Resting and Action Potentials of the Squid Giant Axon *in Vivo*

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ABSTRACT Blood oxygenation and circulation were maintained in *Loligo pealii* for several hours by a strong flow of sea water over both gills on the open, flat mantle. Potentials were measured with a 3 M KCl-filled glass microelectrode penetrating the giant axon membrane. An hour or more after the mantle was opened, the potentials were similar to those observed in excised axons and in preparations without circulation; spike height 100 mv.; undershoot 12 mv., decaying at 6 v./sec.; resting potential 63 mv. However, the earliest (20 minute) resting potentials were up to 70 mv. and 73 mv. Occasional initial action potential measurements (40 to 50 minute) showed a decay of the undershoot that was less than one-tenth the rate observed later. This suggests that in even better preparations there would be no decay, thereby increasing the resting potential and spike height by 12 mv. With the calculated liquid junction potential of 4 mv. the absolute resting potential in the "normal" axon *in vivo* is estimated to be about 77 mv., which is close to the Nernst potential for the potassium ratio between squid blood and axoplasm. The differences between such a normal axon and the usual isolated axon can be accounted for by a negligible leakage conductance in the normal axon.

INTRODUCTION

The resting and action potentials of the squid giant axon have been extensively measured and usually on excised, more or less cleaned axons in sea water (Curtis and Cole (1940, 1942); Hodgkin and Huxley (1939, 1945); Hodgkin and Katz (1949); Grundfest, Kao, and Altamirano (1954)). The useful survival usually has not been more than several hours with a considerably shorter period in which reasonably steady state conditions might be found. Similarly it is well known that squid and *Sepia* axons, under these conditions, exhibit a rather rapid exchange of sodium for potassium in the axoplasm (Steinbach and Spiegelman (1943); Keynes (1951)). The amount of potassium

A preliminary report was made by Moore and Cole (1955). The opinions stated are those of the authors and are not necessarily the opinions of the Navy Department.

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loss is at least about 20 pmoles/cm.^{2} sec. (Shanes (1954)), 20 μa./cm.^{2}, or 4 impulses/sec. (Keynes (1951)) and may be considerably greater (Shanes and Berman (1955)). Both axons have resting potentials of about 60 mv. (Curtis and Cole (1942)), considerably below the potassium potentials near 90 mv. (Hodgkin (1951)), and show a recovery undershoot of the action potential to 10 or 15 mv. below the resting potential (Hodgkin and Katz (1949)). In contrast, carcinous axon and frog muscle have resting potentials nearer the potassium potential and action potentials without undershoot (Hodgkin and Huxley (1945); Nastuk and Hodgkin (1950)).

It was obviously desirable to improve the condition of the preparation. The possibility of damage during careful dissection of the stellar nerve and isolation of the axon could not be ignored and the suitability of sea water as an environment was open to question. After various and not particularly successful modifications of the dissection and the medium, it seemed even more immediately desirable to have some idea of the properties of an axon that was as nearly normal as possible. There was not only the possibility that the resting potential would be increased with an intact circulation as has been found for frog muscle (Ling and Gerard (1949)) but also the hope of answering the long standing question as to whether or not the axon has an undershoot in the normal squid.

The squid had been found able to maintain a good circulation in flowing sea water with the mantle completely slit on the underside and it appeared feasible to gain electrical access to the interior of an axon as it lay near the upper surface of the opened mantle with the Ling and Gerard (1949) type of microelectrode.

The preliminary experiments with an intact circulation in 1953 were promising and were continued in 1954 until the answer seemed far more probable than the chance to prove it conclusively with a reasonable effort and the technique being used. The recent reference by Hodgkin (1958) to his 1952 work with Keynes shows an even closer approach to the expected answer and adds support to the work to be described. On the other hand, Thies (1957) made a preliminary report of considerably higher resting potentials in surviving animals.

Materials and Methods

ANIMAL PREPARATION These experiments were conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts, where live squid, *Loligo pealii*, are available in good condition. It was found useful, both for reference and for improvement of the techniques, to make several *in situ* preparations in which the internal organs were removed and the mantle alone pinned down. These are referred to as "mantle preparations." The *in vivo* or "open chest" preparation attempted to gain access to the nerve with minimal injury to the animal. The mantle was slit ventrally

from the siphon to fin, the single cut artery was clamped, the mantle pinned to the bottom of a plastic dish, and the internal organs covered with sea water. A strong flow of sea water at 12–22°C. from each side of the head passed over the pinned-down mantle, opening and filling the gills, and maintained a deep blue color of the circulating blood. Good circulation was maintained for as long as 3 hours. In contrast to the procedure of Hodgkin and Keynes, the preganglionic nerves were left intact to lessen the amount of injury and possibility of blood loss and oxygenation and low temperatures were not used.

The ganglion was exposed by pulling the siphon and its large retractor muscles to one side with a lead retractor. The next to hindmost stellar nerve was often used because it was more accessible. The thin "skin" covering the nerve fibers radiating from the stellate ganglion for a few millimeters before they enter into the mantle muscle was removed. In some experiments, a short length of the giant axon was dissected clean from the bundle of surrounding small fibers; in most, penetration with a microelectrode through these small fibers was accomplished. The microelectrode and stimulating electrode (stainless steel loop or point) were both positioned by micromanipulators and observed with a binocular dissecting microscope through a small, plane, plastic "window" to prevent distortion by ripples on the water surface.

When the blood color was good, the animal was usually quiet, except for small rhythmic movement of siphonal retractor muscles. Occasionally, without warning, the animal would give a sudden violent contraction of the whole mantle and tear it away from the pins holding it down. This, of course, broke any microelectrode in range. It seemed likely to occur as the needle was ready to penetrate the axon and almost certainly when preparations were being made for action potential measurements. The number of *in vivo* measurements is consequently quite limited.

The time required from the slitting of the mantle to the first resting potential measurement averaged over half an hour and that to the first action potential, nearly an hour.

ELECTRODES The microelectrodes were drawn from 0.8 mm. melting point pyrex glass tubing with a commercial needle puller.[1] In the course of the work it was found that microelectrode tips which were easy to insert into the axon invariably gave high and steady potentials while those tips which could be inserted into the axon only with difficulty gave lower and rapidly declining potentials; this is in agreement with the results of C. Y. Kao (personal communication).

The ease of entry was found to depend more upon the angle of taper of the glass than upon the tip diameter. Microelectrodes with short rapid tapers from the shank to tip had the mechanical rigidity to penetrate the heavy connective tissue sheath surrounding the membrane without the poking, bending, and breaking experienced with long slim tapers. On the other hand it was found that the terminal taper could be too short and sharp. Electrodes with such a shape were usually found to have the thin walled tip broken off after being filled by boiling. It was later observed under a microscope that this breakage occurs whenever such a tip comes in touch with water

[1] Gamma Instrument Company, New York.

(either internally or externally), apparently because of the surface tension forces. Between these two extremes, a rather rapid but uniform taper was found which could survive filling, penetrate the axon, and give satisfactory membrane potential measurements.

Immediately after drawing, these microelectrodes were easily filled with solution, through the shank, from a hypodermic needle and syringe. The squared end of a long close-filling needle was inserted up to the taper of the glass shank and the solution was then forcibly injected with the syringe. The meniscus moved down the taper in a few seconds by surface tension, slowly at first and with increasing speed as the diameter at the meniscus decreased. Although this method was not successful for filling long, thin, hand-drawn electrodes, it was very convenient for this work and eliminated the large inventory of needles usually required. The microelectrodes regularly used were filled with 3 M KCl and had comparatively low electrical resistances, usually between 0.5 and 1.0 megohms. No evidence of appreciable tip potentials as described by Adrian (1956) was noted. The size of our tips and the method of filling just prior to use may have been factors in keeping the tip potential small.

Potentials were measured between one Ag–AgCl electrode with a 3 M KCl bridge to the solution inside the microneedle and another in the external sea water. There was a considerable (0.2 v.) and fluctuating potential difference between the running sea water system and a metallic, building ground. Because a grounded point electronic system was used, the sea water electrode had to carry the ground current. A large electrode area and short electrolyte path were best obtained by Ag–AgCl in direct contact with the flowing sea water. Such an electrode was satisfactory in still sea water but gave disturbing potential variations when the sea water was circulated. Because the solution in contact with the silver chloride should be saturated with silver ion to obtain a reversible potential, the electrode was imbedded in a block of sea water-agar. This combination was reasonably reproducible and constant.

AMPLIFICATION AND RECORDING The measurement of resting potentials with microelectodes requires an amplifier with high input resistance and low grid current. The distribution of the microelectrode resistance and capacitance is such that it is nearly equivalent to a lumped circuit with all the resistance at the tip shunted by a capacitor equal to about a micromicrofarad per millimeter of tip immersed (Woodbury (1952)). This capacitance, of course, attenuates the higher frequency components of the action potential signal. The faithful recording of action potentials with a duration of about one-half millisecond therefore requires that the amplifier and tip input capacitances be as low as possible. At the time of these experiments the conventional solution to these requirements was the use of a cathode follower with the shield on the input lead connected to the cathode to reduce the input to shield capacitance (Moore and Cole (1954)). For most of this work, a 954 acorn type tube was used and, with reduction of the heater voltage from the normal 6 to 4 v., grid currents were maintained in the order of 10^{-11} amp. The later use of microelectrodes of resistances down to 0.5 megohm made the requirement of low input capacity less stringent than it had been for the summer of 1953 when 5 to 10 megohm tips were used. The lower value of tip resistance would allow up to 10^{-9} amp. of grid current to flow with less than a 1 mv. error in the resting potential. An indication that this

current probably would not appreciably affect the axon is obtained from the fact that stimulating currents passed through the recording microelectrode gave a threshold at about 10^{-6} amp. for a 1 msec. pulse.

The output of the cathode follower was coupled to a cathode ray oscilloscope with a high frequency accentuating network and to a buffer amplifier which drove a D. C. pen recorder as shown in Fig. 1. The action potentials were recorded by photographing the pattern on the cathode ray oscilloscope. Time marks were indicated on the action potential records by intensity modulation of the cathode ray beam.

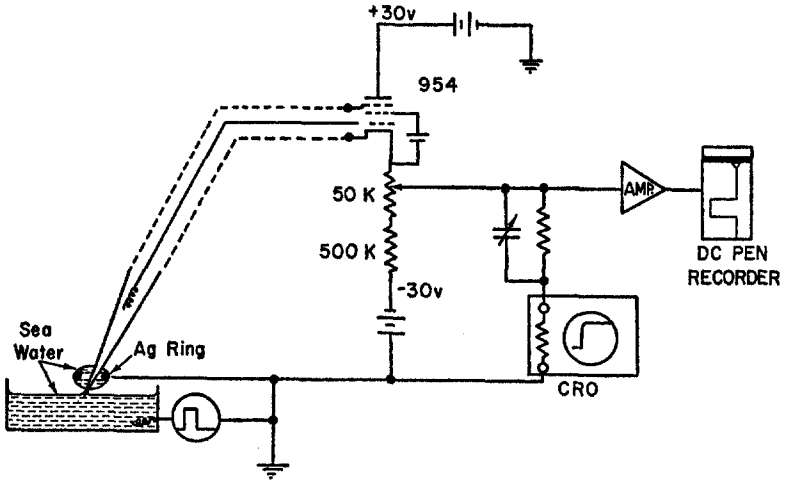

FIGURE 1. Schematic diagram of potential testing and recording system. The sea water droplet at ground potential approximately simulated the high frequency shunting effect when the tip just penetrates an active membrane. The high frequency components of the cathode follower output were accentuated by the coupling network to the cathode ray oscilloscope (*CRO*); the lower resistance is the input resistance of the oscilloscope.

The membrane resting potentials were taken as the difference between the reference potential, recorded with the microelectrode in sea water before entry into the axon and, if intact, after withdrawal, and the internal potential recorded when the tip of the microelectrode had penetrated the membrane and was in contact with the axoplasm.

The response time and the steady state calibration of the over-all system were obtained (Fig. 1) by driving a sea water electrode into which the tip was barely dipped, with a voltage step function from a low impedance generator (Moore and Cole (1954)). A drop of sea water on a small loop of silver wire around the microelectrode near its tip was connected to ground to simulate the operational conditions. If the tip were immersed any appreciable distance and the grounding ring not used, the tip capacity would have given a falsely reassuring impression of brisk response because the capacitative currents would have speeded rather than slowed the preamplifier response. The over-all response time constant was usually 35 microseconds or less. The effect of this on the shape of the action potential was small and the distortion of the slow undershoot phase was negligible.

RESULTS

The resting potentials, without liquid junction correction, averaged 59 mv. in the mantle preparations and were almost the same in the intact animal after the circulation had stopped. These values were the averages of more than twenty determinations and the range of values in each was 7 mv. In the initial experiments, the average of twenty uncorrected resting potentials with circulation was 62 mv. with a range of 10 mv., and in the second series the average was 65 mv. In the first series the maximum value was 66 mv. and in

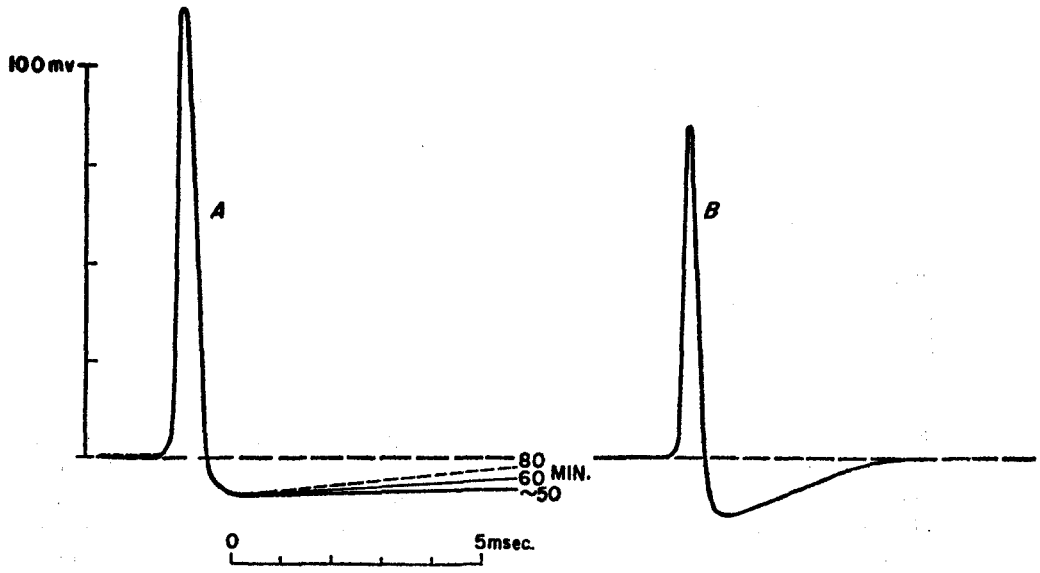

FIGURE 2. A, squid axon membrane action potentials *in vivo*. The rate of recovery from the undershoot increased as shown with time after the initial cutting of the mantle. Temperature 20°C. B, action potential in a mantle preparation showing a somewhat larger and much more rapid recovery from the undershoot. This record was taken during the midsummer slump and gave a typically lower peak action potential.

the second series the largest values, 70 mv. and 73 mv., were also the earliest, obtained at about 20 minutes after the start of the experiment. The action potentials obtained from a mantle preparation were similar to those found after the circulation had stopped and in excised axons. In the example shown in Fig. 2B the resting potential was 57 mv., action potential 80 mv., undershoot 14.5 mv., and maximum rate of recovery after the undershoot 5 v./sec. The average value of the undershoot in mantle preparations was 12 mv. The occasional *in vivo* action potential records taken as early as 40 or 50 minutes showed larger resting and action potentials and a much slower recovery from

the undershoot. The earliest record shown in Fig. 2A was obtained at about 50 minutes in 20°C. sea water. The resting and action potentials were 63 mv. and 118 mv. respectively with an undershoot of 9 mv. The undershoot recovery was estimated to be less than 0.3 v./sec. However, the recovery was completed before the next impulse, 0.1 sec. later, so that the recovery rate was between 0.1 and 0.3 v./sec. The succeeding records from this preparation at 60 minutes, show little change of the resting action, or undershoot potentials, but do show the increased undershoot recovery rates of up to about 1 v./sec. The record shown for 80 minutes is typical of the first action potentials as usually obtained.

During the course of the summer of 1953 there was a progressive deterioration of the height and an increasing duration of the action potential until the middle of July, when experiments were terminated. Measurements made throughout the summer of 1954 showed poor resting and action potentials only during the last 10 days of July.

DISCUSSION

As the work progressed, it became increasingly attractive to assume that the action potential of a truly normal squid giant axon would not show the undershoot so characteristic of the excised axon and that both the action potential and the resting potential would be larger than the excised values by about the amount of the undershoot. Although the earlier, and apparently better, resting potentials were larger, the undershoot recoveries slower, and the peak to peak potential differences rather constant, it was never possible to record an action potential with the markedly decreased undershoot that a close approach to this assumption would require.

Hodgkin and Keynes had informed us that they had obtained *in vivo* results similar to those reported (Moore and Cole (1955)), but it was not realized until the publication of the former's Croonian Lecture either that their experiments had preceded the work reported here or that they had been able to reduce the average undershoot to a value as low as 4 mv. It is encouraging to note the agreement between their average resting potential, 68 mv., and our 65 mv., and between the maximum values, 72 mv. and 73 mv. respectively. Although it is not possible to estimate the relative merits of the differences of detail in the two procedures, it seems probable that the effects of injury and blood loss by the cutting of the preganglionic nerve are significantly less than the advantage gained in speed of access to the axon in the absence of mantle movements of central origin. In any case, these results have added a considerable support to the earlier views and make the extrapolation to an intact animal seem less uncertain.

Thus, it seems very possible that the undershoot is an indication, and a result, of an abnormality not to be found in the normal animal. The pre-

dicted resting potential, without liquid junction correction, is then the average of the observed resting potential plus the undershoot giving 74 mv. for these experiments and about 72 mv. for those of Hodgkin and Keynes. It is to be noted that these values are close to the maximum of the direct observations, 73 mv. and 72 mv., respectively. With the liquid junction correction of 4 mv. (Cole and Moore (1960)) added to the maximum resting potential, a value of 77 mv. is obtained which is quite close to the 76 mv. (at 20°C.) which may be calculated for potassium between squid axoplasm and blood with a potassium content about double that of sea water (Manery (1939) and Robertson (1949)). Although Thies (1957) gave an average of 77 mv. for the uncorrected resting potential *in vivo* at 8°C., there is no certain explanation for the larger values in the reported range from 60 to 111 mv.

These projected potentials are in reasonable agreement for an external plasma potassium concentration, but no estimate is available for an interstitial electrolyte concentration, and they are far below the predicted value for an axon in sea water where the potassium concentration is about half that of plasma. This also raises a question as to the possible effect of the removal of the skin upon the potassium concentration in contact with the giant axon. However, the agreement between the potentials so obtained and those of Hodgkin and Keynes in which the opening of the skin was probably minimal suggests that this was not an important factor.

If it is then assumed that the axon was effectively bathed in an interstitial electrolyte, having the potassium concentration of plasma, the nature of the interface between this and the sea water in which the external reference electrode was placed should be considered. However, at present this can only be ignored in the hope that the transference numbers of sodium and chloride are so high and the separated concentrations so nearly equal as to give a negligible potential.

It should be very helpful to know the underlying causes of the axon abnormality that appears as the undershoot but it is now only possible to conjecture the intermediate effect that they produce. An attractive possibility is that the leakage conductance of Hodgkin and Huxley (1952) is so low in a normal axon membrane as to allow a resting potential within a millivolt or two of the potassium potential and eliminate the undershoot. Then during the first hour of deterioration this conductance would rise from an initial value of perhaps 1 μmho/cm.^{2} to approach the 300 μmho/cm.^{2} found by Hodgkin and Huxley for excised axons in sea water to give a resting potential 12 mv. more positive than the potassium potential and the undershoot of about 10 mv. when the potassium conductance is large compared with those of sodium and this leakage. In support of this, Hodgkin has mentioned that the undershoot is affected by changes of external potassium concentration which have relatively little effect upon the resting potential. The

assumption of a low leakage conductance in a normal axon would thus increase the resting potential to nearly the potassium potential and return the action potential to this value as the sodium current becomes negligible. Although the *in vivo* action and resting potential may be rather well accounted for on the basis of an external plasma potassium concentration, the relatively slight change of the resting potential and undershoot from the equilibrated *in vivo* axon to the usual axon in sea water would require a considerable relative increase of another permeability such as that of chloride or sodium to absorb the 17 mv. increase of "E_K" required by the transfer of the axon from plasma to sea water potassium concentrations. A part of this shift in E_K may also be brought about by the potassium leakage causing an increase in the potassium concentration in the (Frankenhaeuser and Hodgkin (1956)) space just exterior to the active membrane.

In conclusion, although the potentials of the squid giant axon, *in situ* and with an active circulation, deteriorate rather rapidly towards those of the usual isolated preparation, it seems reasonable to extrapolate from the *in vivo* results to those of a normal axon in which the resting potential would be close to the axoplasm–blood potassium potential, and the undershoot would be absent or negligible. These effects would be consistent with a negligible leakage conductance in the normal as compared with that found for the usual isolated axon. A procedure by which an isolated axon could be returned towards these normal properties should be very generally useful and might help in understanding the leakage conductance.

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