

V. Physiological uses of the squid with special emphasis on the use of the Giant Axon

The squid, *Loligo pealei*, is a valuable research animal with regard to several physiological problems. Many investigators have first become acquainted with the squid from studies performed on various parts of the nervous system and in particular the giant axon. Several books have been written on the electrophysiology of the squid giant axon (Cole, 1968; Hodgkin, 1964; Katz, 1966; Tasaki, 1968). The lectures given for the Excitable Membrane Training Program (Adelman, 1971) at the Marine Biological Laboratory at Woods Hole, Massachusetts, are also an excellent source for recent studies on the giant axon. Because of their unusual size, the giant axons are an excellent tool for the measurement of bioelectric phenomena. In fact, the basic knowledge of how the individual nerve fiber or axon conducts impulses has been obtained from the squid. Originally, Williams (1909) noticed the giant axons in the squid, but their significance was lost until Young's accidental re-discovery of them in 1933 (Young, 1936, 1939). Cole (1968) and his collaborators opened a new era of nerve physiology at the MBL by inserting capillary electrodes longitudinally into these giant axons soon after Young reported on their large size. Later techniques were developed to remove the axoplasm and replace it with flowing artificial solutions (Baker, Hodgkin, and Shaw, 1962; Gilbert, 1971; Rosenberg, 1973; Tasaki, Watanabe, and Takenaka, 1962).

Several investigators from laboratories around the world have given the diameters of the giant axons used in their studies.

TABLE I: DIAMETERS OF SQUID AXONS

SQUID SPECIES	DIAMETER IN MICRONS	REFERENCE
<i>Architeuthis dux</i>	137-210	Steele and Aldrich, in press.
<i>Doryteuthis bleekeri</i>	700-900	Tasaki, Watanabe, and Takenaka, 1962.
<i>Doryteuthis plei</i>	290-394	Villegas and Villegas, 1960.
	400-500	DiPolo, pers. communication.
<i>Dosidicus gigas</i>	600-1500	Tasaki and Luxuro, 1964.
<i>Loligo forbesi</i>	700-900	Baker, Hodgkin, and Shaw, 1962.
	500-1040	Cohen, Hille, and Keynes, 1970.
<i>Loligo opalescens</i>	250	Llinas, pers. communication.
<i>Loligo pealei</i>	350-450**	Gilbert, 1971.
	300-700***	Adelman and Gilbert, unpublished data.
	75-350	Joiner and DeGroof, pers. communication
<i>Loligo vulgaris</i>	530-560	Spyropoulous, 1965.
<i>Lolliguncula brevis</i>	75-150	Joiner and DeGroof, pers. communication.
<i>Septioteuthis sepioidea</i>	250-450	Villegas et al., 1963.
<i>Todarodes pacificus*</i>	140-260	Chailaklyan, 1961.
<i>Todarodes sagittatus</i>	400	Mauro, Conti, Dodge, and Schor, 1970.

* Cited as *Ommastrephes pacificus sloanei*, which is probably *Ommastrephes sloanei pacificus* (= *Todarodes pacificus*, (Voss, 1963))

** From squid caught by MBL vessels during July and August

*** Yearly range at the MBL

Table I summarizes some of these findings. The time of year and the sex can affect these values greatly. Thus, *Loligo pealei* caught at the MBL in May and June have larger axon diameters than those caught in July or August. Although some years, the average large-sized axons are bigger than during other years, female squid usually have larger giant axons than males of comparable size. It should be emphasized that Table I probably represents the largest axons available for the particular study quoted. Specimens of *Loligo pealei* in May commonly have axons up to 600 or 700 μ in diameter, and some years the season average can be as high as 500 μ . The giant axons obtained from *Loligo pealei* and *Lolliguncula brevis* at the Duke Marine Laboratory, Beaufort, North Carolina are small as indicated in Table I (Joiner and DeGroof, pers. communication). Thus, the values given in Table I are only approximate and were compiled to give only a very general guide.

Both the axoplasm (Deffner and Hafter, 1959a, 1959b, 1960a, 1960b; Deffner, 1961a, 1961b) and the plasma membrane (Fischer et al., 1970; Villegas and Camejo, 1968) of the squid axon have been chemically analyzed. Isethionic acid and chloride are the major axoplasmic anions; potassium is the major axoplasmic cation (Deffner and Hafter, 1960b). The plasma membrane is composed of lipids and proteins. Phospholipids comprise two-thirds of the lipids (Fischer et al., 1970); in addition, there is a significant cholesterol content (Fischer et al., 1970; Villegas and Camejo, 1968). Enzymes occur in both the axoplasm (Nachmansohn and Steinbach, 1942; Nachmansohn et al., 1943) and the plasma membrane (Cecchi et al., 1971). Sodium and chloride are the major ions in the plasma (Prosser, 1973; Robertson, 1965), which is essentially the same as the natural external environment of the axon.

Using the voltage-clamp technique originated by Cole (1968), Hodgkin and Huxley in their very elegant 1952 studies (see Hodgkin, 1964) have given us the basic knowledge of how the action potential works. Cole received the National Medal of Science in 1967 for his pioneering studies, and Hodgkin and Huxley received the Nobel Prize in Physiology and Medicine in 1963 for their studies on the elucidation of the action potential.

Briefly, Hodgkin and Huxley discovered there are three processes which under normal conditions give rise to the action potential. The "sodium on" process occurs first in time, and permits the sodium ions to move from the external medium, where the sodium ion concentration is high, across the cell membrane to the internal medium where the sodium ion concentration is low. The entry of these positive ions causes the membrane potential to change its value from about -65 millivolts to about $+55$ millivolts. The second process, called the "sodium off" process, then stops this ion movement. A third slower process permits the potassium ions from the internal cellular environment (where the potassium ion concentration is high) to move across the cell membrane to the external medium where the potassium ion concentration is low. This permits the membrane to return electrically to its resting state.

After these electrical events have occurred, there is a slow return of the original ionic environment within the cell by active metabolic processes, so that the cell is ready chemically and electrically for another action potential (Adelman, 1971; Hodgkin, 1964). Thus, the energy for the action potential is derived eventually from the active metabolic processes.

Squid dissection for isolation of the giant axon

Part I: Isolation of the stellar nerve

There are several useful references on the dissection of the squid mantle for neurophysiological studies (Barnes, 1968; Berman, 1961; Bullock and Horridge, 1965; Pierce, 1950; Sherman and Sherman, 1970; Williams, 1909; Young, 1936, 1938, Rosenberg, 1973). Various investigators use different techniques, so only one method will be emphasized for clarity. A special dissection table with a central depression about 25 cm (10 in.) by 30 cm (12 in.) should be hung over the lower tank of a sea water table so a continuous supply of running sea water can be provided over the preparation. A small window of clear plastic or glass (7 cm by 11 cm; i.e., 2¾ in. by 4½ in.) in the center of the depression provides for illumination from below. A battery powered light will avoid the shock hazard of a 110 volt lamp used near sea water. The depression (excluding the window) should be coated with white plastic (General Electric silicon rubber RTV-41) so that pins can be used to secure the squid during the dissection. It is helpful to ink a centimeter and inch scale around the dissection area so the specimen size may be easily recorded.

In netting the squid to be dissected, care should be used not to agitate the remaining squid as this may shorten their lives. For this purpose, a fine mesh nylon bait net (10 in. diameter, 11 in. deep, 19 in. handle) can be used. Decapitate the squid as quickly as possible, recording the time. If the squid has been unduly agitated it may suddenly eject ink during the procedure. Place the body of the squid on the dissecting table so that the bottom or ventral (functional use of term) side is up. The siphon should be facing you and the tail pointing away. Open the animal by cutting through the mantle along a medial longitudinal line on the ventral surface. Record the mantle length and circumference of the collar, i.e., the height and base of the now roughly triangular mantle. Usually, specimens with a long mantle length and large collar circumference possess large axon diameters. Determine and record the sex if you have not already done so (see page 19).

Pin the mantle to the dissecting table, centering it over the window. To remove the viscera, pick up the siphon and cut the visceral attachments back along the mantle on both sides close to the center of the squid. This center is marked by the pen, which is a thin piece of "cartilage" running the entire mantle length. You may notice a muscle contraction in the vicinity of the stellate ganglion while you are cutting out the viscera. Next remove the gills on both sides and turn the animal over.

For the purpose of visibility, remove the skin from the mantle surface. Cut the skin next to the collar, and then strip it away. This very thin tissue contains colored chromatophores which can impair the visibility for dissection of the axon. Turn the animal over and pin down the mantle again. The interior of the mantle cavity should be facing you, with the tail pointing away as before. The pen may now be removed for more convenient access to the stellar nerve. Tear or cut in a straight line through the tissue which lies above the pen. With a forceps, free all the tissue above the pen, scraping tissue back from the cut line. Do this very carefully to avoid tearing the giant axon and its branches. Next cut the pen away from the mantle at the collar end. Place a finger beneath the pen and push gently between the pen and the mantle along its whole length, thus freeing the pen completely.

Note the right and left stellate ganglia which can be easily recognized by about ten stellar nerves radiating from each one (Figure 11). Each stellar nerve contains a

giant post-ganglionic axon, which is easily visible. The largest and longest giant post-ganglionic axon is located just beside the fin nerve, which passes centrally adjacent to the stellate ganglion. Isolate this longest and hindmost stellar nerve. Some people use optical aids for this, such as a microscope or a binocular loop. The retractor capitis muscle was previously cut in removing the pen and lies above the fin and above the longest stellar nerve. This muscle has to be carefully removed. Follow the stellar nerve where it dips into the mantle with the fin nerve.

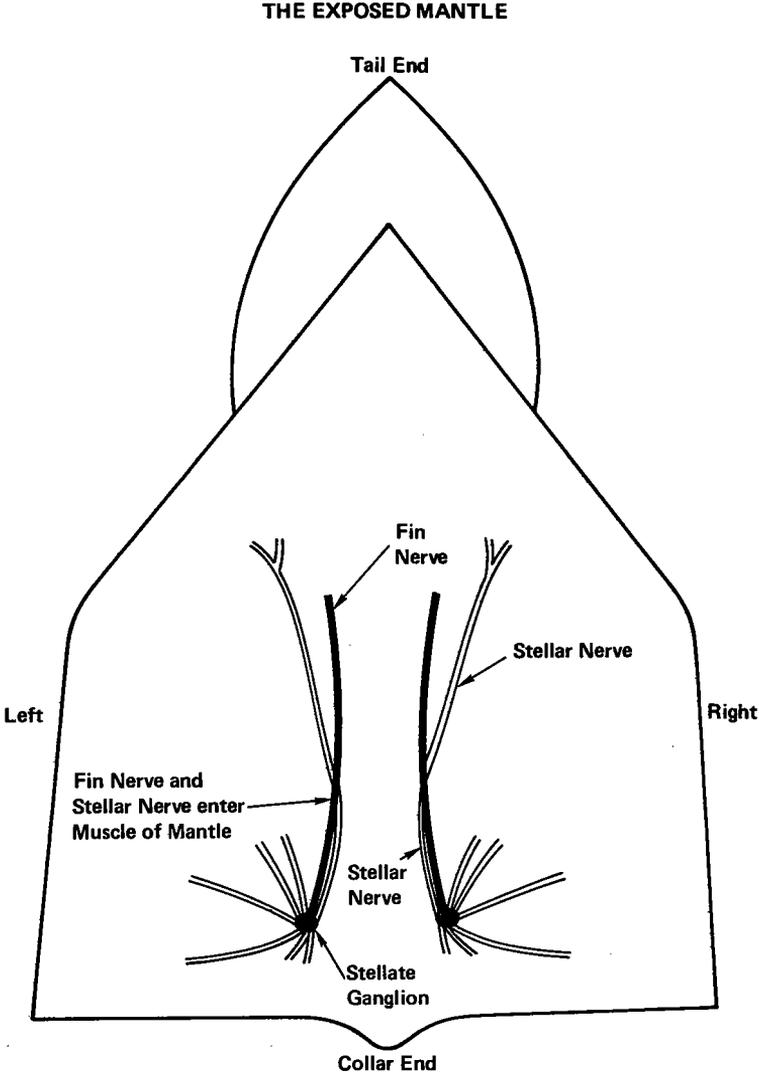


FIGURE 11

At this point, carefully cut, with a fine scissors, the mantle above the nerve. The nerve will deviate about twenty degrees from the pen axis (taking zero as the tail), whereas the fin nerve deviates only about fifteen degrees. Cut through the fin nerve where it deviates from the stellar nerve. Continue cutting the mantle above the stellar nerve until about 5.5 cm of the nerve is uncovered. Make a tie around the stellar nerve at this point in the nerve. Do this by putting some red thread in a curved forceps and putting it underneath the stellar nerve in the slit you have previously made. Tie the thread around the nerve with a square knot and cut one end of the thread close to the nerve.

Make a similar tie using blue thread around the stellar nerve and fin nerve by the stellar ganglion. Pick up the blue thread carefully, and free the stellar and fin nerves from the mantle with a pair of scissors. Using a very slight pull on the blue thread, continue to free the stellar nerve until you have the nerve completely cut away from the mantle along its length between the blue and red ties. Be sure to avoid excessive tugging on the nerve. The different color threads identify the large anterior end from the smaller posterior end of the nerve.

Place the stellar nerve on a plexiglass or glass slide which should be in a 9 cm Petri dish filled with filtered sea water. Two rubber stoppers with a V slit on tip should be glued to the slide by Elmer's contact cement. Then the nerve can be secured easily on the slide by passing the blue thread into the V slit on one rubber stopper and similarly the red thread into the other rubber stopper. Alternatively, wire clips can be used instead of the rubber stoppers. At this stage, the nerve can be refrigerated in the covered dish for a while until your experimental setup is ready. For identification, it should be marked by the squid number and whether the nerve comes from the left or right side.

Part II: Isolation of the giant axon

For this part of the dissection, you should use a dissecting microscope on top of a table with a hole in the center lighted from below. Directly underneath the nerve the light is diverted so the light enters only at the sides of your field of vision, making the outline of the giant axon inside this nerve more clearly visible (Figure 12). The Petri dish can be placed in crushed ice so that the nerve can be kept cool during the dissection.

Cut away the muscle tissue and fin nerve. The remaining tissue consists of the giant axon with fine nerve fibers and connective tissue. The fine nerve fibers are held with a fine forceps and very carefully held away from the giant axon so that they can be cut or teased with a scissors or a needle (Figure 13).

THE STELLAR NERVE

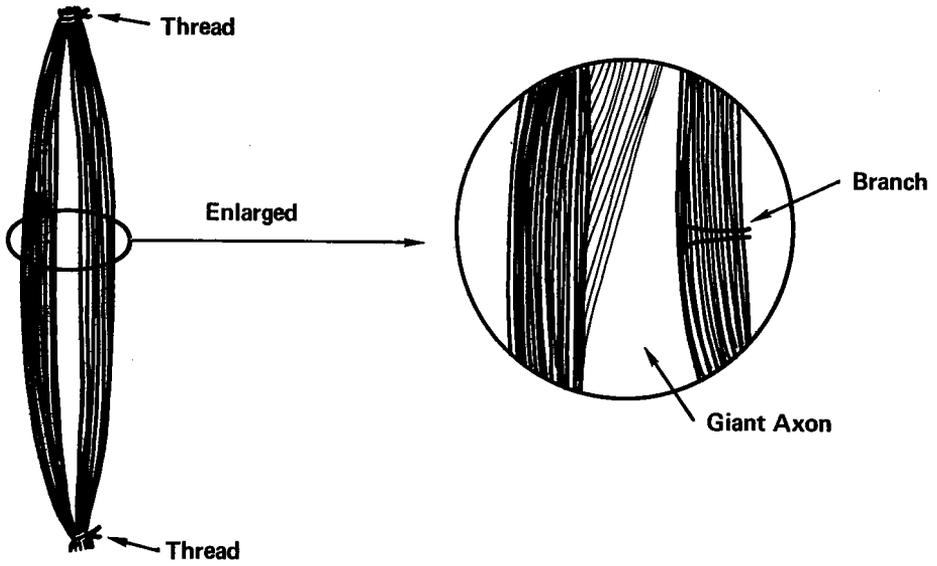


FIGURE 12

ISOLATING THE GIANT AXON

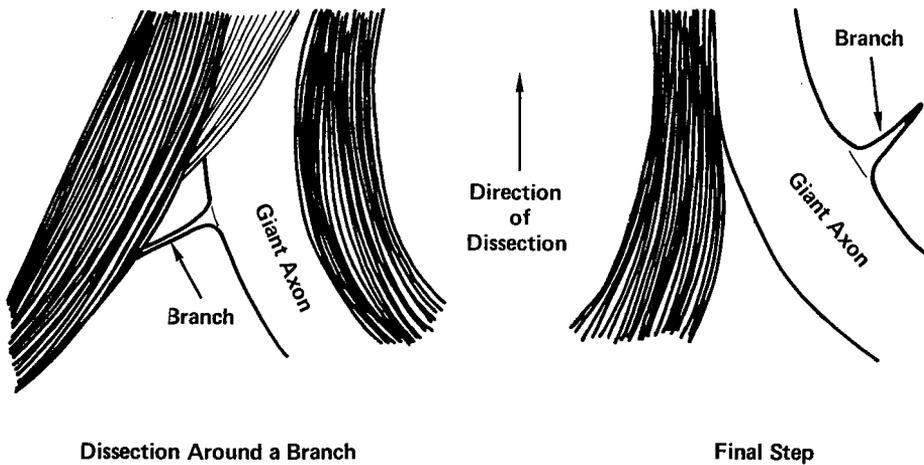


FIGURE 13

Check the experimental requirements to find if you must remove all of the fine fibers, or if you should remove only a few. *You must be extremely careful not to pull away or cut the small branches which originate directly from the giant axon.* It is sometimes convenient to leave a large amount of tissue around a branch so that it can be easily identified. The fine fibers should be cut in the natural cleavages only. For best results try to clean the nerve in two or three sweeps from end to end, rather than going up and down the nerve several times to cut away small fibers. In this way, you can minimize the danger of damaging the nerve by pulling or cutting a branch. *Branches are generally visible only on the sides and usually cannot be seen on the upper or lower axon surfaces.*

Once the giant axon is isolated to a sufficient degree, the Petri dish containing the nerve should be placed in the refrigerator for at least fifteen minutes, at which time, damage due to pulled or cut branches can be observed as white spots or constrictions on the nerve. If there is sufficient length of an undamaged section, then the axon can be tied off to include only the good portion. The nerve is now ready for a neurophysiological experiment.

Physiological saline for the giant axon

The ionic composition of sea water is almost the same as squid plasma; the major inconsistencies are that the plasma potassium concentration is two times the sea water potassium concentration and that the sea water sulfate concentration is three and a half times the plasma sulfate concentration (Robertson, 1965). The sulfate concentration is unimportant for the electrical activity of the axon and can be ignored.

Schmitt, (1955) showed that squid axons survive longer in an external solution depleted of potassium. Thus, it is better to use sea water or a potassium free sea water than plasma for the axonic external solution.

Howell et al. (1973) have shown that in some invertebrates the pH of hemolymph is 0.4 to 0.8 units above the neutral pH at any temperature. Table II gives the pH of water at neutrality. The change in pH divided by the change in temperature is about -0.017 at 25°C and about -0.020 at 5°C . A good buffer should have the temperature dependence of its pK to be the same as these values. Thus, if the pH of a physiological saline is adjusted to be 0.4 units greater than the neutral pH, the saline would have a pH of 7.400 at 24°C and 7.767 at 5°C . The pK temperature dependence of tris (hydroxymethyl) aminomethane or TRIS is -0.031 (Good et al., 1966). However, three buffers which show a pK temperature dependence between -0.014 to -0.020 are N-2-hydroxyethylpiperazine $-N^{\prime}$ -2-ethanesulfonic acid or HEPES, N-tris (hydroxymethyl -2 -aminoethanesulfonic acid or TES, and N,N-bis (2-hydroxyethyl) -2 -aminoethanesulfonic acid or BES (Good et al., 1966).

The chemical composition of a simple physiological saline, which has been successfully used, is 430 mM NaCl, 10 mM KCl, 10 mM CaCl_2 , 50 mM MgCl_2 , and 10 mM TRIS buffered to pH 7.4 at 20 to 25°C . The KCl can be omitted. HEPES buffer can be substituted for the TRIS buffer.

TABLE II: TEMPERATURE EFFECT ON THE pH OF WATER

pH AT NEUTRALITY*	TEMPERATURE IN °C
7.472	0
7.367	5
7.267	10
7.173	15
7.083	20
7.000	24
6.998	25
6.916	30

* Calculated from Weast (1968). (The $\text{pH} = -0.5 \log \text{Kw.}$)

Other physiological uses

Although the predominant physiological use of squid is the study of axons, there are also other important uses, some of which are given in other chapters. In addition, other studies have included the sense organs (Wells, 1966a), the complex behavior and brain (Wells, 1966b), the electrical contacts between embryonic cells (Potter, Furshpan, and Lennox, 1966), the nidamental glands (Atkinson and Granholm, 1968), the chromatophores (Florey, 1969), and the pigments (Ghiretti, 1966; Szabo and Wilgram, 1963).

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