

VIII. Biochemistry of the Squid Giant Axon

The squid giant axon is well known from numerous studies which have contributed significantly to our understanding of the properties of the action potential and the physical characteristics of excitable membranes. It is, of course, the unusually large size of the giant axon found in many species of squid which has made this preparation such a powerful biological tool for membrane physiologists. Although the squid giant axon is unusual, at least in size, many of the observations which have been made upon the membrane properties of the giant axon appear to be generalizable to more typical axons in the nervous systems of organisms other than the squid. This fact raises the possibility that the squid giant axon might also be useful in extending our knowledge of the molecular composition of axons generally and of the molecular events associated with the functional properties of the axon. This is hardly a novel idea, as evidenced by the variety of studies which have been carried out upon the giant axon. These experiments on the molecular characteristics of the giant axon date back to those of Bear, Schmitt and Young (1937) which were carried out shortly after J. Z. Young reidentified and described the squid giant axons in *Loligo*.

The following review of work carried out on the chemistry of the squid giant axon is not meant to be global in scope, but rather to illustrate some of the advantages of this preparation for those interested in the molecular physiology of the axon and its surrounding glial cells. One particularly exciting area of research on the molecular events which underlie the electrical properties of the axonal membrane will not be dealt with here, as they have been reviewed recently by Cohen (1973) and at present fall more naturally into discussion of electrophysiology.

Extrusion of Axoplasm from the Giant Axon.

It is probably obvious at this point that the usefulness of the squid giant axon for molecular studies results from the fact that the axoplasm of the axon can be obtained readily. Thus, most of the studies on the molecular composition of the giant axon have focused on the axoplasm. Giant axons are unique in that they are the only presently available preparation from which relatively pure axonal cytoplasm can be obtained by a simple procedure having only one step. Although this chapter is concerned with the squid giant axon, it should be mentioned that another useful giant axon is that of the polychaete *Myxicola* (Schmitt, 1950). In order to present the methodological considerations for obtaining axoplasm, it is important to first understand the morphology of the squid giant axon.

J. Z. Young's (1939) now classical observations on the morphology of the giant axon should be explored by anyone with a serious interest in the giant axon. A number of stellate giant fibers arise from cell bodies located in the stellate ganglion. These fibers extend from the ganglion to innervate the musculature of the mantle. The two largest fibers are bilaterally paired and located in the stellate nerves which runs parallel to the pen of the animal. These primary fibers are the most useful for obtaining axoplasm as they have a greater diameter, are longer and are more readily dissected free in an undamaged state than the more laterally located giant fibers. The giant axon with its multiple cell bodies of origin represents one of the few known examples of neurons which are true syncytia. However, like other invertebrate neurons, but unlike other syncytial neurons such as those found in annelids, the squid giant axon has a polarized morphology. That is, the axon arises from a single

cluster of cell bodies. Synaptic terminals are found on the proximal segment of the axon in the stellate ganglion and the axon extends out to the periphery to terminate in the musculature of the mantle. In *Loligo pealei* the peripheral portion of the primary giant axon is surrounded by hundreds of smaller axons (50 μ and less in diameter). The primary giant fiber extends 4-8 cm from the ganglion to its first major branch.

Light microscopic and electron microscopic studies (Villegas, 1969 and Metzals, 1969) demonstrate that the squid giant fiber is composed of an outer sheath (approximately 5-10 μ m thick, *Loligo pealei*) and the axon with its limiting membrane, the axolemma. It is important to realize that the sheath adheres tightly to the axolemma and cannot be removed from the axon by conventional dissection or, as far as I am aware, any other methods which are currently employed. The sheath is a complex tissue consisting of an inner layer of Schwann cells (2-3 μ m thick, in *L. pealei*) directly surrounding the axon, and an outer connective tissue endoneural layer containing fibroblasts and collagen. A basement lamina separates the Schwann cell layer from the outer connective tissue layer. The Schwann cell layer is fairly typical of large invertebrate axons. It does not contain any myelin although the processes of the many Schwann cells overlap one another as they envelop the axon. The axoplasm has a fairly typical morphology, containing large number of microtubules, 100 A filaments (neurofilaments), mitochondria, and vesicles and cisternae of varying sizes.

Method for extruding axoplasm and recovering the sheath

The following method for extruding the axon is simple and has been used by many investigators. In order to extrude axoplasm from the giant axon, it should be tied at both ends and carefully cleaned in Millipore filtered sea water as described elsewhere in this book (page 47). The axon must be undamaged along its length or sea water will leak in and produce white spots. An axon with holes punctured into the axolemma cannot be extruded with complete recovery of the axoplasm.

After the axon is cleaned as much as the limitation of time allows, the axon is lifted by the threads at each end and placed on a clean microscope slide. Excess sea water is removed by lifting the axon from the slide and placing it on a dry portion of the slide. If this is done four or five times, the axon will be moist but not surrounded by a pool of seawater. From this point on, one must work rapidly to avoid drying out the preparation. The larger end of the axon is then cut off immediately and the axoplasm extruded by carefully squeezing the axoplasm out of the open end starting from the tied smaller end, much as toothpaste is removed from a tube. I have employed a number of devices to extrude the axoplasm, including a Teflon roller. However, I have found that a short length of Intramedic tubing PE250 (Suggested by Dr. I. Tasaki) is simplest to employ. The portion of the axon (a few mm long) which is adjacent to the open end of the axon should not be extruded, otherwise the axoplasm may be contaminated with material from the sheath. If the deflated sheath is to be analyzed, the unextruded ends of the fiber are discarded and the remaining sheath placed in a test tube at 4°C. The pool of axoplasm on the slide is collected in a capillary pipette (10-25 μ l capacity) and transferred to an appropriate solution for analysis. The axoplasm can be dispersed in solutions of low or high ionic strength and the dispersion is enhanced by the presence of Ca^{++} . The sheath can be homogenized in a small homogenizer (1-2 ml capacity.) Although the giant axons may seem large by conventional standards, all

of the above procedures should be carried out under observation with a dissecting microscope.

Using the electron microscope, Dr. R. Nordlander and I have observed that axoplasm extruded by this method is essentially free of contamination from the sheath and that less than 10% of the axoplasm remains in the sheath. Larrabee and Brinley (1968) have noted that, if giant fibers are presoaked in labeled inulin, very little radioactivity contaminates the axoplasm. Thus, the axoplasm represents a fraction of neuronal cytoplasm, uncontaminated with other cellular components. On the other hand, the sheath is a composite of several things, including residual axoplasm, the axolemma, Schwann cells and connective tissue endoneurium. The entire procedure, starting at the point where the squid are removed from the tank, to extruding the axoplasm takes about 1/2 hour for each axon and approximately 1 ul (1 mg wet weight) of axoplasm is obtained for each cm of axon extruded or 4-6 ul from an average axon.

Molecular Composition of the Squid Giant Axon

A. Small molecules

A number of reports have been concerned with the composition of the dialyzable elements of the axoplasm. Several of these papers have concentrated upon the ions of the axoplasm because they are of direct importance in the interpretation of electrophysiological data on the giant axon. The following sources should be consulted for a compilation of the ionic composition of the axon (Koechlin, 1955; Deffner, 1961; Keynes, 1963; Bear and Schmitt, 1939). A fairly complete analysis of the free amino acids and ionized organic compounds of the axoplasm was carried out by Deffner (1961). His analyses demonstrated that isothiocyanate, taurine and aspartate are the major charged low molecular weight organic compounds in axoplasm. Taurine and isothiocyanate are sulfated, completely ionized at physiological pH and represent the major non-diffusible anions of the axoplasm. Aspartate and glutamate are the predominant amino acids of axoplasm as is the case for nervous systems generally.

Few recent studies on squid axoplasm have appeared which employ the new generation of commonly used but very sensitive analytical tools such as gas chromatography, liquid chromatographic amino acid analyzers, and atomic absorption spectroscopy. As these and other recently developed methods can detect very small amounts of chemical substances and produce specific identifications, an up-to-date analysis of the small molecules of the axoplasm seems warranted.

B. Macromolecules of the giant axon

The awakening of interest in molecular biology with its exponential increase in information regarding the macromolecules of cells has left the squid giant axon relatively untouched. The primary exception to this statement is the work on the fibrous proteins of the axoplasm done at Massachusetts Institute of Technology by several individuals affiliated with F. O. Schmitt (for review see Schmitt and Geschwind, 1957). Schmitt (1950) studied the axoplasm of giant axons of the squid and a polychaete *Myxicola* and demonstrated that the structures which histologists had been calling neurofibrils were, in fact, an artifact of fixation. He demonstrated that neurofibrils are precipitated neurofilaments which are recognized now as 100 Å filaments occurring in many kinds of cells.

Electron microscopic studies demonstrate two primary fibrous components in the axoplasm: 100 Å filaments, and microtubules. The protein tubulin of the microtubules has been described in detail, since the demonstration that this protein binds the antimitotic agent colchicine (Borisy and Taylor, 1967). Tubulin is a dimer having a molecular weight of 120,000 daltons and two different subunits with molecular weights of 55,000-60,000 daltons (Davison and Huneus, 1970). In a comparison of colchicine binding proteins of various tissues, Borisy and Taylor (1967) demonstrated that squid axoplasm had the greatest concentration of colchicine binding protein of the tissues studied.

The protein composition of 100 Å filaments is less well characterized than that of microtubules. However, the studies of Maxfield, (1953) and Huneus and Davison (1970) indicate that 100 Å filaments contain a polypeptide (filarin) with a molecular weight of approximately 70,000. This protein is not soluble in solutions having low ionic strength. The possibility exists that other polypeptides are normally associated with 100 Å filaments, as other proteins appear to cosediment with partially purified filaments. Discontinuous SDS-polyacrylamide gel electrophoresis of axoplasm solubilized in 1% SDS, 8 M urea and 5% B-mercaptoethanol indicate that there are a dozen or more major polypeptides in squid axoplasm (unpublished, Lasek). Polypeptides with an apparent molecular weight of the subunits of tubulin (55,000-60,000) make up a substantial percentage of the total peptides of axoplasm. Other major bands have molecular weights of 68,000 (possibly filarin) and 48,000 (possibly actin). These very preliminary results are tantalizing and suggest that a careful analysis of the axoplasm of the giant axon may yield information regarding the structural basis of the transport of proteins and organelles in the axon (i.e. axonal transport).

In collaboration with Dr. Stanley Twomey, I have made several unsuccessful attempts to study axonal transport in the giant fiber system "in vivo". H^3 -leucine was injected directly into the stellate ganglion and the animals sacrificed after periods ranging from hours to days. Unfortunately, the sheath was always heavily labeled as compared to the axoplasm, and it was not possible to rule out the possibility that H^3 -amino acids simply diffused along the axon and were incorporated locally. As it would be of great advantage to be able to study axonal transport in the squid giant axon, other methods of approach (possibly "in vitro") should be attempted.

Axoplasm of the squid giant axon contains RNA. However, essentially all of this RNA appears to be 4s RNA — most probably transfer RNA (Lasek et al., 1973). This observation makes it unlikely that protein synthesis occurs to any significant degree in axoplasm of the squid giant axon. The possible function of 4s RNA in the axoplasm, which does not contain any detectable levels of ribosomal RNA, raises the question of the function of 4s RNA in the axoplasm. It may be that 4s RNA simply leaks into the axon from the neuron cell bodies and that ribosomes are excluded. Alternatively, the 4s RNA of axoplasm may be involved in some process other than messenger RNA directed protein synthesis. For example, enzymes have been found in a number of cells which add amino acids to the amino-terminal position of certain proteins in the presence of tRNA and ATP. These enzymes add amino acids to proteins in the absence of ribosomes (Soffer, 1973). The location of the DNA template which codes for the transcription of axoplasmic 4s RNA remains to be identified. The most likely candidates are: i) the Schwann cell nuclei and subsequent transfer transcellularly to the axon, and ii) the cell bodies of the giant axon and subsequent axonal transport to the giant axon.

The absence of ribosomal RNA in the axoplasm is of some interest with regard to the question of local protein synthesis at the level of the squid giant axon. If the giant axon is isolated from its cell bodies and incubated in sea water containing H^3 -amino acids, labeled proteins appear in the axoplasm (Giuditta *et al.*, 1968). A case has been made for the synthesis of these proteins in the Schwann cells surrounding the axon and their subsequent transfer transcellularly to the axon (Lasek *et al.*, 1974). This transfer may occur by some form of exocytosis from the Schwann cells and pinocytosis by the axon. This possibility is supported by the demonstration that radioactively labeled bovine serum albumin is actively taken up by the axon into the axoplasm from the surrounding media (Giuditta *et al.*, 1972; Lasek, Gainer and Barker, unpublished). Apparently, pinocytosis is involved in the uptake of the albumin. The squid giant axon appears to be a very useful preparation for studying cell to cell transfer because: i) the axon is large, ii) axoplasm is apparently devoid of a significant protein synthesizing machinery, iii) the isolated fiber actively incorporates readily measurable amounts of labeled amino acids into protein, and iv) the perfusion paradigm can be employed to collect labeled proteins which have been transferred from the Schwann cells to the axoplasm (Gainer *et al.*, unpublished).

In order to incubate the giant axon for incorporation studies, we have used a simple method. The cleaned giant axon is placed in a pool of Millipore filtered sea water on a microscope slide. The pool is contained by an oval barrier of petroleum jelly (Vaseline) made by squirting the Vaseline out of a large bore hypodermic needle. This simple reservoir holds as little as 0.5 ml and, because of the large surface area of the pool of sea water, remains well aerated. The media is kept from drying out by covering the slide with a medium size petri dish and placing a moistened piece of tissue to one side of this "home made" chamber. The axon is kept straight during the incubation by fixing the thread at each end of the axon to bits of clay or wax at each end of the microscope slide.

The metabolic enzymes and pathways present in the axon have been studied by Roberts *et al.*, (1958), Coelho *et al.*, (1960), and Hoskin (1966). Axoplasm has a high rate of anaerobic glycolysis as compared to the sheath. The energy stores in the form of ATP in the giant axon are substantial and remain undepleted in axons subjected to cyanide poisoning for several hours. A ouabain sensitive sodium-potassium ATPase is present in the squid giant axon, which is thought to be located in the axolemma and to be coupled to the Na^+ pump of the axolemma (Bonting, Caravaggio, 1961; and Baker and Shaw, 1965).

The lipids of the giant axon have been described by McColl and Rossiter (1950). Larrabee and Brinley analyzed the uptake of P^{32} into phospholipids of the giant axon and found that unlike mammalian tissues phosphatidylcholine is a minor labeled component in the squid giant axon.

In conclusion, evolution has provided the neurobiologist with a powerful tool in the form of the squid giant axon; and it seems likely that the substantial progress made by electrophysiologists on the properties of the giant axon is only a precursor to our future understanding of the molecular properties of the giant axon and, therefore, axons generally.

BIBLIOGRAPHY

- BAKER, P. F. AND T. I. SHAW, 1965. A comparison of the phosphorous metabolism of intact squid nerve with that of the isolated axoplasm and sheath. *J. Physiol.* **180**: 424-438.
- BEAR, R. S., SCHMITT, F. O. AND J. Z. YOUNG, 1937. Investigations on the protein constituents of nerve axoplasm. *Proc. Roy. Soc. B* **123**:520-529.
- BEAR, R. S. AND F. O. SCHMITT, 1939. Electrolytes in the axoplasm of the giant nerve fibers of the squid. *S. Cellul. and Comp. Phys.* **14**:205-215.
- BONTING, S. L. AND L. L. CARAVAGGIO, 1961. Sodium-potassium activated ATPase in the squid giant axon. *Nature* **194**:1180-1181.
- BORISY, F. G. AND E. W. TAYLOR, 1967. The mechanical action of colchicine. *J. Cell. Bio.* **34**: 525-533.
- COELHO, R. R., GOODMAN, J. W. AND M. B. BOWERS, 1960. Chemical studies of the satellite cells of the squid giant nerve fiber. *Exp. Cell. Res.* **20**:1-11.
- COHEN, L. B., 1973. Changes in neuron structure during action potential propagation and synaptic transmission. *Physiol. Rev.* **53**:373-418.
- DAVISON, P. F. AND F. C. HUNEUS, 1970. Fibrillar proteins from squid axons. II. Microtubule protein. *J. Mol. Biol.* **52**:429-439.
- DEFNER, G. G. J., 1961. The dialyzable free organic constituents of squid blood: a comparison with nerve axoplasm. *Biochim. biophys. acta* **47**:378-388.
- GIUDITTA, A., RETBAIN, W. D. AND M. BRZIN, 1968. *PNAS* **59**:1284-1287.
- HOSKIN, F. C. G., 1966. Anaerobic glycolysis in parts of the giant axon of squid. *Nature* **210**: 856-857.
- HUNEUS, F. C. AND P. F. DAVISON, 1970. I. Neurofilament protein. *J. Mol. Biol.* **52**:415-428.
- KOECHLIN, B. A., 1955. On the chemical composition of the axoplasm of squid giant nerve fibers with particular reference to its ion pattern. *J. B. B. C.* **1**:511-529.
- LARRABEE, M. G. AND F. J. BRINLEY, 1968. Incorporation of labelled phosphate into phospholipids in squid giant axons. *J. Neurochem.* **15**:533-546.
- LASEK, R. J., DABROWSKI, AND R. NORDLANDER, 1973. Analysis of axoplasmic RNA from invertebrate giant axons. *Nature* **244**:162-165.
- LASEK, R. J., GAINER, H. AND R. J. PRZYBSKI, 1974. Transfer of newly synthesized proteins from Schwann cells to the squid giant axon. *P. N. A. S.* (in press).
- MAXFIELD, M., 1953. Axoplasmic proteins of the squid giant nerve fiber with particular reference to the fibrous protein. *J. of Gen. Phys.* **37**:201-216.
- MAXFIELD, M. AND R. W. HARTLEY, 1957. *Biochim. et Biophys. Acta* **24**:83-37.
- MCCOLL, J. D. AND R. J. ROSSITER, 1950. Lipids of squid nerve. *Nature* **166**:185-186.
- METUZALS, J. AND C. S. IZZARD, 1969. Spatial patterns of threadlike elements in the axoplasm of the squid. *J. Cell Biol.* **43**:456-479.
- NICOL, J. A. C., 1948. The giant nerve-fibers in the central nervous system of *Myxicola* (*Polychaeta, Sabellidae*). *Quart. J. of Mic. Sci.* **89**:1-45.
- ROBERTS, NIRA R.; ROBYNS COELHO, ROSE; LOWRY, O. H. AND E. J. CRAWFORD, 1958. *J. of Neurochem.* **3**:109-115.
- SCHMITT, F. O., 1950. The structure of the axon filaments of the giant nerve fibers of *Loligo* and *Myxicola*. *J. of Exp. Zool.* **113**:499-516.
- SCHMITT, F. O., 1957. The fibrous protein of the nerve axon. *J. Cell and Comp. Phys.* **49** Supp 1:165-174.
- SCHMITT, F. A. AND N. GESCHWIND, 1957. *Progress, in Biophysics and Biophysical Chemistry* **8**:165-215.
- SOFFER, R. L., 1973. Post-translational modification of proteins catalyzed by aminoacyl-tRNA-protein transferases. *Molecular and Cellular Biochemistry* **2**:3-15.
- VILLEGAS, G. M., 1969. Electron microscopic study of the giant nerve fiber of the giant squid *Dosidicus gigas*. *J. Ultrastruc. Res.* **26**:501-514.
- YOUNG, J. Z., 1939. Fused neurons and synaptic contacts in the giant nerve fibres of cephalopods. *Phil. Trans. Roy. Soc.* **B229**:465-504.