results essentially the same as those shown in Fig. 9. The other fiber which had been first equilibrated for approximately 1 hr in a Cl-free (propionate) medium showed no volume change when the Na propionate was replaced by K propionate. In fact, some fibers underwent a slow shrinkage when placed in the K propionate medium. Similar data were obtained with muscle fibers (Reuben et al., 1963, 1964). Thus K propionate can serve as an osmotically impermeant salt for both muscle and axon membranes.

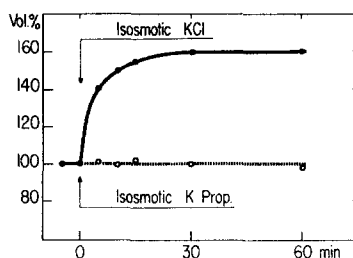


FIGURE 10. Different osmotic effects of K salts with permeant and impermeant anions. Solid line and filled circles, swelling of an axon which had been transferred to a medium in which all NaCl was replaced with KCl. Broken line and open circles, this fiber, which had been equilibrated in an isosmotic Na propionate medium before transferring to K propionate, showed no change in volume.

CHANGES IN AXON VOLUME AND MEMBRANE POTENTIAL INDUCED BY FLUX OF Cl Fig. 11 gives further evidence that the membrane of lobster axons is permeable to chloride. In experiments of this type the fiber was challenged with an isosmotic, but Cl-free (propionate) medium. After an hour in this solution the chloride was reintroduced. Both the membrane potential and the volume of the axon underwent changes when the chloride concentration in the medium was altered. Immediately upon withdrawing the chloride the membrane depolarized by about 20 mv from its initial value (-80 mv). It repolarized gradually, returning to the initial potential only after 40 min in the propionate medium. There was a smaller and briefer hyperpolarization on restoring the chloride. Similar transient changes in membrane potential have been observed in muscle fibers of frog (Hodgkin and Horowicz, 1959), crayfish (cf. Grundfest, 1962, Figs. 3 to 5; Girardier, Reuben, and Grundfest, unpublished data), and lobster (Gainer and Grundfest, unpublished data), and have been ascribed to a passive redistribution of chloride, presumably as KCl.

Since a loss or gain of KCl should result in a maintained shrinkage or swelling of the axon, it follows that the observed transient volume changes are "anomalous" in nature. Similar phenomena have been reported for crayfish muscle fibers (Reuben et al., 1964), except that a steady volume displacement ranging from 5 to 10% occurred in the axon following the transient response due to removal of chloride from the medium. Both the potential measurements and the osmometric data indicate that the axon membrane is permeable to Cl.

LOCALIZED VOLUME CHANGES ASSOCIATED WITH APPLIED CURRENT In crayfish muscle fibers an applied transmembrane current induced readily detectable volume changes in the fiber (Reuben et al., 1964). In similar ex-

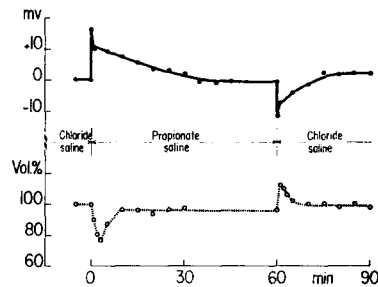


FIGURE 11. Changes in membrane potential and volume of lobster axon caused by movement of Cl. Transfer of the fiber to a Cl-free medium caused a transient depolarization (solid line and filled circles). Restoration of the Cl medium caused a smaller transient hyperpolarization. The shift to and from the propionate saline also caused transient changes in volume (broken line and open circles).

periments performed with lobster axons any volume change that might have occurred during an applied current of 5×10^{-7} amp for 10 min was not detectable. However, during the period of cathodal current flow the fiber changed in appearance as observed under the light microscope. A local granulation developed at the point of insertion of the microelectrode and spread longitudinally with increased amount of current. Electron micrographs of axons fixed in this condition revealed localized enlargements or "gapping" of the normally uniform periaxolemmal space (Brandt et al., 1965 *a*). Consistent with the light microscope observations the gaps increased in size and frequency with greater amounts of current. These effects were minimized or absent with anodal currents or when cathodal currents were applied with a K propionate-filled microelectrode.

OSMOTIC EFFECT OF GLYCEROL Squid axons have been reported to be relatively impermeable to glycerol (Villegas and Barnola, 1961; Villegas et al., 1962). Experiments were performed to test the permeability of lobster

axons to this nonelectrolyte. Fig. 12 shows volumetric data on axons which were exposed to isosmotic (0 NaCl + 900 mM glycerol) and hyperosmotic (450 mM NaCl + 900 mM glycerol) media. The volume changes are of the same type as those that have already been seen in connection with changes in the medium made by increasing the level of KCl (Figs. 7 to 10). The fiber which was exposed to the isosmotic glycerol (Fig. 12, filled circles and solid line) swelled more slowly at first than fibers which were exposed to isosmotic KCl (Figs. 9 and 10). In both cases, however, the steady new level was reached within about 30 min, but the swelling in the glycerol medium was somewhat less. In the hyperosmotic glycerol medium (Fig. 12, open circles and broken line) the initial shrinkage was of the same order as in an equally hyperosmotic KCl medium (Figs. 7 to 9) but the steady-state volume repre-

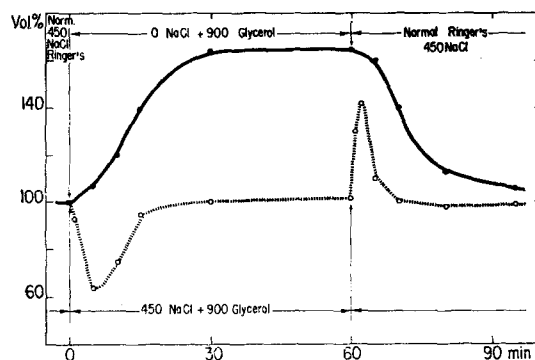


FIGURE 12. Volume changes in two lobster axons, one of which was exposed for 1 hr to a medium made hyperosmotic by addition of 900 mM/liter glycerol (broken line, open circles) and the other to an isosmotic medium with 900 mM/liter glycerol substituting for the NaCl (solid line, filled circles). Further description in text.

sented a return to the initial volume of the axon (Fig. 12). This level was obtained within 30 min, whereas in the hyperosmotic KCl medium the fiber attained a steady state only after about 1 hr and was then considerably swollen. On returning the axons from the glycerol medium to the control solution the fiber exposed to the hyperosmotic glycerol swelled rapidly, but soon thereafter returned to the control volume. The fibers exposed to the isosmotic glycerol medium also returned to the control volume, but much more slowly. Thus, there was an initial "latency" both in the beginning of the swelling and at the beginning of the return to the control volume.

The treatment with glycerol was not only reversible with respect to volume of the axon, but it also did not eliminate conductile activity. The fiber remained excitable while in the hyperosmotic medium. The excitability was abolished in the Na-free isosmotic glycerol medium, but was restored immediately on reintroducing NaCl. Thus, the irreversible changes which were

noted on exposing the fiber to KCl-enriched media apparently are due to the large ionic influx or to exposure of the axoplasm to a high concentration of Cl. Morphological alterations were noted in the fibers exposed to glycerol as well as in those exposed to KCl (Brandt et al., 1965 *a*, and unpublished data) and gapping between the Schwann cells and the axolemma was severe in both cases. However, the membrane was disrupted in axons which were exposed to the high KCl media, but this condition was not seen after exposing the fibers to glycerol.

B. Squid Axons

BEHAVIOR AS AN OSMOMETER The axoplasmic compartment of the squid fiber shrank to about 60% of its original volume when the fiber was exposed

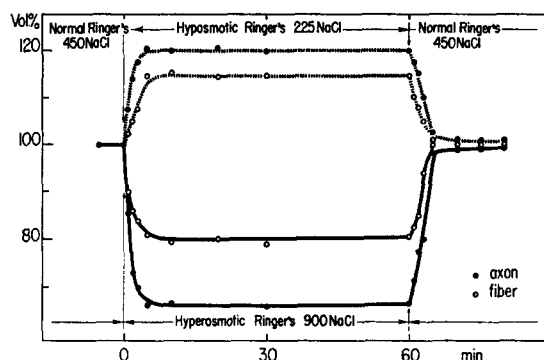


FIGURE 13. Volume changes in two squid giant axons, one of which was exposed for an hour to a hyperosmotic medium (solid lines) and the other to a hyposmotic medium (broken lines). As was the case also with lobster fibers (Fig. 3) the axon proper (filled circles) underwent larger changes in volume than did the whole fiber (open circles) in response to the osmotic challenges. The volume changes were reversible.

to a medium made hyperosmotic by doubling the NaCl (Fig. 13, filled circles). The whole fiber, however, shrank only by about 20% (open circles). On exposing the fibers to a medium made hyposmotic by removing half the NaCl the axoplasmic compartment swelled by about 20% while the volume of the whole fiber increased by only about 15%. These changes reached a steady state within about 5 min and were reversible, although rather more slowly than they had developed.

For a comparable change in osmotic pressure the shrinkage in the hyperosmotic media was considerably larger than the swelling in the hyposmotic. There was also a marked difference in the relation between the axonal volume and that of the whole fiber. This difference signifies that the two compartments, axon and connective tissue, behave qualitatively in a manner similar to that described for lobster axons. The difference in behavior of the two

compartments is shown particularly clearly when the data for a number of fibers are plotted in terms of their pressure-volume relation (Fig. 14). The axon proper shrank in hyperosmotic media, losing about 35% of its volume when the osmotic pressure was doubled. The limits of shrinking and swelling observed were 45 and 20%, respectively. Thus, the squid axon while responding somewhat more rapidly than the lobster axon to osmotic forces does so to a smaller extent and over a more limited range, particularly with respect to the increase in volume in hyposmotic media.

The connective tissue compartment of the squid axon appears to behave passively in response to the osmotic challenges. It swelled slightly in hyperosmotic media. The swelling appears to be related to the fact that the

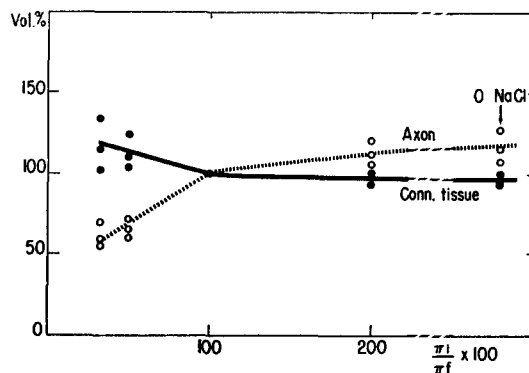


FIGURE 14. *P-V* relation for squid axons (broken line, open circles) and for the connective tissue (solid line, filled circles). Abscissa, relative changes in osmotic pressure made by adding NaCl to or subtracting it from the medium. Ordinate, relative volume changes calculated as was done in connection with Fig. 5.

axoplasmic volume had diminished, the connective tissue then expanding somewhat into the space formerly occupied by the axon. The insignificant change in volume of the connective tissue in hyposmotic media may account for the small degree of swelling of the axon in such media.

Villegas and Villegas (1960) have reported that the connective tissue sheath does not form a significant barrier for the access of the external medium to the axolemma. This is confirmed in the present work since the axonal volume changes in response to changes of somatic pressure occurred rapidly (Fig. 13) and were independent of the changes in volume of the connective tissue sheath (Fig. 14). The volume of the axoplasmic compartment as calculated from our data is about 80% of the total fiber volume. This is in good agreement with estimates based on electron microscopic observation (Baker et al., 1962; Villegas and Villegas, 1960). The osmotic "dead space" estimated from extrapolation to the intercept on the volume ordinate is about 40% or about double the value for lobster axons (Fig. 4).

OSMOTIC RESPONSES TO INCREASING KCl IN THE MEDIUM That the squid axon membrane is impermeable to KCl was shown in experiments like that of Fig. 15. On transferring an axon from the control medium to one made hyperosmotic by doubling the NaCl (i.e., to 900 mM/liter) the relative axonal volume decreased to about 70% of the original level. After 30 min in this medium the bathing solution was changed to one containing 450 mM/liter KCl, in addition to the standard level of 450 mM/liter NaCl. With the exception of a further small transient shrinkage, no significant change in volume occurred in this condition, indicating that the membrane is as impermeable to KCl as it is to NaCl. The fiber returned to its original volume on restoring the normal medium.

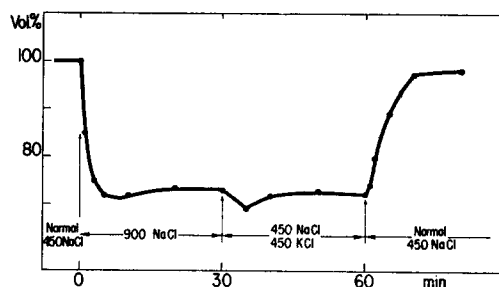


FIGURE 15. Evidence for impermeability of the squid axon to KCl. The axon was first subjected to a medium made hyperosmotic by increasing NaCl to 900 mM/liter. After 30 min the medium was changed for one containing 450 mM/liter KCl as well as the normal level of NaCl. The volume remained essentially constant, indicating that the axon is effectively as impermeable to KCl as it is to NaCl.

Since the membrane potential of the squid axon changes approximately according to the thermodynamic (Nernst) relation on changing the K concentration in the medium (Curtis and Cole, 1942), the resting membrane must be regarded as permeable to K. It may be concluded therefore that the impermeability to KCl is due to the effective impermeability of the membrane to Cl. Further evidence that the squid axon is impermeable to Cl is provided by the absence of a transient change in membrane potential when the axon is placed in a Cl-free (propionate) medium or when the normal NaCl medium is restored.

DISCUSSION

Chloride Permeability of Lobster and Squid Axons The fact that lobster axons swell in isosmotic KCl media (Figs. 9 and 10) is direct evidence for the permeation of this salt (Boyle and Conway, 1941). The steady displacement of the volume obtained upon altering the NaCl concentration in the medium must therefore arise from the effective impermeability of the membrane

toward Na ions. Squid axons, however, exhibited steady displacements of volume when they were exposed to KCl-enriched solutions (Fig. 15), as well as to media made hyperosmotic or hyposmotic by changing the concentration of NaCl (Figs. 13 to 15). Thus, the permeability of the squid axon membrane to K cannot be determined by volume measurements. However, a high relative permeability of the membrane to K has been established by electrophysiological measurements (Curtis and Cole, 1942; Hodgkin and Katz, 1949). On the other hand, electrophysiological measurements indicate a very low Cl permeability of the squid axon membrane (Grundfest et al., 1954; Baker et al., 1962; Baker et al., 1964). Total replacement of Cl in the bathing medium with propionate did not alter the membrane potential of the squid axons in contrast with the transient depolarization observed in lobster axons (Fig. 11). Thus, both the electrophysiological data and the present osmometric findings agree that the squid axon membrane is effectively impermeable to Cl while the lobster axon is permeable.²

The "Transport Number" for Cl A transient depolarization occurred in lobster axons when the Cl of the control medium was substituted with propionate, and the fibers hyperpolarized on restoring the Cl (Fig. 11). Depolarization was also observed in circumesophageal axons by Julian et al. (1962), but the change was not followed for sufficiently long times to observe its transient character. The changes in potential have been ascribed to a diffusion (liquid junction) potential arising from an alteration of the Cl gradient across the cell membrane (Hodgkin and Horowicz, 1959).

A "transference" or "transport" number for Cl (t_{Cl}) may be evaluated from the change in membrane potential according to the following equation which is applicable to liquid junction potentials (MacInnes, 1961):

$$-E_L = \frac{RT}{F} \int_I^{II} \sum^n \frac{t_i}{Z_i} d \ln a_i \quad (6)$$

where E_L is the liquid junction potential, a_i is the activity of the i^{th} ion, t_i is its transport number, and Z_i its valence. Assuming that there is no interaction between different ions, and substituting concentrations for activities,

² While Baker et al. (1962), in agreement with earlier data (Grundfest et al. 1954) had noted that chloride ions "do not make a substantial contribution to the resting potential" of squid axons, and Baker et al. (1964) had assigned a value of $P_{Cl} = 0.02$, a subsequent paper from the same laboratory (Chandler and Hodgkin, 1965) reported a value of $P_{Cl} = 0.7$ for one axon (No. 9, Table 5) which was studied in a K-free medium. The authors do not comment upon the discrepancy with the earlier findings. Their permeability measurements of different axons show considerable variability. Assuming, however, that the new estimate for P_{Cl} is also valid, it is possible that the absence of K in the medium may have increased the permeability for Cl. Removal of K makes the membrane more permeable to Na in vertebrate cardiac and skeletal muscle fibers (Carmeliet, 1961; Reuben et al., 1963; Grundfest, 1966 *a, b*).

t_{Cl} can be obtained by transforming the above equation into the form:

$$\frac{-dE_L}{d \ln Cl} = \frac{RT}{F} t_{Cl} \quad (7)$$

The validity of this equation depends upon the measurement of the change in membrane potential prior to the occurrence of any redistribution of ions.

In lobster axons the peak transient depolarization on removing all but 1 meq/liter Cl, or on total substitution of this anion with propionate varied from 15 to 20 mv (Fig. 11). For a depolarization of 20 mv the calculated value of t_{Cl} would be 0.11. In frog muscle fibers the transient depolarizations were larger and yielded a value of t_{Cl} of 0.6 (Hodgkin and Horowicz, 1959), but in crayfish muscle fibers t_{Cl} has a value of approximately 0.1 (cf. Grundfest, 1962, and Girardier, Reuben, and Grundfest, unpublished data). However, it is questionable whether the transport numbers obtained from the electrophysiological measurements are truly representative of the ionic permeability of the cell membrane. On reintroducing Cl the transient hyperpolarization was about half the value of the peak transient depolarization (Fig. 11). The same asymmetry is also observed in the transient hyperpolarizations of frog (Hodgkin and Horowicz, 1959) and crayfish (cf. Grundfest, 1962, Figs. 3 to 5; and Girardier, Reuben, and Grundfest, unpublished data) muscle fibers. A liquid junction potential should be symmetrical for a given concentration gradient (MacInnes, 1961). Indeed, the hyperpolarization might be expected to be larger than the depolarization, since the return from depolarization to the initial level (Fig. 11) presumably reflects ionic redistribution with loss of Cl from the cell. Thus on reintroducing Cl into the medium the ratio Cl_{out}/Cl_{in} must be larger than was the initial level.

The asymmetry of the transient changes may reflect asymmetrical boundary conditions, such as might be caused by a region of fixed charges on the axoplasmic side of the membrane (Chandler et al., 1965). The asymmetry might be caused also by regions of restricted extracellular spaces immediately surrounding the axolemma, as was suggested by Frankenhaeuser and Hodgkin (1957) to explain slow changes in the membrane potential of squid giant axons. In lobster axons evidence which indicates the importance of considering such spaces is provided by the swellings which develop in the periaxolemmal space under various conditions (Brandt et al., 1965 *a*).

Our doubts about the validity of the electrophysiological measurements of t_{Cl} are supported by the results of parallel measurements of t_K which will be reported elsewhere. Briefly, small changes in the level of the external K from the normal values also yield values of t_K which are low and the sum of the electrophysiologically determined values $t_K + t_{Cl}$ is far less than unity both in crayfish muscle fibers (Girardier, Reuben, and Grundfest, unpub-

lished data) and in lobster axons (Freeman, Reuben, Brandt, and Grundfest, unpublished data).³ Thus, while the electrophysiological and the volumetric data provide convincing evidence that the membrane of lobster axons is permeable to Cl, other types of data are required for a quantitative evaluation of the degree of the Cl permeability.

Osmotic Pressure-Volume Relationship As defined by a steady displacement of volume when the concentration of NaCl in the bathing medium is changed, both squid and lobster axons are effectively impermeable to NaCl. It is clear, however, that the lobster axon can gain or lose greater amounts of water for equivalent variations in osmotic pressure. The "dead space" obtained by extrapolation of the π - V curve to the ordinate (Figs. 4 and 14) is 20% for lobster axons and about 40% in squid axons. The latter value is in agreement with the results of Villegas and Villegas (1960).

The extrapolated limit value for dead space has been ascribed to the total content of osmotically inactive intracellular material (Conway, 1957). As measured on single fibers, both muscle and nerve, this dead space shows considerable variation for the different types of cells. In crayfish muscle fibers it was about 40% of the resting volume (Reuben et al., 1964). The value obtained (Reuben et al., 1963) in frog muscle fibers (35%) was identical with the total intercept obtained for whole muscle (Conway, 1957). However, 20% was ascribed to the intracellular component after allowing about 15% for extracellular space (Conway, 1957). This allowance is not valid for the single fiber preparations.

The axons could swell to only a limited degree in hyposmotic media (Figs. 4 and 14). The maximum increase in the volume of squid axons was only about 20% and of lobster axons about 60%. The changes in over-all diameter and volume were even smaller, since the sheaths swelled much less than did the axons. In a preliminary note, Shanes (1948) reported that squid and crab nerves underwent very small changes in volume in response to osmotic challenges. Although crayfish muscle fibers can swell by about 300% (Reuben et al., 1964), swelling is also limited in frog muscle fibers and the degree of swelling or shrinking can be modified by various experimental conditions (Reuben et al., 1963, and data to be published). Volume changes of red blood cells may also be modified by numerous factors (cf. Davson, 1959). In the course of the present work it was found that volume displacements of lobster axons for a given osmotic challenge are highly dependent on the ionic composition of the medium (Freeman and Grundfest, 1966). Thus volume regulation of cells is a far more complex process than that called forth by simple osmotic effects.

³ Another example of the unreliability of the electrophysiological measurements of transport numbers is given by Chandler and Hodgkin (1965, Table 6, axon 14). In that case t_K was almost 1.5, which, of course, is theoretically impossible.

Rates of Solute Permeation In the determination of rates of permeation of solvents or solutes across the axolemma the complex morphology of the whole system must be considered. Villegas and Villegas (1960) have concluded that the sheaths and cells surrounding the squid axon offer no measurable diffusion barrier to small molecules. This conclusion was drawn on the basis that the swelling of the fibers in a solution containing a permeable solute was initially at a constant and maximal rate. Whereas the initial rate of swelling of lobster axons in KCl-enriched media fulfilled that criterion, this was not the case with glycerol (Fig. 12). It is possible that the complex layers surrounding the axolemma act as a selective diffusion barrier towards this solute, but not for KCl. However, as was indicated in the foregoing section, regulation of cell volume appears to be a complex process and the form of the curve describing influx and efflux of a solute may be due to the interplay of a number of factors.

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