Further Studies on the Nature of the Excitable System in Nerve

I. *Voltage-induced axoplasm movement in squid axons*
II. *Penetration of surviving, excitable axons by proteases*
III. *Effects of proteases and of phospholipases on lobster giant axon resistance and capacity*

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The question of major interest for this laboratory in the past ten years or so has been—What is the nature of the molecular level structural changes or rearrangements which take place in an axon when it goes from the resting to the active state and back again? What, in terms of ultrastructure, *i.e.*, molecular form, position, or orientation, differentiates the active from the resting state? Three avenues of approach have been used so far: (*a*) An attempt has been made to mimic the advancing and trailing ends of an impulse with a cathode and anode applied to a nerve or axon, and, after rather long passage of current, to examine the cathodally and anodally polarized regions for changes which might be extrapolated to the corresponding ends of a normally propagating impulse. (*b*) An attempt has been made to detect rapid and transient changes in nerve structure accompanying normal impulse propagation, in the hope that once having detected such changes the participating molecules might be identified. (*c*) An attempt has been made to produce structural changes in nerve molecules whose identities are already known, and to determine the effects of such changes on electron microscopic level structure and on functional properties of the axon. In the second approach one studies changes in structure produced by known changes in function, *viz.* normal activity; in the third approach one does the opposite and studies changes in functional properties produced by known changes in molecular level structure.

The following several matters have bearing on this general problem.

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I. *Voltage-Induced Axoplasm Movement in Squid Axons*

(A) OLDER OBSERVATIONS From work on plant cells (1), on whole nerve (2), and on single axons (3, 4, 5) the evidence is strong that cellular colloidal or macromolecular structure is dispersed at a cathodal region whereas it is condensed at an anodal one, and that shifts in the K:Ca ratio produced by the electrodes in the adjacent parts of the nerve (6) are the mechanism determining which way the change will go, *i.e.*, a cathodal increase in K:Ca ratio permits dispersion of the structure whereas an anodal decrease in the K:Ca ratio condenses it. The hypothesis has been that excitation at a cathode is fundamentally a matter of such molecular structural dispersion produced by the residual transsurface voltage, when favored by an increase in K:Ca, whereas block at an anode is molecular level condensation due to a decrease in K:Ca, and that qualitatively similar changes, on a microscale, occur at the advancing (cathodal) and trailing (anodal) ends, respectively, of the normally propagating impulse. Table I enumerates some of the older observations supporting this view.

The writer has also argued that cathodally induced swelling, stickiness, transparency increase, and structural dispersion are accompanied by and partly due to increased hydration resulting from water movement toward the cathodal site, the reverse being true at the anode, and that similar hydration changes on a smaller scale should occur with the propagating impulse. In a different context, Finean notes that the "state of hydration of a lipoprotein structure such as is envisaged in myelin structure and in membrane structures may be expected to have an important influence on the electrical and permeability characteristics, and it is a state which might readily be modified in relation to function" (7). Indirect evidence for water movement transversely across axonal surfaces has been adduced (8, 9, 10). Although there is still no incontrovertible proof of such hydration changes with normal impulses, we have now objectively demonstrated transport of axoplasm, or at least of some of its components, by electrodes applied to the squid axon, and have crudely quantitated it.

(B) NEW OBSERVATIONS Freshly excised squid axons were placed under mineral oil with their open ends contacting sea water soaked gelatin films on clean glass plates. Pieces of photographic plates, cleared and washed free of silver salts, were used. Calomel electrodes, contacting the plates by way of KCl bridges and wicks, were used to pass current through the axons *via* their contacts with the gelatin films. The open axonal ends were observed with a dissecting microscope for exuding axoplasm which was then picked up between oil seals in a micropipette for measurement of volume and specific gravity.

Among a number of interesting qualitative observations only a few will be

mentioned: Anodally the axon contents were seen to contract away from the sheath; this contracted, opaque structure then became surrounded by a clear area within the sheath. It may be imagined that this represents a shrinkage of the axon proper within the sheath, an electrically induced sort of syneresis, leaving expressed water in the vacated space. Cathodally there was a decrease in opacity. These findings are consistent with earlier observations (5).

In addition, as seen in Table II, in 15 out of 19 cases, more axoplasmic material flowed out of the cathodal than out of the anodal end. In some cases

TABLE I

EFFECTS OF ELECTRODES AND IONS ON NERVE OR AXON STRUCTURE AND PERMEABILITY

Cathode	Anode
1. Nerve or axon swells	1. Nerve or axon shrinks
2. Nerve or axon becomes soft and sticky	2. Nerve or axon becomes stiff and non-sticky
3. Nerve or axon scatters less light	3. Nerve or axon scatters more light
4. Nerve or axon becomes more transparent	4. Nerve or axon becomes less transparent
Therefore, cathode disperses colloidal level structure. This structural dispersion is accompanied by decreased transsurface resistance; *i.e.*, increased transsurface ion flow, lowered threshold, and excitation	*Therefore*, anode condenses colloidal level structure. This structural condensation is accompanied by increased transsurface resistance; *i.e.*, decreased transsurface ion flow, raised threshold, and block
5. Excess K produces comparable structural and functional changes	5. Excess Ca produces comparable structural and functional changes
6. Excess Ca inhibits both these structural and functional changes.	6. Excess K inhibits both these sturctural and functional changes
7. A cathode increases the local K:Ca ratio	7. An anode decreases the local K:Ca ratio
8. Outflowing current probably produces a local increase in K:Ca ratio	8. Excess Ca decreases axonal permeability to NaCl and probably also to KCl
Therefore, cathodal excitation with its attendant decrease in transsurface resistance is thought to be due to local, colloidal level, structural dispersion produced by an increase in K:Ca ratio	*Therefore*, anodal block with its attendant increase in transsurface resistance is thought to be due to local, colloidal level, structural condensation produced by way of a decrease in K:Ca ratio

there was no outflow at all anodally. This may have been due to mechanical blockage produced by anodal shrinkage of the axon. The anodal extrudate, when it appeared, was seen to contain stringy, solid material which could well have been agglomerated protein, since cell protein at a pH probably above its isoelectric point would move toward an anode; some water would be expected to move with it. The cathodally migrating, more transparent material had, as was expected, a lower specific gravity (1.0512 ± 0.0019) ($N = 16$) than that obtained at the anodal end (> 1.0692) ($N = 13$). Also, the anodally extruded material was of higher specific gravity even than pieces of whole axon (1.0559 ± 0.0014) ($N = 10$). The differential effect may disappear at low enough voltages.

Clearly, anodal polarization results locally in an increased concentration of certain, as yet unidentified, axon solids. How much of this is due to water removal and how much to the anodalward migration of some negatively charged solid (6) is not yet known. However, the finding is consistent with the earlier supposition that certain cell solids are condensed by an anode. Exactly

TABLE II

AXOPLASMIC FLOW PRODUCED BY APPLIED LONGITUDINAL CURRENT FLOW

			Rate of flow		Total volume	
Diameter axon,	Average current	Applied voltage	Cathodal end	Anodal end	Cathodal end	Anodal end
mm.	*ma.*	*v/cm.*	*cc* × 10^6/*sec.*		*mm.*³	*mm.*³
0.401	0.804	36.4	0.564	0.023	1.298	0.032
0.400	1.104	27.9	1.14	0.000	1.855	0.000
0.417	0.813	23.2	0.307	0.000	0.77	0.000
0.368	0.330	21.9	0.090	0.000	0.179	0.000
0.362	0.406	22.0	0.144	0.000	2.526	0.000
0.468	0.128	20.4	0.127	0.047	0.313	0.031
0.400	0.423	15.4	0.532	0.446	0.396	0.222
0.368	0.265	15.3	0.348		1.057	0.102*
0.593	0.122	12.3	0.127		0.619	0.198*
0.356	0.114	10.4	0.033		0.389	0.084*
0.401	0.300	10.0	0.225	0.204	0.788	0.925
0.434	0.316	9.7	0.232	0.207	1.590	0.642
0.434	0.180	9.1	0.127	0.107	0.312	0.333
0.484	0.410	9.1	0.177	0.308	0.835	0.995
0.368	0.138	6.6	0.232	0.255	1.098	0.745
0.384	0.186	6.5	0.110	0.000	0.498	0.009
0.434	0.129	4.2	0.052	0.082	0.212	0.206
0.434	0.095	3.7	0.028	0.095	0.132	0.323
0.400	0.070	3.0	0.015	0.000	0.064	0.000
Average			0.243	0.111	0.786	0.255

* Values marked with an asterisk are not accompanied by corresponding flow rate because total flow yielded only a single sample.

what happens to the degree of hydration of the cathodal region is not clear because the absolute water content is not known. However, it is clear that a more dilute material moves cathodally than moves anodally, and this together with the change in appearance suggests strongly that there is probably a real increase in degree of hydration cathodally. Water movement cathodally would imply that the bulk water behaves like a positively charged particle, and that the confining surfaces along which it moves, *i.e.* the inner surfaces of the axoplasmic tube, have a net negative charge. This would be the case if these surfaces were covered by a protein, phospholipid or phospholipoprotein at a

pH above its isoelectric point (11). Such phenomena are, of course, well known in classical colloid chemistry.

Further experiments should be directed at determining the chemical nature of the specific materials being dispersed or condensed at these electrode regions. That a calcium-complexed phospholipoprotein may be a key substance is discussed elsewhere (11). Experiments are planned to evaluate this point of view.

II. *Penetration of Surviving, Excitable Axons by Proteases*

Lobster giant axons exposed to proteases by immersion remain functional even though their surface structure, as examined by electron microscopy, seems to have been markedly modified by proteolysis (11, 12). It has been concluded from these earlier studies that proteases can penetrate all the way into the axoplasm, therefore necessarily also crossing the hypothetical membrane region, and that they hydrolyze certain peptide bonds on their way in, even though no change has been observed in the resting potential, action potential, threshold, or conduction velocity. It has been argued that function persists, even though such penetration and hydrolyses occur, because the polypeptide fragments in the axonal surface structure are held approximately in normal position by their association with phospholipid in phospholipoprotein lamellae. If the phospholipid component, on the other hand, is hydrolyzed by phospholipase at either the A or at the C position then the axon fails functionally, and by electron microscopy it is found that the phospholipid residues (diglycerides) liberated by hydrolysis have become detached from protein and coalesced randomly among the still intact but now tortuously stretched and distorted remaining protein layers of the lamellae.

Therefore, apparently normal conductive function can survive certain hydrolytic changes in protein all the way in through the hypothetical membrane, but phospholipid integrity is mandatory.

It has been suggested that the proteases may not penetrate all the way into the axoplasm even though electron microscopy indicates that they do. Therefore, explicit tests have been made, and it turns out that the original conclusion is supported.

Squid giant axons were excised and hung, by threads attached to each end, as a catenary, in an appropriate vessel, in such a way that the ends could be in air while the central part was submerged in an experimental solution, the solution being either sea water or sea water containing protease. Armour, crystalline, salt-free trypsin and alpha chymotrypsin, 1 mg. of each per cc. of sea water, were used. In a few cases Worthington enzymes were used. After exposure for several hours the axons were removed, washed with a large volume of sea water, gently blotted on filter paper, and tested for excitability. All those reported upon were excitable.

The axons were then laid on a plastic plate with one end hanging over the edge of the plate and were rolled, as by a rolling pin, with a bit of glass tubing, from the other end. This extruded the axoplasm from the dependent end, and it could be picked up for assay. Before extruding any axoplasm a small piece of whole axon was cut from the dependent end and assayed for protease activity to make sure that none of the enzyme had diffused up to the end segment held in air. This being the case it could safely be assumed that extruded axoplasm which might show activity could not have picked up that activity by contact with the edge of the cut end from which it flowed.

Protease assay was carried out by a method described by De Robertis (13) as follows: Eastman Kodak photographic plates were cleared and washed. This yielded glass plates covered with a thin film of gelatin. Then, after drying, small pieces of the plates were stored in the refrigerator over distilled water. This hydrated the gelatin without wetting it grossly, and this gelatin film was then used as a substrate for the protease assay. Small pieces of axon, small droplets of axoplasm, and small droplets of enzyme solution were placed on the gelatin films in a moist chamber, and to each of them was added a droplet of artificial sea water buffered with glycylglycine. After 8 to 12 hours the plates were rinsed with distilled water and stained with hematoxylin. Wherever there has been protease activity one either saw no staining at all because a hole had been digested through the gelatin, or one saw fainter staining than in the control region because some gelatin had been removed. Although the method was not used for quantitative assay it was found that one could detect a few micrograms, and perhaps fractions of micrograms per milliliter. Various technical improvements could probably make the technique extremely sensitive.

In 23 out of 29 cases, whole pieces of unsubmerged axon from the part from which axoplasm was to be extruded showed no protease activity. In five cases there was a questionable trace of activity which probably was not enzymatic, but which, from its appearance, was more likely due to mechanical adherence and tearing of the gelatin film by the piece of solid axon. In only one case was there obvious contamination. Therefore the cut end from which the axoplasm was to be extruded was not contaminated by protease. In all cases, the immersion medium containing enzyme was shown to be strongly proteolytic for the gelatin film. In 26 out of 28 cases, whole pieces of unsubmerged axon cut from the other end after rolling also showed no protease activity. This was in keeping with the above results, and, in addition, indicated that rolling the axon did not of itself appreciably spread the enzyme. The artificial, glycylglycine-buffered sea water used as a diluent was inactive. In three preliminary experiments, axoplasm from freshly excised axons had no effect on the gelatin film; normal axons may well contain protease and almost surely do, but fortunately the method did not detect them. Therefore there was no confusing background activity.

Now then, extrusion by rolling usually yielded from two to four samples of axoplasm per axon. Table III shows the results of assays on 19 axons exposed to the proteases. The first sample of axon to come out often showed little or no

activity. This is in keeping with the earlier finding of no activity in control axoplasm, since the first sample would be axoplasm coming primarily from unsubmerged axon. In some cases there was activity, but it must be remem-

TABLE III

ASSAY OF AXON PIECES AND OF AXOPLASM FOR PROTEASE ACTIVITY

	Duration of exposure to enzymes	Assay for protease activity: Piece of axon from extrusion end	Consecutively obtained samples of axoplasm				Piece of axon from end which was rolled
	hrs.						
Trypsin plus alpha chymotrypsin, 1 mg. each per ml. sea water	1	0	tr.	+++	tr.		0
	1	0	+++	+	+	+	0
	3	0	+	++	+++		0
	3	0	tr.	++			0
	3	?tr.	0	+	+	+	0
	3.5	0	tr.	+	+		0
	3	?tr.	++++	+++	++		?tr.
	3	0	tr.	tr.	+		0
	3.	0	0	0	+	++	
	3	?tr.	0	++	+	+	0
	4	0	0	tr.	tr.	+	0
	4–	0	0	0	tr.		0
	4.5	0	tr.	+	++		0
	4.75	0	tr.	+	+		0
	5	?tr.	++				0
	3	?tr.	++	++	+++		0
	3.5	0	++++	++++	++++		0
	3.5	0	0	++++	++++		0
	3	0	+	++++			0
Alpha Chymotrypsin alone	3.75	0	0	0	?	?	0
	3.25	0	tr.	+			0
	3.5	0	?tr.	?tr.	?tr.		0
	3	0	0	0	0	0	0
Trypsin alone	3.75	++	+	tr.			0
	3.75	0	++++	++++	++++	+	0
	3.5	0	++++	++	++	++++	0

bered that it was not possible to control the amount of axoplasm which would emerge as the rolling process began and progressed. Subsequent samples from all of the axons showed variable amounts of activity, and all these axons were excitable. In three cases the activity seemed to decrease again in the last samples to emerge. It seems clear that proteases penetrated into the submerged segment of axon. A few experiments suggested, though the data are not con-

clusive, that trypsin was probably the main penetrant. It was also noted that the axoplasm changed consistency with the protease treatment. Normal axoplasm can easily be picked up with a forceps and can also be pulled out of the cut end of the axon. In addition, it is quite elastic. After axons are in sea water for some time the axoplasm becomes more fluid. However, after protease treatment of the intact axon the expressed axoplasm is so fluid that it can no longer be picked up with a forceps but must be sampled and transferred with a pipette. This also speaks for entry of the protease. In addition, it has bearing on the question of possible axoplasmic binding of ions since it is hard to see how such drastic changes in axoplasmic state could fail to inactivate the axon if its ion distribution depended upon binding to axoplasmic protein.

One control requires special mention: The squid axon has branches, and it might be argued that the proteases penetrated by way of cut branches. However, in all cases the medium was sea water, and when sea water penetrates a branch which has been left open or cut off too close to the axon then the axon becomes cloudy and brownishly discolored. In no case did this happen, and it is hard to imagine that protease would have entered such a branch without sea water going in and producing the characteristic signs. In addition, it was shown that when the axonal surface was purposely damaged in the enzyme solution then axoplasm opacity and discoloration did develop.

It is concluded, therefore, that protease, perhaps only trypsin in these experiments, was able to penetrate the entire thickness of the squid axon surface, therefore also passing the hypothetical membrane, without inactivating the axon electrically, *i.e.* the axon was still excitable and could conduct impulses. Correlating with the earlier experiments (11, 12) it is concluded that this is true even though the protease hydrolyzes certain peptide bonds and produces certain destructive changes on the way in. Excitability and conduction can therefore survive transsurface holes, preexisting and/or enzymatically produced, large enough to permit the entry of molecules of about 20,000 molecular weight. Other experiments on water transfer suggest that holes with "effective pore radii" of 16 $\pm$ 4 A.U. may normally exist in the axons of the lobster walking leg (14). Any simple sieve or pore hypothesis for ion selection, or any postulated simple change in so called permeability as a basis for excitation must take such data into account.

There are other observations in the literature which are consonant with these findings. Thus, bacterial polysaccharides (molecular weight 140,000 to 500,000) and also egg albumin, bovine plasma albumin, and human alpha globulin have been shown to penetrate *in vivo* into mouse epithelial cells in liver, capillaries, adrenal cortex, and synovial membrane, and into cartilage cells, osteoblasts, and osteocytes (15). Proteases have also been applied to isolated frog axons and shown to be functionally relatively innocuous as compared to the phospholipases (16) even though trypsin can penetrate frog

nerve myelin lamellae and produce granular dissociation of the myelin (17) much like that shown for lobster axons (11).

III. *Effects of Proteases and of Phospholipases on Lobster Giant Axon Resistance and Capacity*

Phospholipases, which hydrolyze lecithin at either the A or the C position, produce characteristic structural modifications in lobster axons which result in changes in threshold, conduction velocity, and potentials, leading finally to inexcitability. Proteases produce quite different structural changes as a result of peptide bond hydrolysis in the cell surface but these, unlike the changes due to phospholipases, do not seem to alter threshold, conduction velocity, or potentials, and do not lead to inexcitability. Having, therefore, a method for at least semiselective chemical dissections producing different functional consequences, it would seem that observations of the effects of these enzymes on so called membrane constants might allow one to give molecular class names to the structural substrates primarily responsible for maintaining transsurface resistance and capacity.

The method used was, in principle, that described by Hodgkin and Rushton (18), and need not further be detailed here. The biological preparation, the giant axon of the lobster nerve cord, the enzymes used, and their administration are described elsewhere by Tobias (11, 12). Minor modifications permitted the isolated axon to be lifted from sea water into overlying mineral oil for measurements. In all cases, after isolating the axon in sea water, an initial set of measurements was made in oil; the axon was then lowered into the sea water with or without enzymes for a measured time, usually about 15 minutes, and then again raised into the oil to be followed for as long as desired. The proteases used were Armour trypsin and alpha chymotrypsin, usually in a concentration of 1 mg. per ml.

Control data are shown in Table IV for comparison with the values obtained by Hodgkin and Rushton on lobster leg nerve axons. As is well known, there is great variability in all of these measurements, but the averages for the two sets are comparable. The paper of Hodgkin and Rushton does not indicate at what time after isolation their measurements were made. In the present series the measurements were made at from 10 to 240 minutes after isolation ot the axon. In a number of experiments the values for length constant and for transsurface resistance fell gradually with time whereas transsurface capacity seemed less vulnerable to *in vitro* aging. This would fit with the notion that the capacity is, in general, more stable than the resistance. The decline in transsurface resistance was rapid enough in some experiments as to suggest that extrapolation backwards in time to the *in vivo* situation might yield higher values than any recorded with excised tissue.

TABLE IV

LENGTH CONSTANT, AXOPLASM RESISTIVITY "MEMBRANE" RESISTANCE AND "MEMBRANE" CAPACITY OF ISOLATED, SURVIVING LOBSTER AXONS

Length constant			Axoplasm resistance			"Membrane" resistance			"Membrane" capacity			
No. axons	No. measurements	Mean	No. axons	No. measurements	Mean	No. axons	No. measurements	Mean	No. axons	No. measurements	Mean	Source
		mm.			*ohm cm.*			*ohm cm.*$^{-2}$			*μF. cm.*$^{-2}$	
10	13	1.61	10	13	60.5	10	13	2290	10	13	1.33	Hodgkin and Ruston (lobster leg axon)
32	69	1.68	26	55	94.8	26	55	2434	26	48	0.84	Present series (giant axon of lobster nerve cord)

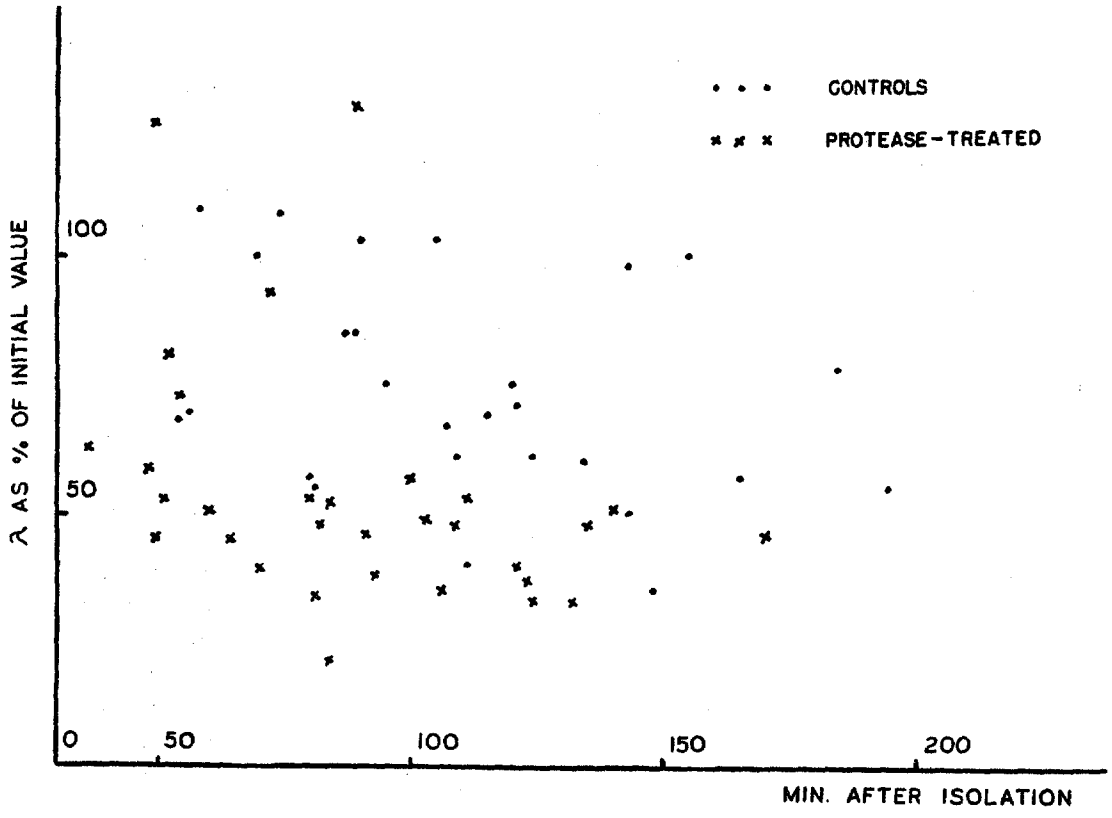

FIGURE 1

The results of protease treatment are summarized in Figs. 1 to 4, and can be stated rather briefly: (*a*) Exposure to proteases accelerated the rate at which both the length constant, λ, and the "membrane" resistance decreased with time. On the average, such treatment depressed "membrane" resistance about 20 per cent more than occurred simply with the passage of time. "Membrane" resistance in one axon fell to as low a level as 5 per cent of its initial value

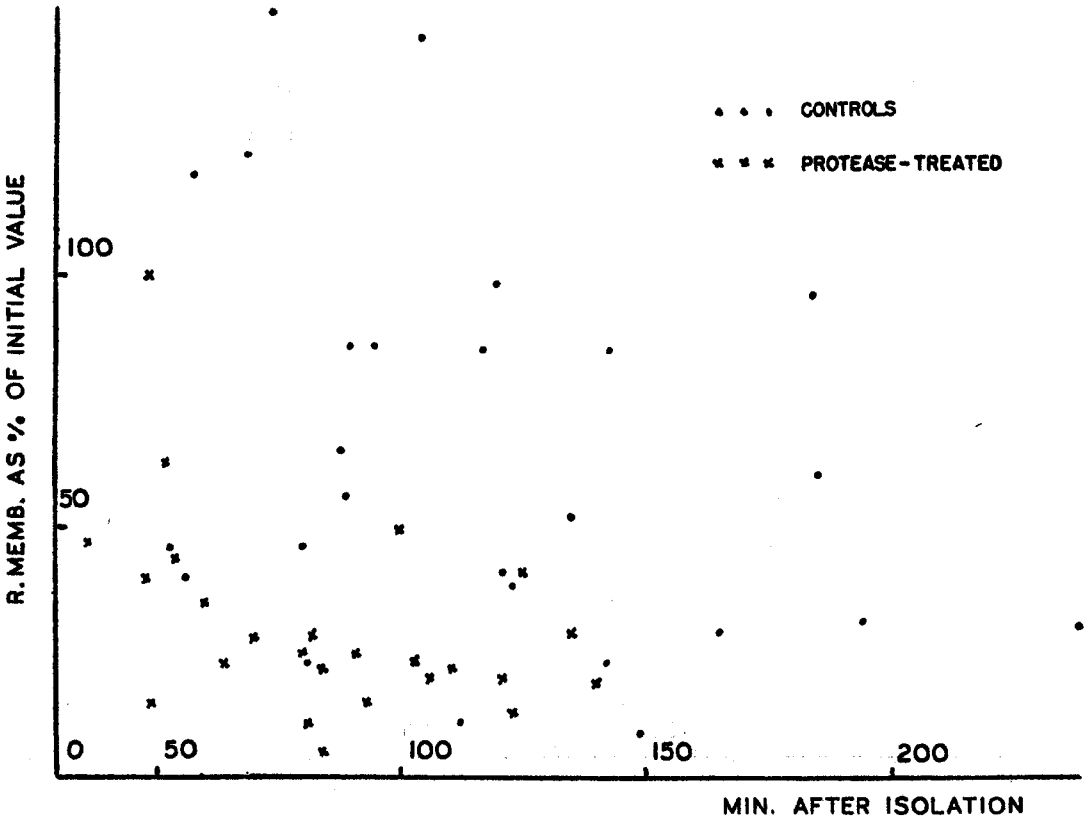

FIGURE 2

(from 3370 ohm cm.$^{-2}$ to 154 ohm cm.$^{-2}$) just before failure of excitability; capacity at the time was 0.69 μ *F*.cm.$^{-2}$. It appears that whatever the system is that is responsible for the high resistance to ion movements in these axons, it is present in considerable excess over the amount needed to insure excitability. Thus, it is clear that although resistance can change greatly with time in both control and protease-treated axons, any specific effect of the protease itself is a small one. (*b*) Axoplasm resistance was not convincingly changed by exposure to proteases. This is not surprising, as axoplasm resistance is not high to begin with, and, barring such effects as specific ion binding or unbinding,

even large structural changes in gels have little effect on conductance. (*c*) The data on "membrane" capacity are poor, but no effect of proteases on capacity was detected.

The results of phospholipase action are even more simply stated, and require no graphic display. Length constant, "membrane" resistance, and

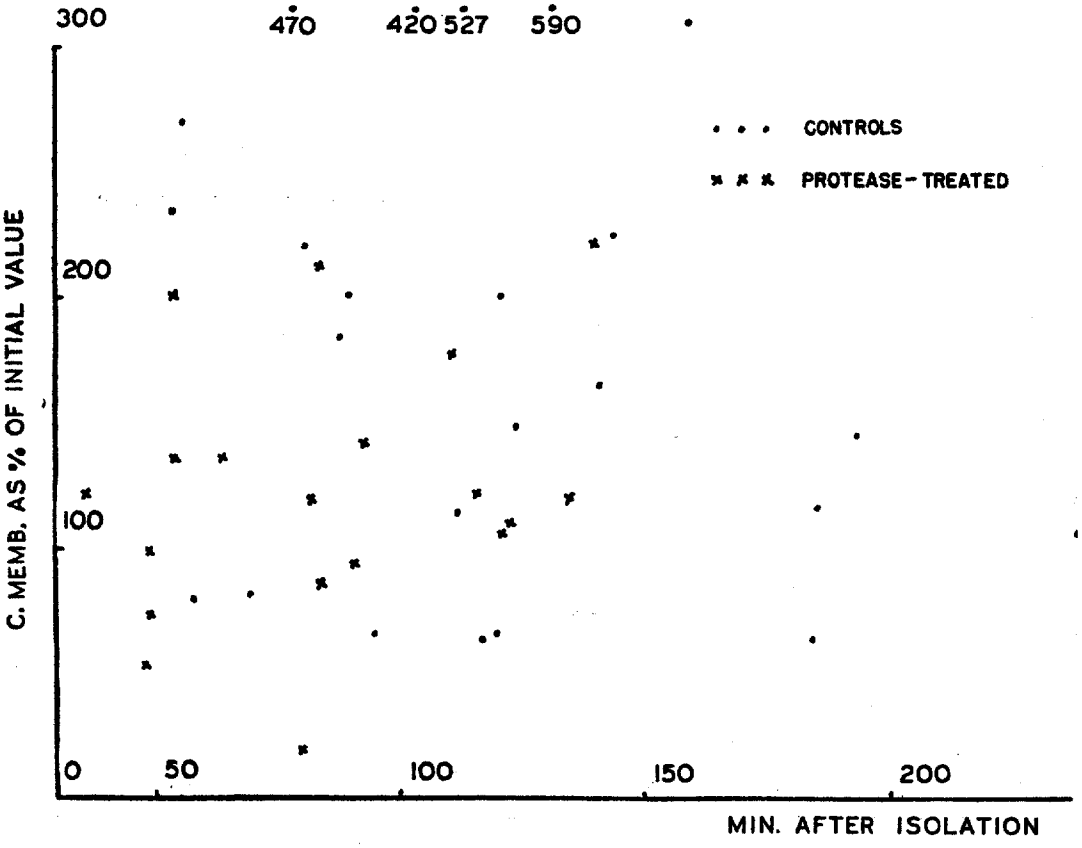

FIGURE 3

"membrane" capacity fell quickly to unmeasurable levels after exposure to these enzymes. Digitonin, which precipitates cholesterol and some phospholipids, and which produces electron microscopic changes similar to those produced by the phospholipases (11), also precipitously drops the axon constants to values too low to measure.

It is tentatively concluded that since (*a*) proteases penetrate all the way through the hypothetical membrane region (see Part 2), and (*b*) probably hydrolyze certain peptide bonds on the way in, and (*c*) still have only a relatively small effect on the "membrane" constants, if any, whereas (*d*) the phospholipases or digitonin drastically change these quantities, therefore the molecular

name of transsurface capacity and resistance is more likely to be phospholipid than protein. This, of course, fits with the fact that the proteases have little if any effect on excitability whereas the phospholipases destroy it.

These experiments are not yet considered final. They should be repeated with penetrating microelectrodes, and without the need for moving the axons

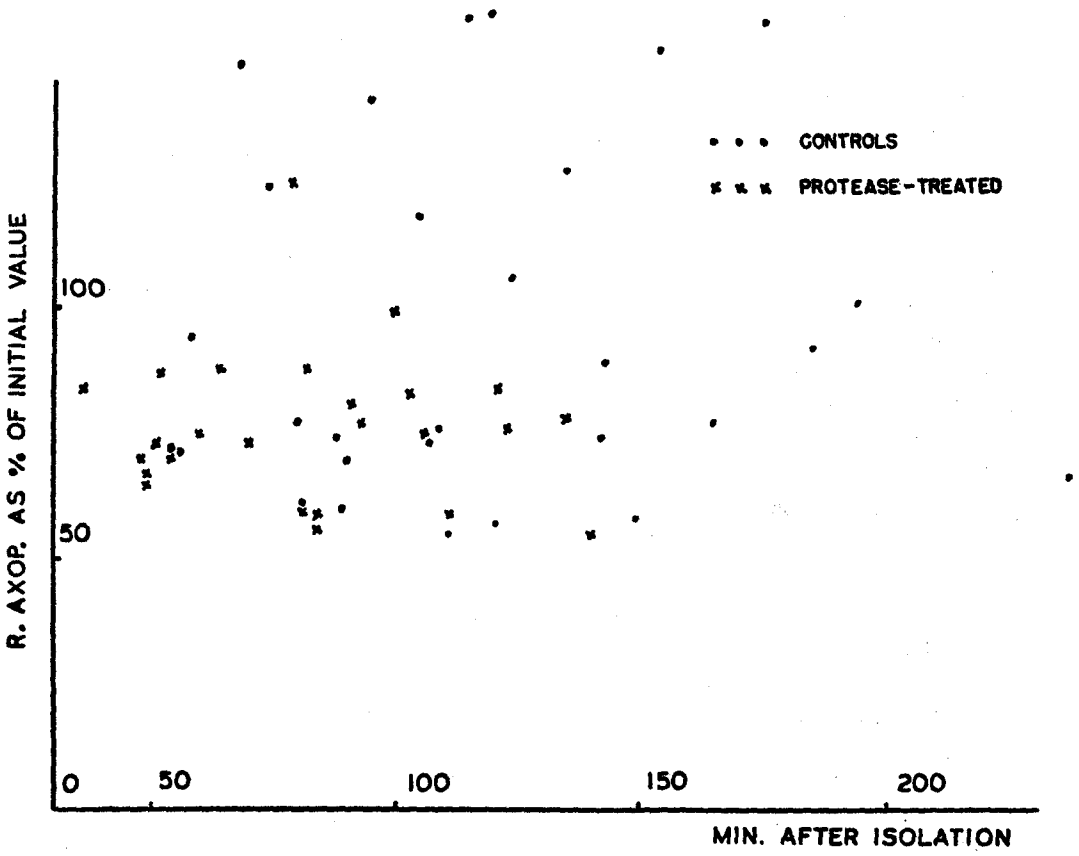

FIGURE 4

from water into oil. Going through the oil-water interface is a strenuous procedure, and many axons, seemingly more from spring and summer lobsters, are broken by such treatment after exposure to proteases. It was apparently simply a matter of luck that enough resistant axons were accidentally encountered to allow the accumulation of these data. The effect of proteases *per se* may therefore be even less than indicated above. The 20 per cent effect noted may well be due to the strain of lifting the axon, mechanically weakened by protease, through the oil-water interface. At any rate, it is unlikely that the proteases would have produced changes greater than those measured. For these

reasons the data are qualitatively acceptable, but the conclusions are considered to be quantitatively tentative.

CONCLUSIONS AND INFERENCES

1. In a long-axis voltage gradient, produced *via* non-polarizable electrodes applied to the squid giant axon, more axoplasm solid moves anodally than moves cathodally, whereas more axoplasm water moves cathodally than moves anodally.

It is suggested that, in a similar fashion but on a microscale, water may also move preferentially toward the advancing cathodal end of a normally propagating impulse, whereas the trailing or anodal end may undergo a relative dehydration. Such changes may contribute to the structural dispersion postulated to occur at a cathode or at the advancing end of an impulse, *i.e.* excitation, and to the structural condensation postulated to occur at an anode or at the trailing end of an impulse, *i.e.* block. It is also inferred that the bulk water bears a net positive charge relative to the cell surfaces along which it moves which will be negatively charged. This would be the case if the surfaces were coated with an ampholyte above its isoelectric point.

2. Protease activity, though not detected in control axoplasm from squid giant axons, is found in the axoplasm of surviving, functional axons which have been exposed to the enzyme from outside. Therefore axons can remain excitable in the presence of holes, preformed and/or enzymatically produced, which are large enough to admit substances of molecular weight about 20,000, which traverse the entire surface structure including the hypothetical membrane.

This finding supports an earlier conclusion based on electron microscopic evidence which held that proteases applied from outside could destructively penetrate all the way through the surface structures of the giant axon of the lobster nerve cord without functional inactivation of the axon.

3. Proteases applied to the giant axon of the lobster nerve cord have rather minor effects, if any, on "membrane" resistance; they may lower it slightly. No effect was detected on axoplasm resistance or "membrane" capacity, but the former is low to begin with, and the data on the latter are poor. "Membrane" resistance can fall markedly in excised axons simply as a function of time, and still the axons remain functional. "Membrane" capacity seems less vulnerable to *in vitro* aging. Contrariwise, phospholipases and digitonin produce a fall of the axon constants to unmeasurably low levels.

These findings are consistent with earlier observations on the effects of these enzymes on axon function which showed that proteases applied from outside did not destroy function whereas either phospholipase A or C did. It was concluded that a certain amount of axon surface proteolysis can be tolerated, but

phospholipid integrity is mandatory for function. It is now tentatively concluded, in addition, that the chemical name of "membrane" resistance and capacity is more likely to be phospholipid than protein.

4. Axoplasm is normally viscous and elastic. Both the passage of time after axon isolation and exposure of the excised axon to proteases make axoplasm extremely fluid. This can occur even though excitability and function remain intact.

It is sometimes argued that potassium may be accumulated in cytoplasm by some sort of binding to negatively charged fixed sites. If so, such binding would presumably have to be able to persist in spite of the drastic change in state implied by the above mentioned shift from a quite rigid, elastic gel to a very fluid sol.

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