THE FACTORS CONCERNED IN THE LEADING OFF OF ACTION POTENTIALS FROM SINGLE CRUSTACEAN NERVE FIBERS

Thesis by

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I. Introduction

The action potential of nerves has been studied since the time of du Bois-Reymond and Hermann, starting in 1848. Its main characteristics,--that of being negative, having a relatively slow velocity compared to other electrical changes,--and the conditions for excitation were being studied at this early period. The currently held conception that the physical mechanism of the action potential results from the breaking down of an electrically polarized membrane during activity was also being evolved by these investigators and their contemporaries.

Their recording methods were galvanometers, which were slow and not sufficiently sensitive to obtain faithful reproductions of rapid and minute electrical changes. Later, the capillary electrometer, which had the advantage of being fairly sensitive and which, of course, did not draw current, was used. It had the disadvantage, though, that it possessed considerable inertia and that elaborate corrections in the records were necessary. The string galvanometer of Einthoven also was widely used; it was much more sensitive to rapid changes in potential, but, on the other hand, drew current.

In more recent years, the development of the vacuum tube amplifier allowed minute potential changes to be detected, and several new types of oscillographs to record these amplified responses were invented. One of these rapidly moving oscillographs (the mechanical one devised by Matthews) consists of an electro-magnet, connected to the output of the amplifier, which moves an iron

tongue bearing a small mirror. The second type, the cathode ray oscillograph, introduced for this work by Gasser and Erlanger (1922), reproduces most faithfully the potential variations since it is without inertia. It furthermore has the advantage of allowing direct visual observation.

With the aid of this latter oscillograph much new information has been gained within the last two decades regarding the action potentials of myelinated nerves. The older data has been checked, amplified, and a deeper analysis made of the underlying mechanisms. In most of this work the sciatic nerve of the frog has been used. The methods of leading off have been fairly simple: the nerve was dissected from the animal, washed in Ringer's solution, and placed upon non-polarizable silver-silver chloride or calomel electrodes in a moist chamber. Long lengths of nerve - up to 13 cm. - have been obtained from this preparation, thus enabling the distance between the stimulating and lead-off electrodes to be large. In the main, single-ended amplifiers have been used, of which the proximal lead (the one nearest the stimulus) has been connected directly to ground, while the distal one went to the grid of the first stage of amplification.

The most obvious electrical sign of nerve activity is the action potential spike; as recorded, this amounts to several millivolts in amplitude. Indeed the spike was the only sign known until the present methods were introduced. It turned out to be a much more complicated phenomenon than had been expected. As recorded

from frog nerve, the first, main, "A" spike consists of four parts, the α, β, γ and δ waves. These are followed by two slower effects, the "B" and "C" waves. See Gasser and Erlanger, 1927; Erlanger and Gasser, 1930.

In these papers it was shown that the nerve is composed of groups of fibers which have different rates of conduction, from 42 to 0.3 m. per sec. The fibers of a nerve range in diameter from 20 down to 1 μ, and it was found that the larger the fiber, the faster is the rate of conduction. However, since the fibers of a nerve are not present in a uniform distribution of sizes, a continuous "spectrum" of action potentials is not recorded, but instead the series of waves already mentioned.

A correlation between fiber diameter and conduction velocity attempted by Gasser and Erlanger in 1927, when only the Aα, β, and γ waves were known, indicated that the velocity was proportional to the fiber diameter. This view was changed in 1930 with the discovery of the additional waves and upon a more careful reconstruction of the action potential from fiber size data, and it was stated that the velocity varies with the square of the diameter. Douglass, Davenport, Heinbecker and Bishop (1934) believe that the truth lies between these two possibilities. Recently, however, Gasser and Grundfest (1939) and Hursh (1939) have concluded from careful reconstructions of mammalian action potentials that the conduction rate is really proportional to fiber diameter.

Further investigation of nerves from the frog and of both myelinated and unmyelinated mammalian nerves have pointed to the conclusion there are only quantitative differences between the conduction velocities of fibers of different diameters. It is the opinion of Duncan (1934) that whether a fiber in these animals is myelinated or not depends only on its size; fibers acquire a medullary sheath when attaining a diameter of 1 to 2 μ. And it can be concluded from Bishop (1933) that conduction rate declines gradually in the smallest myelinated fibers to that found in those without a marked myelinated sheath.

Blair and Erlanger (1933), using small nerves from the frog, have compared other fiber properties against conduction rate. They found that the threshold of stimulation decreased continuously with increasing velocity, and that, indeed, for any one duration of a constant stimulating current, the voltage required decreased smoothly as the conduction rate increased. The absolute and relative refractory periods also gradually decreased as the velocity of conduction increased. However, the chronaxie and the summation interval did not vary in a simple manner. The chronaxie, for instance, remained almost constant until the fiber velocity decreased to 5 m. per sec. and then began to increase sharply. The authors do not assume that this indicates that there are two types of fibers with different chronaxies, but rather that the methods of measuring the chronaxie, and also the summation interval, were at fault.

Not only has the action potential from a whole nerve been found to be complicated, but also the action potential from a single fiber. This single fiber potential is obtained, in the case of vertebrate myelinated nerve, either by stimulating the whole nerve with shocks so weak that only a few of the most excitable fibers are affected, or by the method developed by Adrian and Bronk (1928) in which a section of nerve between the stimulus and the lead-off electrodes is dissected until the continuity of only one fiber is preserved.

When a stimulating current is applied to a fiber, the first result is a movement of ions which initiates two processes. One of these processes is directed toward excitation, while the other, called accommodation or cathodal depression, is opposed to the first. However, if the sum of these two processes attains a certain minimal value, the fiber responds after a latent period with a sudden, large negative potential change known as the spike potential (see Hill, 1936, for theories of excitation). This potential is propagated down the fiber, and in both directions unless blocked, at a uniform conduction velocity (Erlanger and Gasser, 1937). The spike potential follows the all-or-none relation; that is, it rises to the greatest extent possible under the conditions existing at a given point or else does not occur at all (see Kato, 1934, for his experiments demonstrating this relationship, and for earlier work referred to there).

The spike in frog "A" fibers reaches its crest in 0.3 msec. and declines to within a few per cent of its height within a further 0.6 msec. The spike does not end here but continues in a small, negative after potential which lasts for 20 to 30 msec. in a freshly mounted frog nerve (Gasser and Graham, 1932). After the nerve has been stimulated for some time or if it is poisoned with veratrine, this after potential increases in size and duration and may become as large as 10 per cent of the spike height. (Gasser and Grundfest, 1936).

Following these potentials there appears a small but delayed positive after potential (Gasser, 1935; Gasser and Grundfest, 1936). In freshly prepared, fast frog and mammalian fibers this potential attains a maximum of about 0.2 per cent of the spike height in 30 msec. and then declines. In "C" fibers and after a tetanus in the faster fibers this positive phase becomes much larger and earlier, canceling the negative after potential and being followed by a second negative after potential. This last potential may then be followed by a second positive potential. Therefore it seems that separate positive phases follow both the spike and the negative after potential. This conclusion is supported by experiments with veratrine which accentuates the negative after potential.

The after potentials can be modified in still other ways. Schmitt and Gasser (1933) and Lehmann (1937b) have found that they are abolished during carbon monoxide poisoning and asphyxia, even

though the spike remains. They have been correlated with the oxidative metabolism of nerve and with heat production. Thus they are considered an expression of the recovery process, but Gasser believes that recovery attended by large after potentials is expedited at the cost of an extra expenditure of energy and is not often called into play in the animal. Lehmann (1937a) found that removal of calcium from the solution bathing nerves had the effect of decreasing the negative phase and increasing the positive one, while removal of potassium had the opposite effect. Graham (1933) has also found that calcium ions, as well as warming, increase the negative after potential while potassium and cooling decrease it. She has later found that yohimbine increases the positive after potential.

Finally, the excitatory effects accompanying the various potential effects should be mentioned. There is the well known absolute refractory period which lasts until the spike is almost over, during which it is impossible to excite the fiber. This is followed by a relative refractory period of slightly longer duration, upon completion of which the excitability has returned to normal (Adrian, 1921). A spike elicited during the latter phase is not as large and is not conducted as fast as a normal one. But the end of the relative refractory period does not complete the excitability cycle. Adrian and Lucas (1912) found that a period of supernormal excitability ensues for a brief period. This period was correlated with the negative after potential by Gasser and

Erlanger in 1930. In nerves with a large positive after potential, especially those poisoned with yohimbine, a subnormal period follows; this decreased excitability was shown to accompany the positive phase by Graham and Gasser (1934). Only after all potential changes have ceased can the fiber return to its normal state of excitability.

These are the principal facts, then, describing the electrical changes occurring in nerves and nerve fibers possessing a myelinated sheath. Further references to research on this subject may be found in Davis (1926), Erlanger and Gasser (1937), and the Annual Review of Physiology (1939).

It has never been possible to lead off action potentials from isolated, single, myelinated nerve fibers. It is possible, however, to obtain large, single unmyelinated fibers, which are free from all other tissue, in crustaceans and cephalopods. These fibers seem very promising material for investigation of the action potential, but have only been studied within the last few years. Experiments on unmyelinated fibers previous to this have all been done on whole nerve bundles.

Levin (1927), and after him, Furusawa (1929), investigated "the retention of the action potential" in the leg nerve from Maia. They used a sensitive galvanometer of fairly long period, which recorded the sum of the monophasic action potentials produced by tetanic stimulus; the activity under their live electrode was compared to the injury potential by this method. They found that after several minutes of stimulation at the rate of 50 per sec. the

action potential was reduced by 50 per cent or more. It was even possible that after such a period of stimulation the galvanometer remained at its maximum position and only gradually returned to the initial one. This was taken to indicate that the nerve became completely "depolarized" and then slowly recovered, taking 10 min. or more. Furusawa found that the injury potential could be reversibly abolished by asphyxia, and he concluded that the "action and injury potentials are two aspects of the same fundamental phenomenon, viz. of an active membrane potential existing at the surface of the nerve fiber and maintained by oxidative processes."

Action potentials resulting from a single stimulus were led off from the leg nerves of several crustaceans and of Limulus by Monnier and Dubuisson (1931) using a cathode ray oscillograph. Their records were complicated by spurious electrical responses which were not satisfactorily explained; much importance was paid to chronaxie measurements of the constituent fibers.

Lullies (1934) studied the action potentials of the leg nerves of Maia with a string galvanometer. He attempted in particular to search for fiber groups, and reported three such groups which he compared to the slowest three in vertebrate nerve. Lullies maintained that these groups were distinguished by having different thresholds, refractory periods, conduction velocities, and resistance to narcosis (with urethane). He investigated fiber sizes, finding 40 to 50 fibers with a diameter of 10 to 20 while the diameter of the remaining fibers ranged from 8 down to 1 .

Bayliss, Cowan, and Scott (1935) also led off action potentials from Maia nerve. They used a three stage amplifier and a mechanical oscillograph adapted from a loud-speaker. These authors were not able to find the three waves reported by Lullies; they found two, however, conducted at a rate of about 2.5 and 1.0 m. per sec., respectively, at 12° C. The question of the existence of after potentials was raised, especially in regard to the retention of the action potential which Levin had found. Their results are of interest since they found that veratrine produces a negative after potential reaching a maximum at 125 msec. and lasting as long as half an hour, though they were not sure that repeated discharges of the fibers did not complicate the picture. Yohimbine produced a positive after potential of 200 msec. duration. Their results with these drugs are analogous to those already mentioned in regard to myelinated nerve.

Again using Maia nerve and a mechanical oscillograph, Bogue and Rosenberg (1936) reported on the various waves appearing in their action potential records. They found one main wave and three smaller ones; the first of these later waves was regarded as possibly due to a repeated discharge, for which crustacean nerve is notorious, while the other two were looked upon as the result of definite fiber groups. They also reported a negative after potential lasting 30 to 60 msec. Velocity measurements ranged from 5.3 to 1.4 m. per sec. They studied the effect of frequency of stimulation on the size and form of the action potential. Rapid stimulation

(200 per sec.) produced a record on which the main waves were superimposed on the after negativity but followed the rhythm of the stimuli; with still higher frequencies the response became irregular. Their preparations were readily fatigued after 2 min. of such stimulation and only partially recovered. No positive after potential was seen.

The electrical response of vertebrate unmyelinated nerve, from the rabbit's vagus, was investigated by Bishop (1934). He obtained records that were neither monophasic nor were they satisfactory to him. His explanation of their complexity is incomplete and doubtful.

All these investigators used whole unmyelinated nerves which were led off in moist air. They all obtained records which were highly complex and between which there was little agreement. The nature and relative importance of the complicating factors was not at all clear.

One of these factors may very well have been, as the present work will point out, the conditions of leading off. The characteristics of the amplifier and recording systems are also of prime importance. With single-ended amplifiers the potential from the active locus will affect the leading off even after it has left the grounded lead, though of course decrementally (Erlanger and Blair, 1935). The use of a differential amplifier, in which only the differences in potential of the two leading off points is recorded, would seem advisable. Here a third, reference potential, ground, is available for reduction of the stimulus artifact and the elimination

of external disturbances. For reference to such amplifiers see, for instance, Schmitt (1937) and Toennies (1938).

The work on unmyelinated fibers so far reviewed has evidently been done with the view of correlating the experiments in this field with those done on vertebrate, myelinated nerve. Since it is not difficult to prepare either single large fibers or fine bundles of the smaller fibers from crustacean nerve, for instance, it has become both wasteful and unsatisfactory to use whole nerves, yielding necessarily complicated records, in the study of this type of nerve.

The first recording from single crustacean fibers* was done by Hodgkin (1938), using an amplifier built by Toennies. He worked with large motor axons from the leg nerves of Carcinus. Instead of leading off in air, his fibers were submerged in a layer of aerated mineral oil. He recorded action potentials from these fibers that were similar to those from single medullated fibers published by Gasser and Grundfest (1936). The impulse traveled at a uniform rate of 3 to 5 m. per sec. and the duration of the spike was 0.8 to 1.0 msec. with a wave-length therefore of about 4 mm. as compared to one of about 40 mm. in frog "A" fibers. The absolute refractory period lasted for about 1 msec. and a fiber could transmit as many as 500 impulses per sec. However, in this paper he was particularly interested in the sub-threshold potentials which arose at the grounded stimulating electrode when the excitation was just sub-liminal. This sub-threshold potential was shown to be distinct from the well known polarization potential which arises, when a potential difference is

* But see van Harreveld and Wiersma (1936), pp. 94-96.

applied along a nerve fiber, from the capacitative action of its membranes and which can be easily imitated with physical models. Hodgkin's potential was negative, arose locally at the cathode stimulating electrode, and merged into the action potential if the stimulus was sufficient. The maximum height of this potential, though, was at most one-seventh that of the spike. This result is of some interest because such a local or unpropagated response has never been observed in myelinated nerve.

The same author (1939) has also shown that the velocity of impulses in these fibers are greater by 40 per cent when the impulse travels through a portion of fiber submersed in sea water solution as compared to air. This finding is taken as evidence that currents generated by, but outside, the fiber are necessary for conduction and supports the polarized membrane theory of nerve action to be discussed later.

The purpose of the present work has been not only to study the shape and properties of the action potential from the larger single unmyelinated fibers and from fine bundles of the smaller fibers, but especially to consider the factors concerned in the leading off of these action potentials. In doing this it also became necessary to re-examine the meaning of the action potential in the light of current views as to its nature.

The thesis is divided into three main parts. In the first, the preparation of the fibers and the methods of leading off will be described. These latter were of two types: in moist air and in solution.

The second part will deal with the results obtained, and especially the effect of varying the environment of the fiber. The form and shape of the action potential will be described, and data concerning the velocity of conduction in different fibers will be presented.

The final section will review the theories and facts currently believed in regard to bio-electric potentials, and a critical analysis of the important factors pertaining to the leading off of action potentials from unmyelinated fibers and the conclusions to be drawn will be given. A new method for such leading off will be offered.

II. Methods

A. Preparation of nerve fibers

Most of the experiments here reported were performed on single motor axons from the claw of the crayfish, Cambarus clarkii. The method of preparation was essentially that developed by van Harreveld and Wiersma (1936), and subsequently used in this laboratory.

The chitin was removed from the top of the ischio- and meropodites. The muscles contained in the latter segment were removed. Sometimes the shell of the carpopodite was partly removed in order to obtain a longer length of fiber. Following these operations, the claw was clamped above a Petri dish with the exposed portions just lying under van Harreveld's (1936) crayfish solution. Dissection was carried out with the aid of a low-power binocular microscope.

A precaution should be mentioned here. It seemed wise to remove as much blood as possible from the claw and particularly from the region of the nerve before dissection was begun. Furusawa, (1929) and others have believed that blood clotted about nerve fibers may depress their excitability.

All of the nerve which obviously did not contain motor axons was immediately removed. The remaining portion was teased into smaller bundles with fine needles. Micro-manipulated platinum electrodes were used to test for the function of the axons contained in the

bundles. The stimulus was the shocks from the secondary of an induction coil, occurring at the rate of about 50 per sec. A bundle suspected of containing the desired fiber was raised upon these electrodes and the claw was observed as to whether or not stimulation caused a contraction.

In this manner the axon and its other large neighboring fibers were isolated. Usually the large motor fibers of the claw occur in two groups, the fibers of each group being closely connected along their length by connective tissue. Removal of this tissue was, indeed, the most delicate step, for the fiber had to be prepared free for at least 20 mm. without injury at any point. In the early part of the work this was not always done; instead a "bridge" preparation was made, the axon being single only at one point just distal to where the stimulus was to be later applied. However axons prepared in this manner seldom survived long. Hence the main necessity for long lengths of isolated fibers, but it will appear later that there are other important reasons as well.

With practice the preliminary isolation could often be made in a few minutes. The complete isolation of the fiber from all other tissue and mounting it in the lead off chamber required more time (30 minutes or more). The fiber, together with a short length of nerve attached at either end for ease in clamping, was then removed from the claw and transferred to the moist chamber for leading off action potentials.

This was accomplished by stretching the fiber out on a small bent plate of celluloid dipped into the Petri dish, and then quickly transferring it to the moist chamber, which had been previously filled with solution. There it was floated off the plate and its ends clamped. After the fiber had been properly adjusted in respect to the electrodes, the solution was pipetted out, leaving the axon either stretched across the electrodes in the first arrangement of the chamber or lying in the trough in the second (see II, B). A cover was sealed to the top of the chamber with vaseline. The fiber was then ready for the experiment.

The smaller nerve fibers, of less than 10 μ diameter, were almost impossible to prepare for long distances because of the close attachment of connective tissue. Furthermore, there was no way, during preparation, of checking whether they still conducted impulses, since they did not cause a contraction. For these reasons it was impractical to use them. Inhibitor fibers were used in a few instances, but since their action potentials showed no qualitative differences from those of the motor axons, the latter were used almost exclusively because of greater convenience in preparation.

B. Description of lead off chamber

It was desirable to construct the lead off chamber of a material possessing the following properties: (1) the material must be a good insulator electrically; (2) it should be capable of being easily and accurately worked and should be sturdy; (3) it should be transparent for ease in orienting and measuring the fibers; and (4) it should not liberate substances toxic to the preparation. There are a number of materials, transparent plastics, which satisfy the first three conditions; these materials include cast and pressed Lucite, cellulose acetate and nitrate, cast Bakelite, and Catalan. Their suitability in respect to the fourth condition was tested by extracting milled shavings from each of the above substances with physiological solution at 50° C. for 24 hours. The extracts were placed in a small celluloid chamber through which several prepared nerve fibers passed. Since these fibers still innervated some of the muscles of the claw, conduction of nerve impulses through the extract could be conveniently tested by stimulating the fibers, proximal to the chamber.

Of all the substances tried, cast Lucite was the most acceptable. Conduction through its extract was maintained for one and a half hours, and it met the other conditions satisfactorily. Therefore this methyl-methacrylate resin, manufactured by Du Pont, was used for constructing the chamber and, especially, the trough described under (2) below.

It may be noted at this point that freshly killed nerve tissue does not seem to be poisonous to living fibers when placed in close contact in solution. The chamber used in testing the plastic extracts was packed with lengths of nerve which had been thoroughly crushed; the prepared fibers were led through the chamber and covered with more crushed nerve. Conduction of impulses through this mess was unimpaired after more than an hour.

(1) The chamber for leading off in moist air

The chamber consisted of an outside box 27 mm. wide by 83 mm. long and 15 mm. deep, cemented to a larger sheet of Lucite and open at the top and one end. Into this open end a second similar but smaller U-shaped box of the same depth was fitted so that it blocked the open end of the first box. This second piece was held and its position varied by means of a screw threading a block mounted on the base of the apparatus. Electrodes ran from one side of the boxes to the other at a level 9 mm. above the base, and were connected to binding posts mounted on the base. The ends of the fiber were clamped at the center of the ends of the two boxes. Therefore the nerve fiber lay perpendicularly across the electrodes. A photograph of the chamber with a nerve bundle in place is shown in Figure 1.

The pair of stimulating electrodes was always at the end of the outer box, which will be called the "proximal" end, while the lead off electrodes were then "distal."

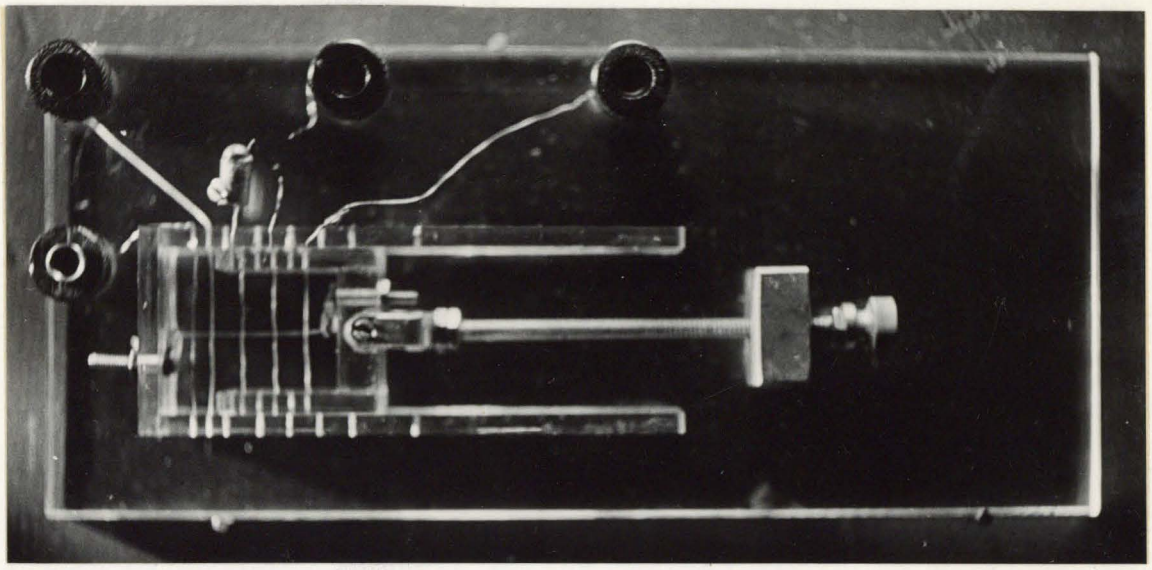


Figure 1. A photograph of the chamber as arranged for leading off in moist air. The two electrodes running across the box at the left are for stimulation. The electrode at the extreme right is the distal lead off, while the other two are optional for the proximal lead off. The screw arrangement at the right is for varying the length of the chamber. A lucite cover fits on the top when in use.

The electrodes were, at first, silver wire of .015" diameter, plated with silver chloride. These were later discarded, for reasons to be noted below, and platinum wire of .010" substituted. The fibers were held at their ends by small clamps made of fine rubber bands stretched about a small curved piece of celluloid. The clamps were attached to the box by fine brass screws.

It became apparent that the method of mounting the fiber and its orientation to the electrodes was extremely important in determining the form of the electrical record. While the arrangement described above gave results of value, it was later abandoned for one which allowed the leading off process to be more satisfactory.

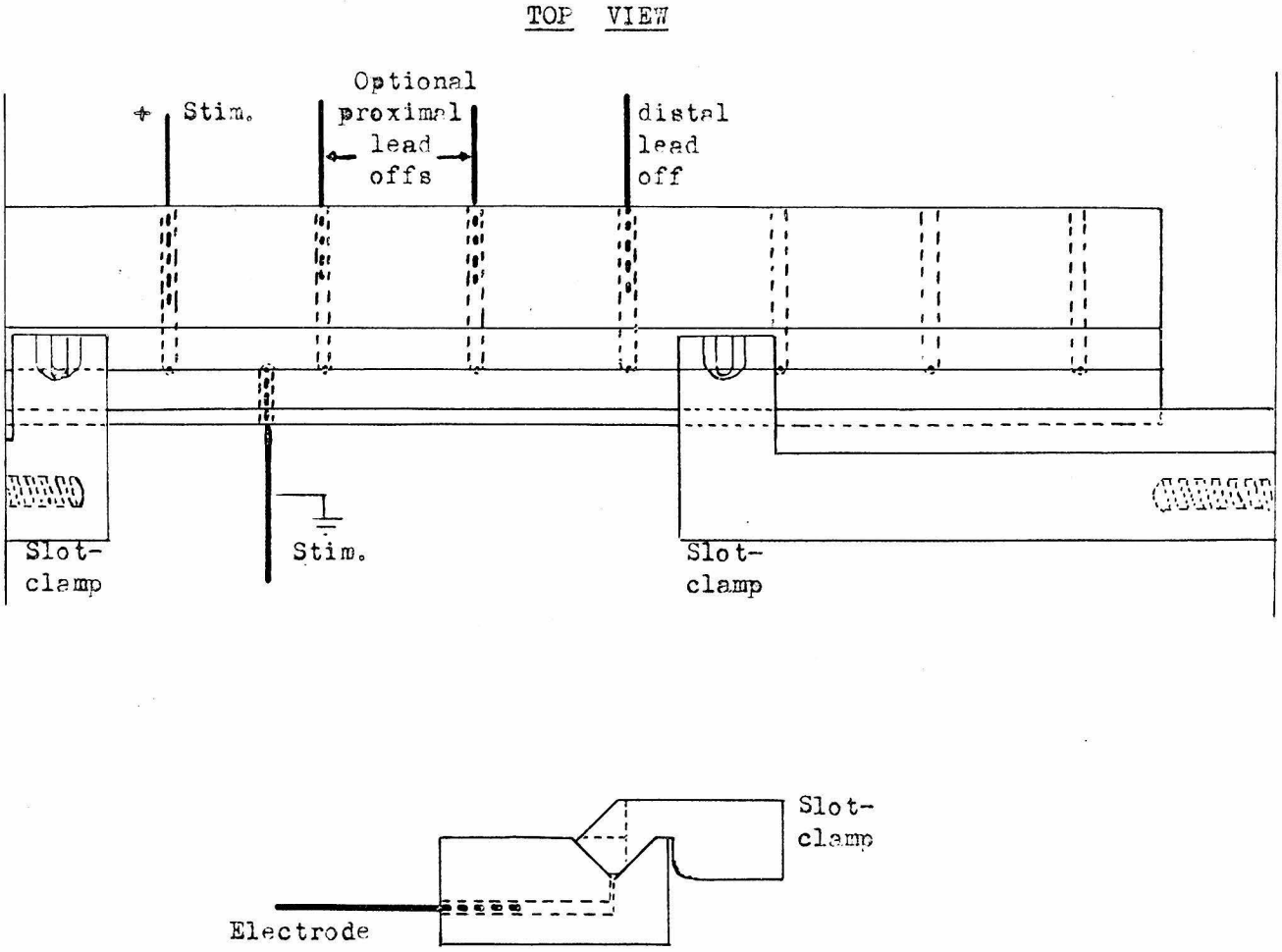
(2) For leading off in solution

This latter method involved the use of a V-shaped trough, which was fitted into the chamber, and in which the fiber lay surrounded by a small quantity of physiological solution. The trough with a 90° angle at its base was cut into a 1/4 inch strip of Lucite. A series of fine holes were drilled from the side of the strip to a point about 1/2 mm. below the angle of the trough; these were met by a corresponding series of smaller holes (90 μ diameter) bored from the base of the trough with a fine needle. This formed a system of fluid leads to the nerve fiber, for these tubes were filled with physiological solution which was in contact with wire electrodes inserted a short distance into the holes at the side of

the strip. This arrangement has the advantage of removing the fiber from contact with any metal and allows the use of non-polarizable electrodes, such as silver-silver chloride.

The trough was made 40 mm. in length. The fibers were held at their ends by "overhead" V-shaped blocks of Lucite into which small slots were cut. These blocks were mounted on the ends of the inner and outer boxes, respectively, in such a way that the angle of the V could be swung down into the angle of the trough, making a snug fit. The bundles of nerve left at either end of the fiber were wedged securely into the slots, resulting in the fiber lying evenly along the bottom of the trough when the blocks were swung into place. Since the distal block was mounted on the movable inner box, it could be shifted along the trough. This allowed the fiber to be adjusted to any desired length, and also permitted the use of fibers varying from 15 to 32 mm. in length.

Figure 2 gives a top view and cross-section of the trough and shows the relation of the overhead blocks. The position of the stimulating and lead-off electrodes is also indicated. A photograph of the chamber with the trough in place is shown in Figure 3.



CROSS-SECTION of trough through a lead

Figure 2. Top view and cross-section of the trough

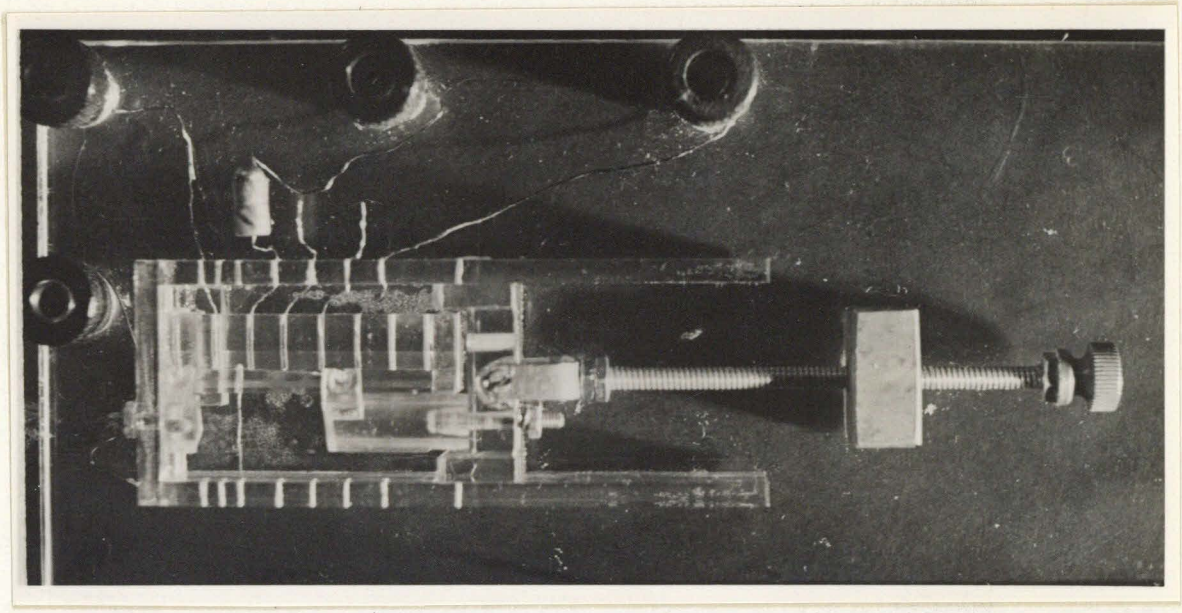


Figure 3. The chamber as arranged for leading off in solution. The trough is seen down the center of the box. The slot-clamps are are raised slightly out of the trough. See Figure 2 for other details.

C. The amplifier, cathode ray oscillograph, and stimulator

The action potentials were led into a five-stage resistance-coupled push-pull amplifier. The frequency response of the amplifier system was down 20% at 10 kilocycles and 35% at 15 kilocycles at the high frequency end and less than 30% at 1/2 cycle per second, referred to the response at 1 kilocycle. This range was ample to record faithfully the form of the led-off action potential. A wiring diagram of the first two stages is given in Figure 4.

The lead-off electrodes were connected to the two input grids, and neither of them was grounded. It will be noted that no grid leaks were used on the first stage. They were unnecessary because the grids were prevented from flating by being connected to the grounded stimulating electrode by means of the fiber and its surrounding solution. Grid leaks were shunned to avoid the possibility of any current flow along the axon. The possible current flow, from the grid through the capacity to ground in the tubes and back through the fiber, that could be caused by the action potential would amount to less than 10^{-10} amperes; therefore there was no danger of the fiber's subjecting itself to electrotonus.

Good insulation between the electrodes was insured by care in the construction of the chamber and trough, and particularly by coating the electrodes with glyptal and paraffin at the points where they entered the chamber. The leading off of an appreciable action potential from an idle electrode due to its capacity to ground was quite impossible with this set-up (see III, B (2)).

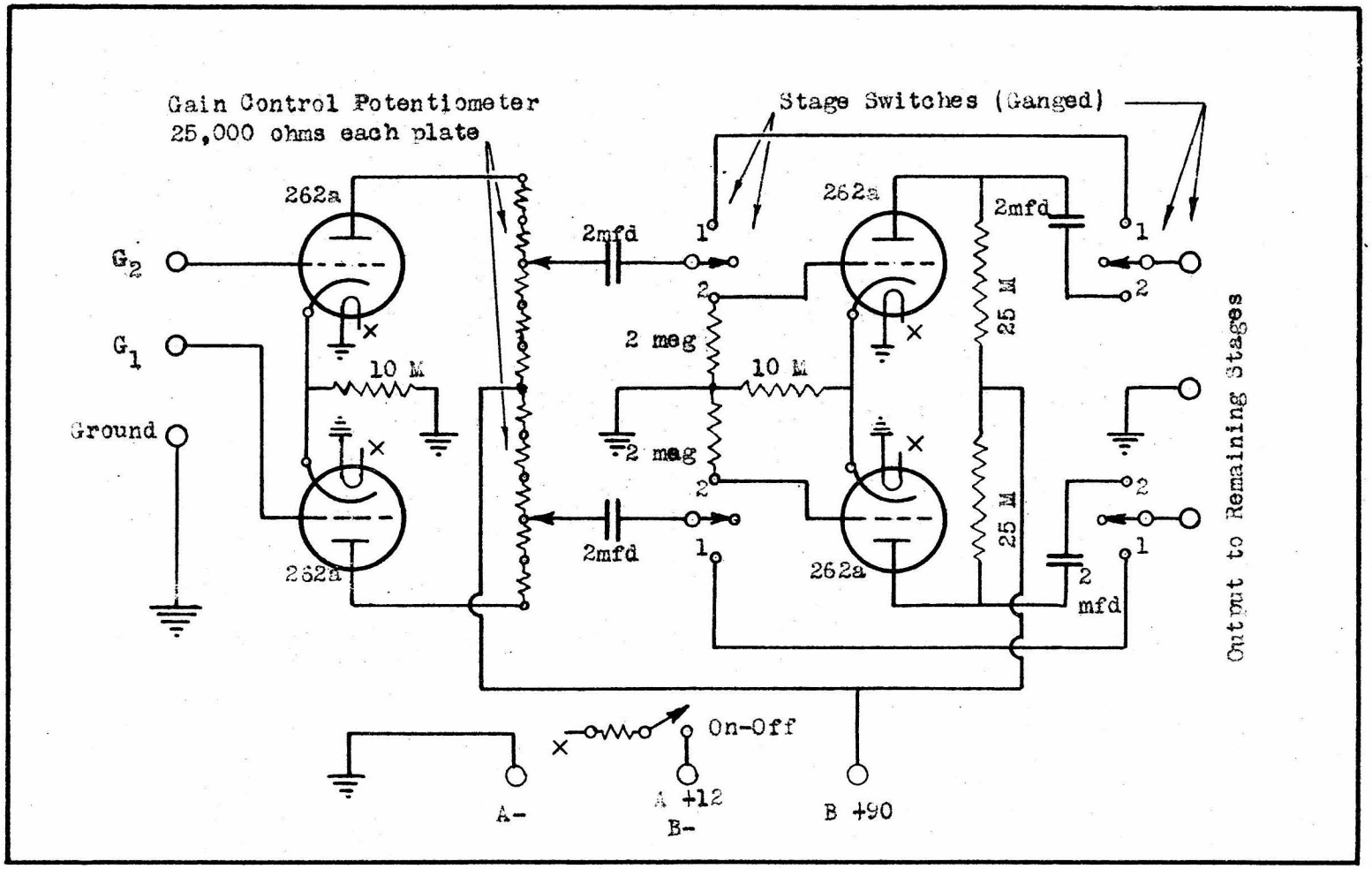


FIGURE 4.

Wiring Diagram of First Two (Differential) Stages of Amplifier.

The amplifier operated differentially; that is, it responded only to differences in the potential of the two lead off electrodes. The response to a potential applied between both grids shorted together and ground was about 0.5% the response when the same potential was applied across the grids. Such an amplifier is essential in this type of work for a number of reasons. Since it is necessary to ground one of the stimulating electrodes, a single-ended amplifier would lead off a large stimulus artifact, whereas in the present case the stimulus tends to affect both grids equally and this artifact is minimized. There are several other very important considerations for the use of a differential amplifier which had best be discussed later (see IV, B).

The response of the amplifier to 1 mv. between the grids was 1 cm. on four stages and 5.5 cm. on five stages. This amplification could be further varied by means of a gain control consisting of two ganged potentiometers placed in the plate circuits of the first stage.

The signal was fed into a cathode ray oscillograph with an accelerating anode potential of 3000 v. The sweep circuit, producing the horizontal deflection of the spot on the fluorescent screen was constructed to produce an equal deflection per unit of time; in other words, it was quite linear. The vertical deflection caused by the signal fed into the amplifier was also proportional to that signal. Therefore the led-off action potential was not distorted either in respect to time or height.

The screen of the oscillograph was coated inside with a material which produced a highly luminous spot, especially suitable for photographing, when the beam of electrons from the cathode impinged upon it. The horizontal deflection was synchronized to sweep across from left to right 30 times a second. This allowed photographs of the screen to be made with a Leica camera, with the shutter adjusted to 1/30 sec. Using one of the new fast films, very clear records were thereby obtained.

Stimulation was obtained from an oscillator generating rectangular waves. Briefly, the frequency was determined by a thyratron relaxation oscillator which fed into a peaking amplifier. This in turn tripped a multi-vibrator circuit which reblocked itself after a time determined by the setting of the duration control. The output of this circuit controlled the plate of the output tube, the cathode lead of which was inserted into the output potentiometer. This apparatus gave sharply rectangular waves, the frequency and duration of which could be varied over as wide a range as desired.

It was necessary to make a number of minor changes in the electrical equipment to adapt it for the present work. The input cable to the first stage of amplification was removed, and the wires from the lead off electrodes to the grids were made as short as possible to reduce the capacity between the leads. The filament bias resistors were increased to produce differential amplifier operation. Also a more convenient gain control was installed on the first stage.

The stimulator contributed an undesirable AC hum until the heater on the output stage was grounded. The output was also made more reliable by replacing the thyratron at this point with a triode and by rewiring the output potentiometer. In most of the experiments a 0.5 mf. condenser was inserted in the positive lead to eliminate the electrotonic action on the nerve of a direct current component in the stimulus. The stimulator was altered so that it could be synchronized to frequencies that were multiples or sub-multiples of 60 cycles. Thereafter it was set synchronous with the sweep circuit of the oscillograph, usually at 30 per sec.

III. Results

A. From leading off in moist air

(1) The action potential

When leading off was accomplished with the method first described, with the fiber suspended across the electrodes in moist air, the resulting action potentials were very large. The spike, in the case of the motor fibers of Cambarus, often ranged from 5 to 25 mv. In the larger fibers of Panulirus, the rock lobster, it could even amount to 50 mv., corresponding to what Hodgkin found in Carcinus. However it was noticed that the height of the spike under these circumstances was rather variable; furthermore the records were seldom simple but, instead, beset with complications which will be discussed presently. No correlation between fiber diameter and action potential amplitude could be made except the qualitative one that the larger the fiber, the greater the spike height.

On the other hand, the duration of the spike was usually fairly constant at about 1 msec., the ascending phase requiring about 0.4 msec., while the descending part took the remaining time. The spike dropped sharply toward the base line, and was followed by a very small negative after potential lasting about 2 msec. No positive after potential was ever observed. Therefore when leading off took place under favorable conditions, the action potential was simple, negative and monophasic. It should be stated here that

monophasicity, or the recording of the potential from one electrode only, was accomplished in all experiments by killing the fiber—by crushing at a point just prior to the distal leading off electrode.

Figure 5 is the record of an action potential obtained from the fiber producing abduction of the claw in Cambarus. I believe that this record gives a fairly accurate picture of the potential changes that occur at a given point on the surface of a crustacean single fiber when this method of leading off is used.

The velocity of conduction was measured by leading off at two points 5 to 10 mm. apart. The action potential at point A was recorded, then at point B, followed by another measurement at point A. The same strength and duration of stimulus was used in all three instances. The elapsed time between beginning of the stimulus artifact and the peak of the spike was evaluated from each of the three records. These times were required to check in the first and last case. The distance between A and B divided by the time required for the impulse to travel between these points, obtained by subtracting their "latent periods," gave the velocity. In the various motor fibers of Cambarus this velocity was found to range from 3.0 to 3.5 msec. at 20° C.; see Table III. Since the duration of the action potential is a bout 1 msec., the wave-length is about 3.0 to 3.5 mm.

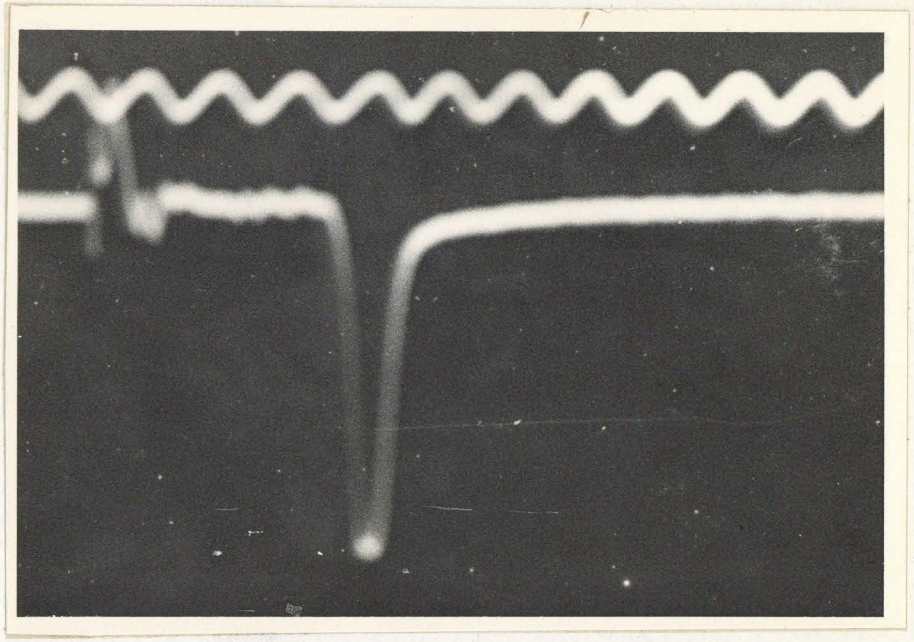


Figure 5. An action potential of the axon causing abduction of the claw in Cambarus. The proximal lead off electrode was 12 mm. from the stimulus, the artifact from which is seen at the left. Time is 1 msec.

(2) Objections to this method

There were many objections to this method. First, metallic electrodes caused damage to single fibers. Silver-silver chloride electrodes were found to be very poisonous. The silver ion was the injuring agent notwithstanding the small concentration possible. This concentration, of course, lies within the range allowed by its very small solubility product. Control experiments showed that a precipitate of pure silver chloride suspended in crayfish ringer will irreversibly abolish conduction within one minute after a drop of this suspension was placed on the surface of a single fiber.

After this factor was discovered, platinum electrodes were substituted. They were satisfactory for leading off action potentials, but the products of electrolysis appearing at the stimulating electrodes seemed to depress the excitability, for the threshold usually rose.

A second and even greater drawback to the method was the action of surface tension. This factor entered when the solution was pipetted from the chamber after the axon had been mounted. The fiber clung to the fluid while it was being withdrawn. This produced stretching until the restoring tension in the fiber exceeded the surface tension of the solution. Then the axon snapped back to a horizontal position across the electrodes. However, since this stretching was not uniformly applied along the length of the fiber, a flow of its viscous contents often occurred. Irregularities in

the diameter of the axon thus arose and it will be shown later that these variations were in part responsible for the spurious effects encountered in the electrical record. See Section IV, B, for a discussion of this. Figure 6 is a drawing of a Panulirus motor axon of 65 μ diameter in which these irregularities are shown. The axons also tended to become flattened over the area in contact with the electrodes.

Variations in the amount of the fluid layer surrounding the fiber were also suspected to be another cause of the spurious electrical effects. These variations arose because small beads of fluid would collect along the length of the fiber. Evidence that such beads would produce large positive artifacts will also be given later. Figure 8 is a record led off from a Panulirus axon which illustrates well the baffling array of spurious electrical effects often encountered and in which the action potential recorded at the proximal electrode is smaller than some of the spurious effects.

Finally, single fibers suspended in moist air seldom survived longer than fifteen minutes. The cause of their death may have been largely the fact that potassium ions were liberated at the surface of the axon whenever a wave of activity passed. Evidence of this liberation of potassium in crustacean nerve has been found by Cowan (1934) who, working with Maia, observed that the potassium concentration inside the fiber is about thirteen times that in the blood, that it is liberated only during activity, and that it reversibly abolishes conduction. In the present case it was discovered

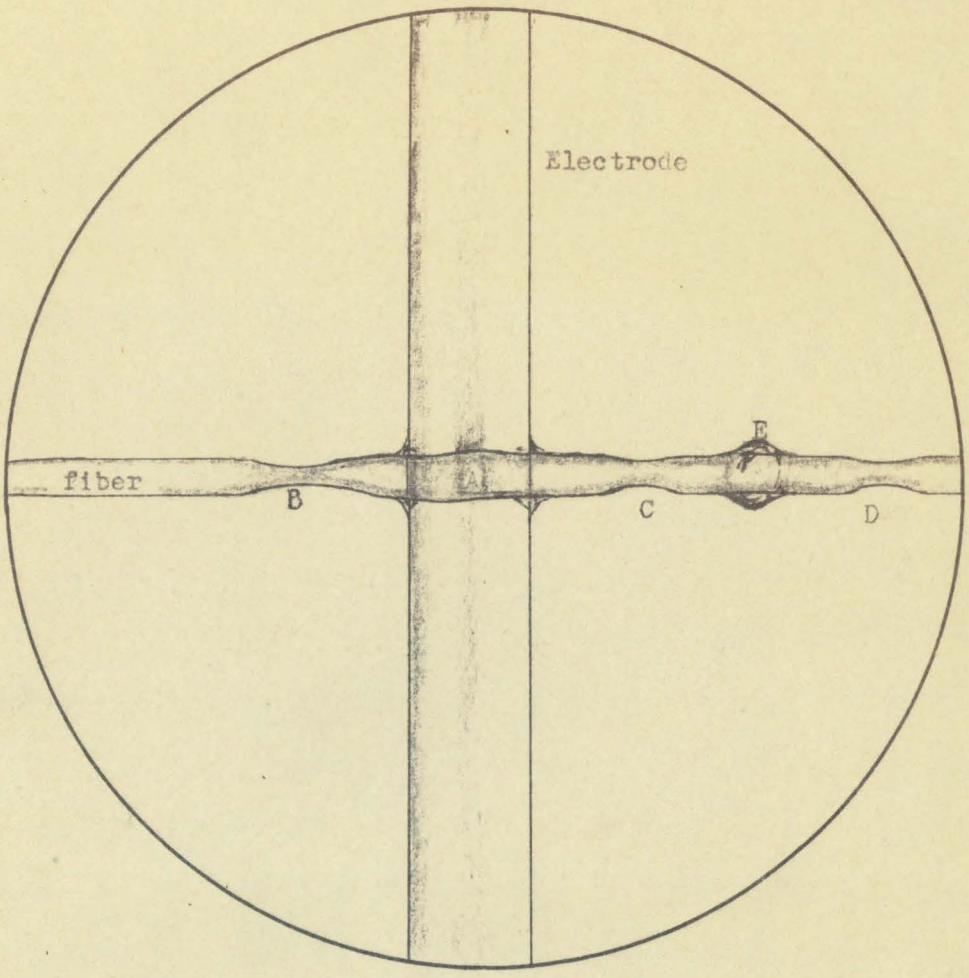


Figure 6. Irregularities seen along the course of a fiber from Panulirus of about 50 μ diameter. At A is seen the flattening over the electrode; at B, C, and D, the constrictions in the fiber; and at E the presence of a bead of fluid. All these would tend to cause a variation in the conductance along the fiber.

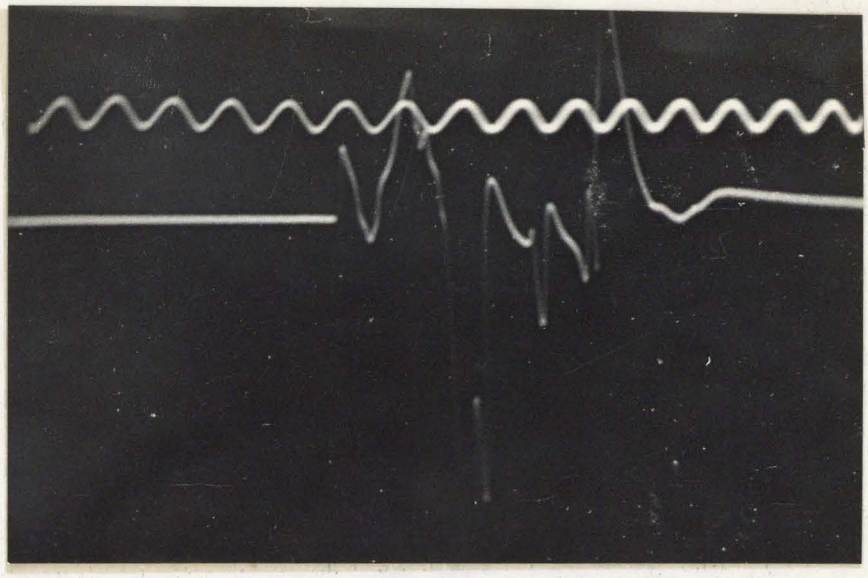


Figure 7. The array of spurious electrical responses often recorded when leading off took place in moist air. The fiber was from Panulirus, of 60 μ diameter; the proximal lead off electrode was 3 mm. from the stimulus and the first negative variation at the left corresponds to the action potential led off there. Time, 1 msec.

that washing the axon with physiological solution would often restore activity. It was also observed that a 0.2% solution of potassium chloride in crayfish ringers would abolish conduction within 2 1/2 minutes.

An attempt was made to overcome these difficulties by surrounding the fiber and electrodes with mineral oil while leading off took place, after Hodgkin's method. This was accomplished by introducing a layer of the oil above the fluid in the chamber and then sucking the physiological solution out from under the oil layer, which was thereby lowered to the level of the fiber. This method did reduce the action of surface tension but had the disadvantage of introducing irregularities in the amount of fluid surrounding the fiber; insulation between the electrodes was rendered less certain; and it was generally messy.

The above enumerated factors and probably others which escaped detection made this whole method very unsatisfactory. Therefore it was abandoned.

B. Results from leading off in solution*

(1) Advantages

The second method, that of leading off in solution, was then adopted. Its advantages may be mentioned at the outset. First, the axon is surrounded by the solution contained in the trough and is therefore in an environment more similar to that

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* All the following results were obtained from the crayfish, Cambarus clarkii.

obtained in the animal. The fiber itself cannot be damaged by the withdrawal of fluid. Second, the use of fluid leads between the metal electrodes and the bottom of the trough removed the fiber from contact with metal, and also permitted the use of non-polarizable electrodes. If care was taken that solution from the fluid leads did not circulate into the trough during the experiment, preparations could easily conduct for an hour or longer, using silver-silver chloride electrodes. Under these conditions, the decline of the spike height under continuous stimulation is very small and the "retention of the action current" (which is really another way of saying that the action potential becomes smaller) found by Levin (1926) and Furusawa (1929) does not occur.

It should be stated, however, that something perhaps akin to the "retention" described by these authors was seen during the late spring and the summer months. Then prepared fibers seldom survived longer than half an hour. Their behavior was interesting though exasperating: the action potential would remain at an almost constant level for 15 to 30 min. and would then begin to decline, invariably starting at the distal end. The distally led off potential would drop out while one recorded more proximally was still declining. Within a few mimutes after this phenomenon started the fiber would become completely and irreversibly inexcitable. A tentative explanation of this effect is that the injury potential occurring at the ends of the fiber, and which is known to be an oxidative process, depletes the fiber's energy reserve at its locus, progressing down the fiber as it does so. Conducting the experiment at a lower temperature was of doubtful benefit; rather the temperature at which the animals themselves were kept seemed to be of importance. This was shown by storing them in an aquarium at 10 to 15° C. for one, or better, two weeks prior to the experiment. Preparations then became as durable as they had been in winter months. That some change occurs in the metabolism of the crayfish during warm weather seems certain, for quick fatigability of another physiological process, that of peripheral inhibition, was noted under similar circumstances (Marmont and Wiersma, 1938).

Furthermore, one did not have to worry about the fiber's liberating of potassium during activity. This was shown by the

control experiment described in the last section where crushed nerve, which also liberates potassium according to Cowan (1934), did not embarrass the function of single fibers in solution even though the damaged tissue was placed in contact with them.

(2) The problem of spurious electrical responses

However, when the trough method was first used, spurious spikelike electrical responses were recorded at points corresponding to the position of idle electrodes if these electrodes were between the two actual leading off electrodes. Indeed, spikes were obtained corresponding to the points where the fluid lead holes were placed even though the metal wire to these leads had been removed, or even if the holes were filled with air! Furthermore, the base line on the screen was greatly disturbed between these spurious action potentials, and positive variations appeared.

There were several possible hypotheses for these effects, namely: insufficient insulation between the lead electrodes with each other and ground, capacity between the electrodes, variations in external conductance along the fiber, and finally, unequal tensions or pressures on the surface of the fiber.

Resistance measurements showed that insulation between the electrodes was sufficiently great so that leading off from an idle electrode was impossible from this cause.

Hodgkin (1938) also had trouble with the leading off of action potentials from idle electrodes and blamed this difficulty on the capacity of the electrode to ground. The possible capacity

between electrodes in the present case was absurdly small, as shown by calculations. This possibility was tested, however, by orienting a platinum wire at different angles to the trough. The wire was held at one end by a glass rod and the other end was just allowed to contact the solution about the fiber. An electrical variation arose at this point, but its magnitude was independent of whether the wire was parallel to the electrodes or at right angles.

It had long been suspected that variations in the conductance of the fluid along the fiber could be the cause of the spurious responses and an analysis was made of how this could occur (see IV, B). To test this hypothesis a finely drawn glass rod with a small rounded point was used to change the amount of fluid at a definitely localized point. The rod was applied by hand, the fiber and trough being observed under the microscope.

It was found that a negative artifact followed by a smaller positive one arose at the point where the glass was being inserted into the solution surrounding the fiber even though it did not make contact with the latter. On the other hand, when the point was being withdrawn a positive potential followed by a smaller negative one appeared at this place. The latter potentials appeared just at that moment when the point had drawn up a small peak of fluid above the surface of the solution before breaking contact with it. It must be understood that the fiber had to be stimulated in order for these effects to occur; furthermore they only occurred if the rod was inserted between the two leads to the grids. The same effect was observed when a fine bundle of myelinated fibers from a

frog was substituted for the crayfish fiber. Figure 8 is a drawing of the position of the glass point in the two cases and the resulting spurious response seen on the oscillograph screen.

Therefore it is really a local variation in conductance that produces these spurious electrical responses. This was further tested in several ways. Small agar-crayfish ringer blocks were made and inserted at a point along the trough. When the action potential reached this place, a positive potential appeared: this was a point of locally increased conductance. Conversely, if the cross-section of fluid about the axon was decreased by local application of vaseline or a drop of mineral oil, a negative potential arose. That the effect was really limited to the point in question could be demonstrated by first killing the fiber just distal to the place in question. No change in the electrical response resulted. If it was killed just proximally, the effect immediately disappeared.

The cause of the spurious responses at the idle electrodes can now be explained. When the electrode holes were bored into the vertex of the trough, a very small mound of material was forced up on either side. This was sufficient to raise the fiber slightly out of the solution which remained in the trough, or at least to decrease the amount of solution present at the site of the electrode. Therefore a negative variation appeared when the action potential reached the idle electrode and this was followed by a usually smaller positive one as it passed this point. When the irregularities were removed from the trough the artifacts no longer appeared at the idle electrodes.

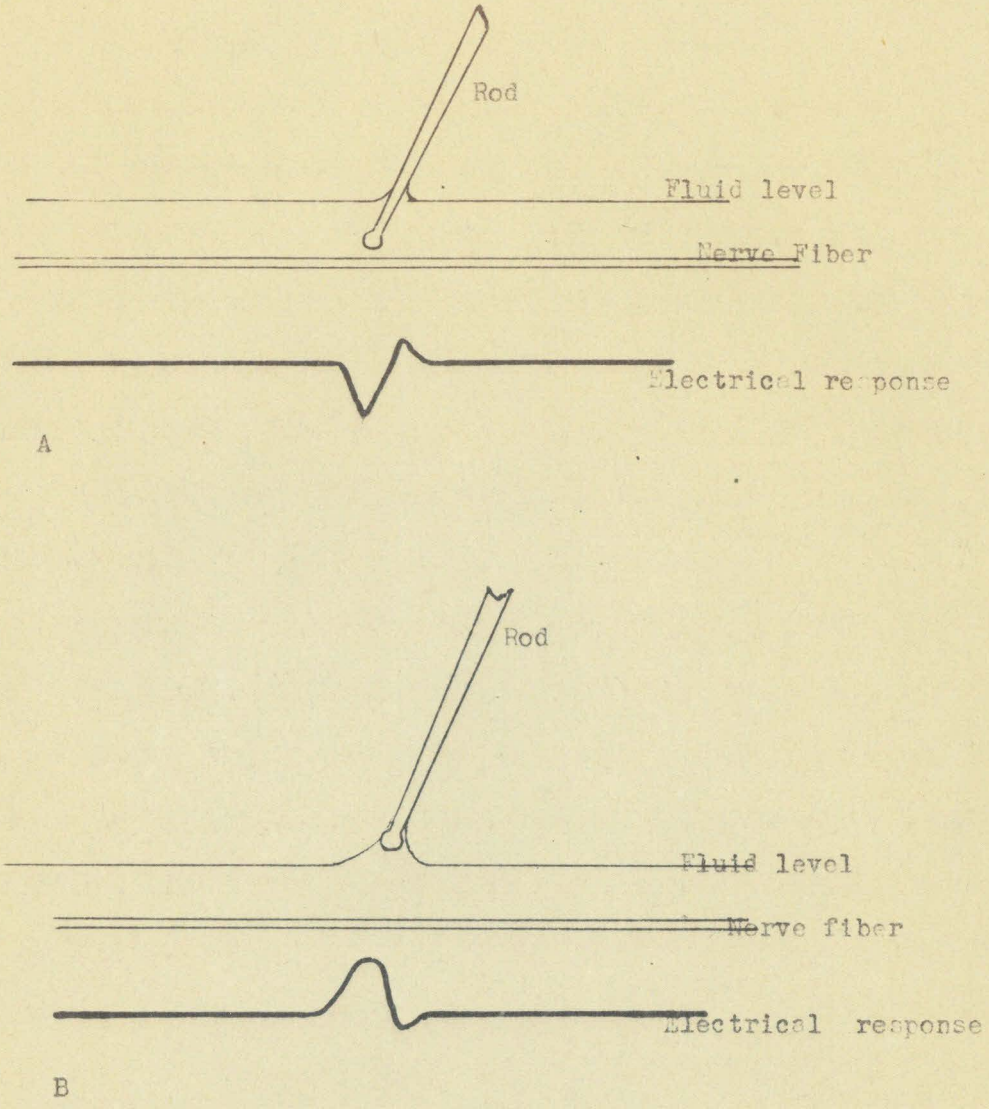


FIGURE 8.

A. Negative artifact resulting from local decreased conductance of region in vicinity of glass rod.

B. Positive artifact resulting from local increased conductance of region beneath small fluid peak.

It has been said that these effects always appeared between the electrodes connected to the two grids, and never between the first grid and ground. This fact arose from the action of the differential amplifier and will be discussed later. Experiments to be described presently show that the external action potential is larger in a region of decreased conductance and smaller in a region of increased conductance. It will then be demonstrated that this will produce a negative or a positive response as the action potential crosses the boundary into a region of lower or higher conductance, respectively.

The local pressure hypothesis no longer seemed very likely. It had been made in the first place because preliminary experiments in which the fiber had been gradually stretched during leading off indicated that the action potential increased with tension. This result was dependent on a change in conductance rather than tension (see III, B (6)). That pressure on the fiber membrane may produce a change in the action potential still is a possibility. However, this would be very difficult to test without altering the conductance of the surrounding medium and the effect would probably be small.

(3) Conditions for obtaining satisfactory records

To obtain satisfactory records it was then necessary to insure that no variations in conductance could occur between the two lead off electrodes. This was accomplished by doing two things.

It appears that there are two critical factors involved in leading off by this method. First, the size of the pore from the fluid lead to the trough; and second, the amount of solution surrounding the fiber. Not only must the amount of surrounding solution not vary along the fiber or fibers, but these must also be uniform. This uniformity is most readily obtained in single fibers.

The first factor is analogous to that encountered in the recording of sound on film. Light from a fine slit is focused on the moving film and the sound registered by variations in the intensity or width of the image. The finer the image of the slit the more faithful will be the recording. The same consideration will apply here. Another, very important consideration is that the action potential of the fiber will always become smaller (as will be shown in the next part of this section) as it passes the region of increased conductance which must occur at the pore electrode. The change in the action potential may be minimized if the electrodes are as small as possible, though these will, in practice, be larger than the diameter of the fiber.

(4) Experiments in which the conductance about the fiber was varied

It was of interest to study the effect of changing the conductance of the solution about the fiber. This could be done by varying the volume of the solution or its conductivity. Both methods were tried. The first, by varying the volume, was achieved by using a number of lids which were beveled to different depths so that each

First, another trough was built on the same design but with greater care being taken that there were no irregularities in its walls. Second, a "lid" was made to fit into the trough. This lid consisted of a rectangular strip of Lucite with one of the long edges beveled at 45°. The beveled edge was fitted into the angle of the trough after the nerve fiber had been set in place there but before the fluid had been withdrawn from the chamber. Therefore the preparation was made to lie in a small, uniform triangular space completely filled with physiological solution. See Figure 12, A, for a drawing of this lid.

Since there was always a thin film of fluid between each side of the lid and the corresponding side of the trough, it was important that the lid did not cover the stimulating electrodes; for the current flowing between the stimulating electrodes upon excitation would then also flow in this sheet of fluid. This current flow would produce a tremendous positive artifact during the stimulus and thus disturb the amplifier response for a few milliseconds. This action is shown diagrammatically in Figure 9. It will be seen that the equipotential lines in this current flow pattern extend over into the region between the leading off electrodes and thus establish a potential difference between the two grids which can be very large compared with the action potential, especially if the proximal lead off point is near the stimulus.

Any variations in conductance along the fiber up to the proximal grid lead cannot induce any difference of potential between the grids, and therefore cannot disturb the record.

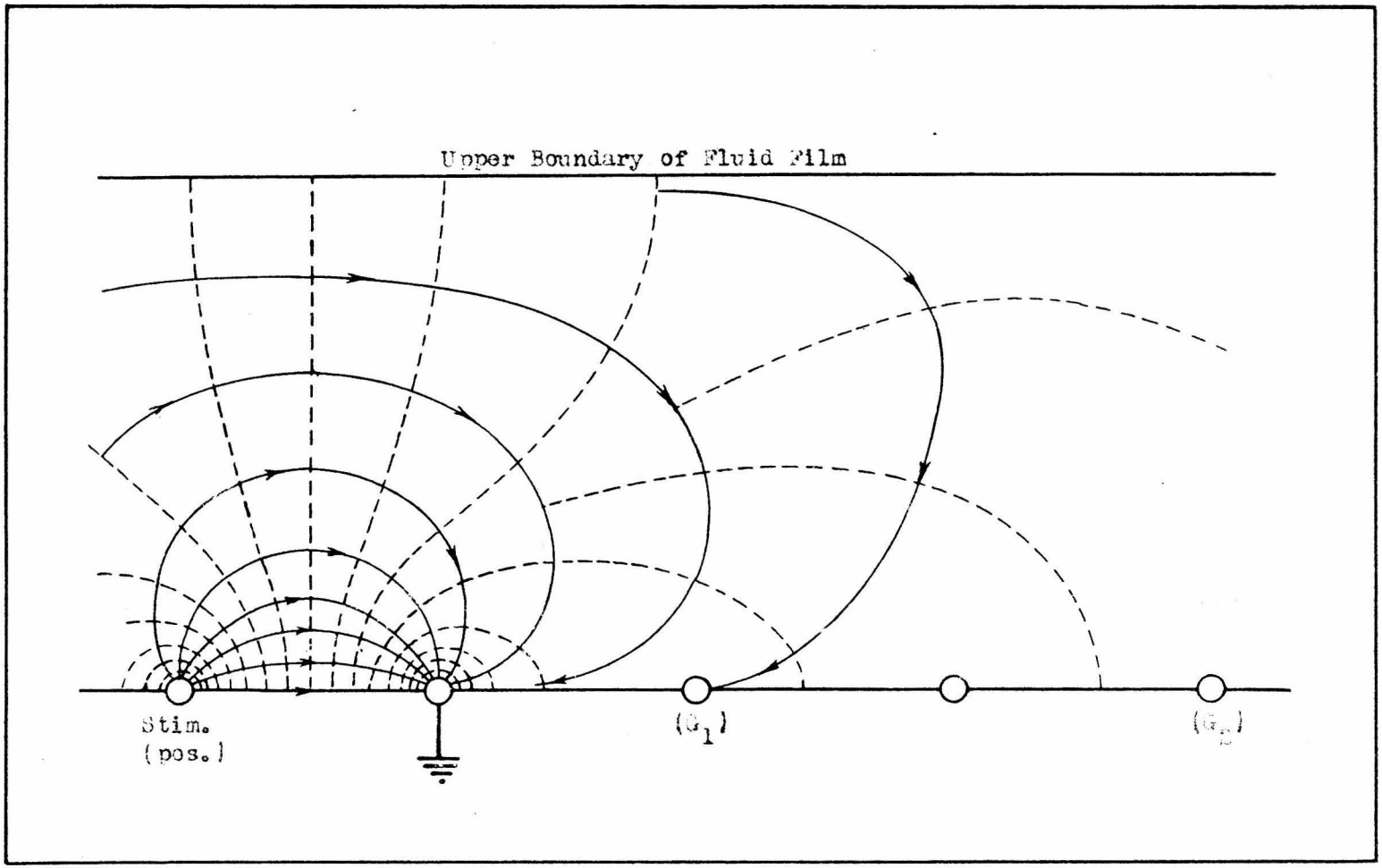


FIGURE 9.

Stimulus Artifact Production due to Current Flow in Fluid Film.

gave a different cross-section of solution, which was, of course, constant along the length of the fiber.

It was found that increasing the cross-section of the fluid had the following principal effects on the action potential: the spike height decreased, apparently according to a hyperbolical relation, the duration of the impulse was shortened, but no appreciable change in velocity was detectable in the range of variation which was practicable. Figure 10 illustrates the different types of action potential led off from one point during the course of such an experiment, and Table I gives the data from these experiments.

There were several difficulties to this experiment. First, it was impossible to have the fluid uniformly placed about the fiber, since this would require that the axon lie in the center of a circular tube. Second, the number of points on any one curve was limited by the fact that changing the lids was a delicate task: when the lid was withdrawn, the resulting circulation of fluid tended to displace and damage the fiber. Several experiments were invalidated for this reason. Platinum lead off electrodes were used so that Ag^+ would not be carried to the fiber by this circulation of fluid.

Figure 11 is a graph of the spike height against conductance, plotted log-logarithmically. It will be seen that the points of each experiment fall roughly along a straight line. This suggests that the relationship is a hyperbolic function, and indicates that the action potential external to the fiber varies with the conductance

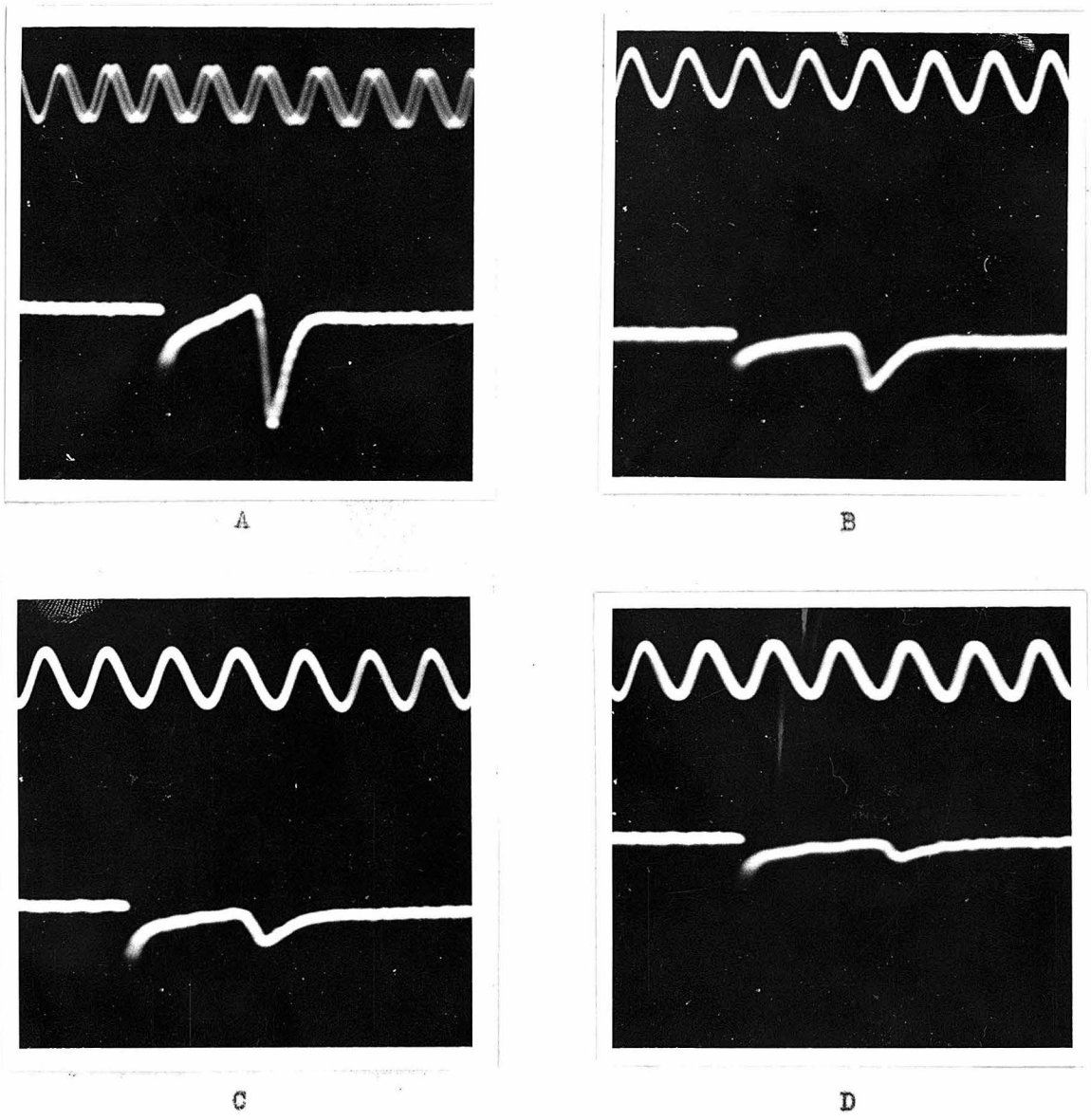


Figure 10. Four action potential records taken from the abductor fiber of Cambarus. The potentials were obtained from fiber at an electrode 7 mm. from the stimulus. In A the cross-section of the trough was .037 mm.^{2}; in B, .137; in C, .209; and in D, .610. Time, 1 msec.

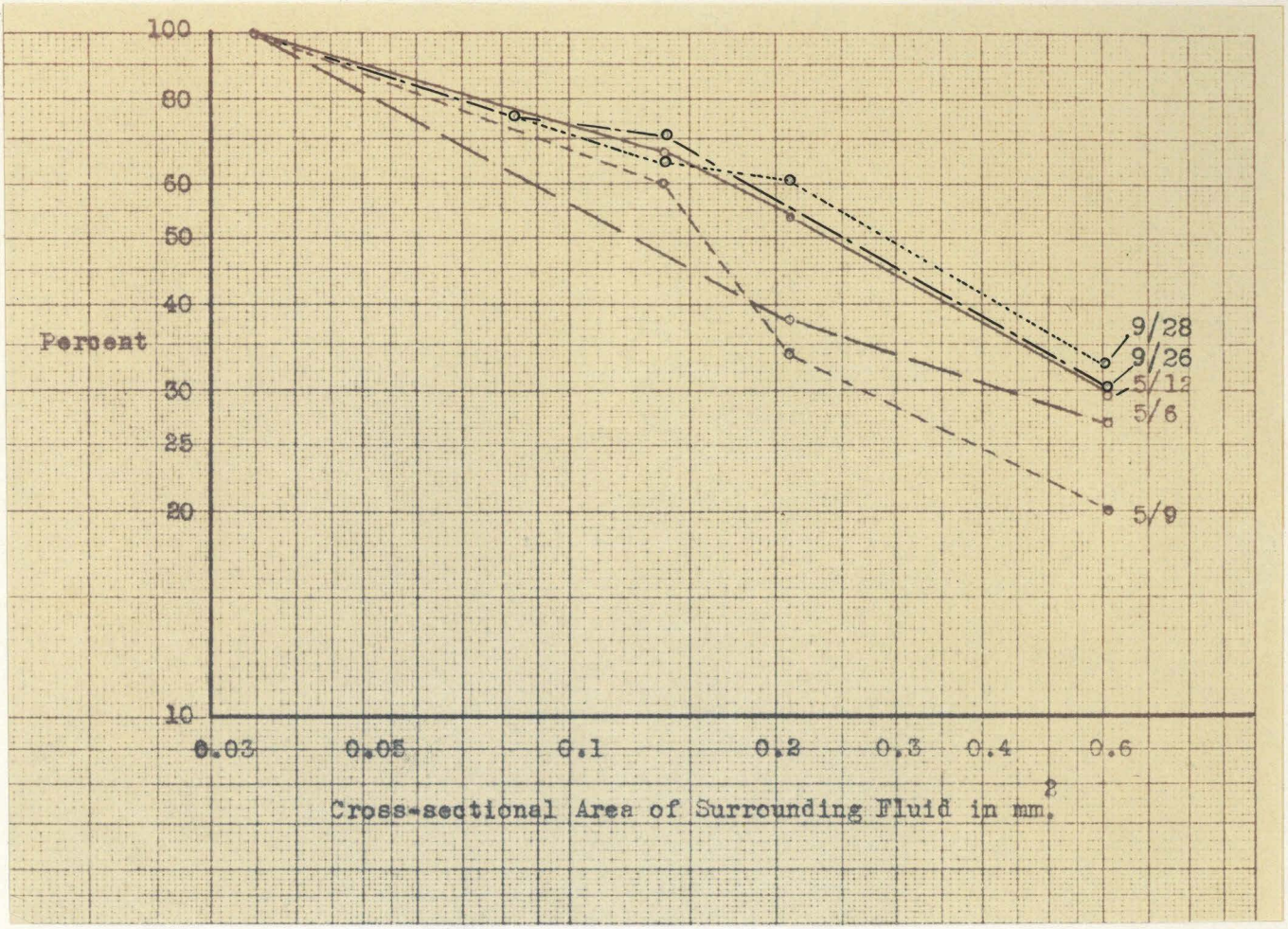


Figure 11. Graph of the data given in Table I. The ordinates represent action potential heights in percentages of the height of that obtained from leading off with the trough of .035 mm.² cross-section. The abscissae represent the trough cross-section corresponding to each point on the curve.

according to such an equation as:

$$(h + a)(c + b) = k$$

where h is the height of the spike, c the external conductance, and a, b, and k are constants.

It will be seen from Figure 10, as well as Figure 5, that the rising and falling phases of the spike are remarkably linear, i.e., are straight lines. This was always the case. The ratio of the duration of these two phases, that of the rising to that of the falling, ranged between 0.4 and 0.7, and did not appear to vary significantly with the conductance. However, the total duration of the spike seemed to decrease with increasing conductance (see Table I). The short negative after potential present when leading off took place in air was usually either very brief (1 msec. or less) or absent entirely.

Changing the conductance by changing the conductivity of the solution surrounding the fiber in the trough was done with the aid of solutions made by mixing the ordinary physiological solution with one having the same osmotic pressure as the former but which was not ionized. Glycine, asparagine, glucose, sucrose, arabinose and urea were among the substances used for the un-ionized solution. Of these glycine, asparagine and arabinose gave the best results; these would probably be less involved in the metabolism of the fiber than the others and therefore would be less likely to depress its function. When the physiological solution was diluted up to

half with the solutions of these substances, the action potentials increased over those in the plain solution by as much as 45 per cent; see Table II.

However, if the proportion of un-ionized solution was increased to three-fourths or more reduction of the action potential was observed; its duration became longer and the conduction rate decreased. Decrease in the latter was seen in cases where the spike was heightened: in the preparation of 6/24 in the Table, the velocity was 5 m. per sec. in physiological solution but only 3 m. per sec. in half arabinose.

Why the greater concentrations of these solutions depressed the action potential is not at all clear. It may be due partly to interference with the osmotic relations of the fiber which would disturb its surface membrane where the action potential is believed to arise, and partly to disturbance of its metabolism. It should be said that the effect of warm weather on the animals, already mentioned, also entered here and made velocity measurements difficult since the fibers conducted their full length for only a short time after they were mounted. The solutions of the other substances all produced smaller action potentials, even in half proportion. Glucose, which is probably metabolized, was the worst of the lot.

If the assumption be made, and it seems fair to do so, that the only effect of the weaker solutions of glycine, asparagine and arabinose was to lower the conductance outside the fiber, then the larger than "normal" action potentials obtained in the experiments

with these solutions are additional evidence that the height of the action potential is a function of the conductance of the medium surrounding the fiber. To show this was the purpose of the experiment.

Another interesting phase of the effect of conductance on the action potential is the production of the spurious responses already described. Four cases were studied: (1) where the action potential crosses a sharp boundary from a region of lower to one of higher conductance, (2) the converse of this case, (3) where the action potential travels through a region where the conductance sharply increases for a short distance and then sharply returns to its previous value, and (4) the converse of (3). From the results already described and from theoretical considerations, which will be presented in the next section, one should expect a pure positive artifact in the first case, a pure negative one in the second, a diphasic positive-negative one in the third, and a diphasic negative-positive artifact in the last case.

This experiment was easily performed with the aid of special lids, which are pictured in Figure 12. It will be seen there that these lids are milled so that the amount of solution about the fiber sharply increases or decreases as required. Figure 13 presents pictures of the electrical response recorded in each of the instances. The proximal lead off electrode was 2 mm. from the stimulating electrode in each case while the discontinuities were placed 5 to 8 mm. distal, as is noted in the

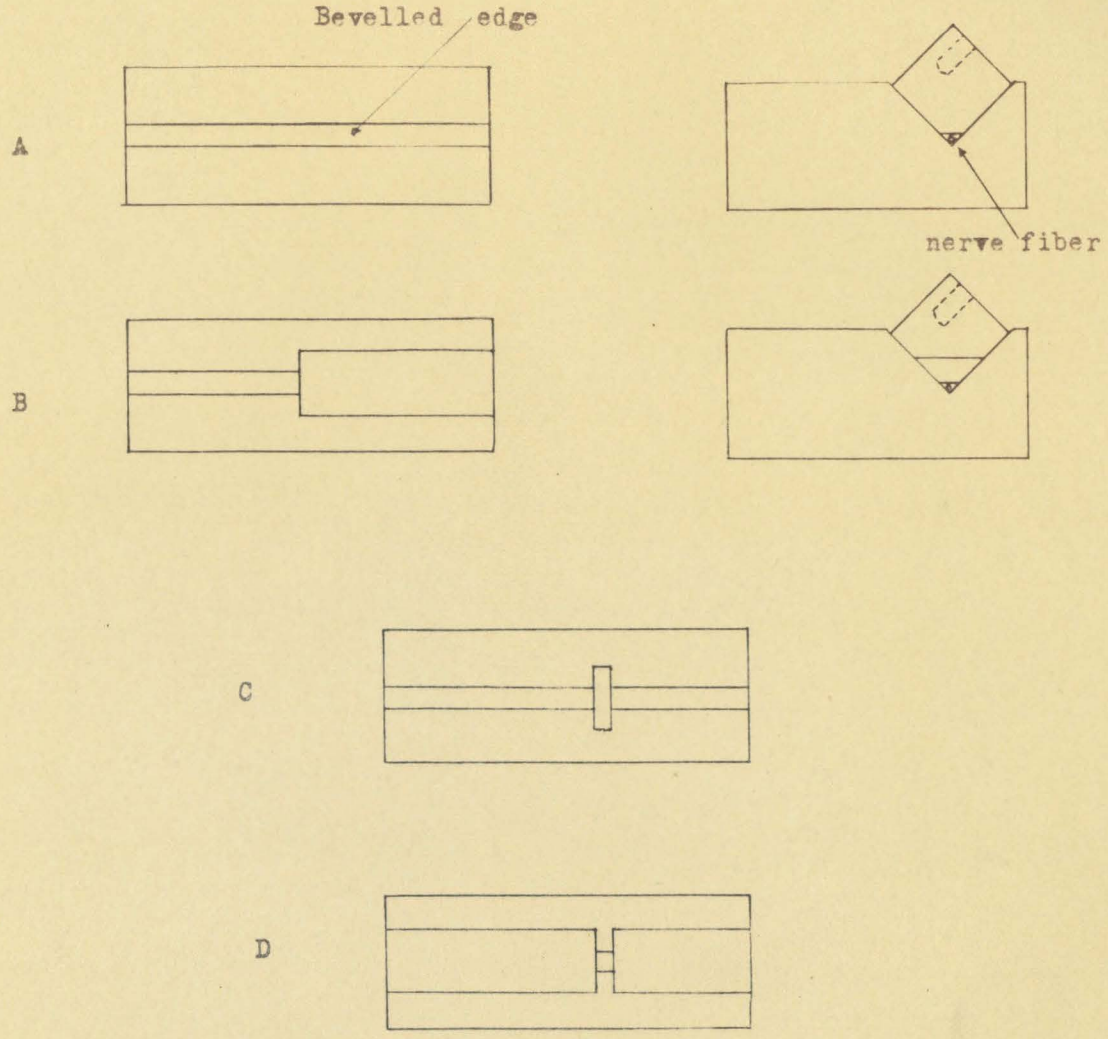


Figure 12. Drawings of trough lids

A. Looking down on the bevelled edge of the ordinary, uniform lid and as placed in the trough for leading off.
B. Lid for production of pure positive or negative "spurious" responses by a sudden change in the conductance.
C. Lid for producing a positive-negative diphasic artifact.
D. Lid for producing a negative-positive diphasic artifact.

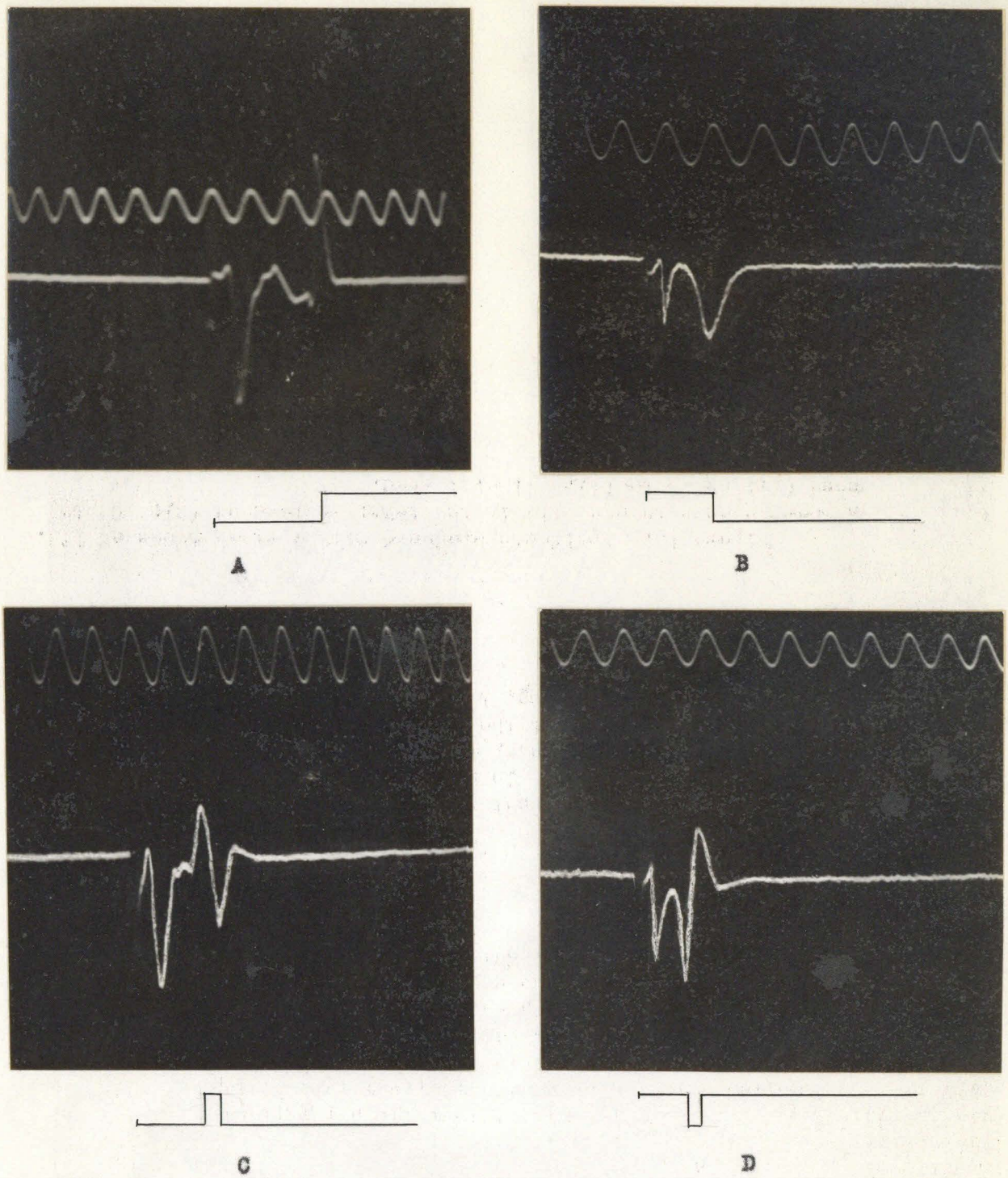


Figure 13. Artifacts produced with various types of lid. A corresponds to case (1) in the text; B, to case (2). C, to case (3); and D, to (4). Changes in conductance are indicated under each picture. C and D are retouched. Time, 1 msec.

legend. In cases three and four the artifacts remained until the length of the region of increased or decreased conductance was cut down to zero, although the response became very small when the region was shorter than a quarter of a millimeter. Such a small increase in conductance as was afforded by an electrode hole (90 μ in diameter) produced no detectable artifact.

(5) The velocity of conduction

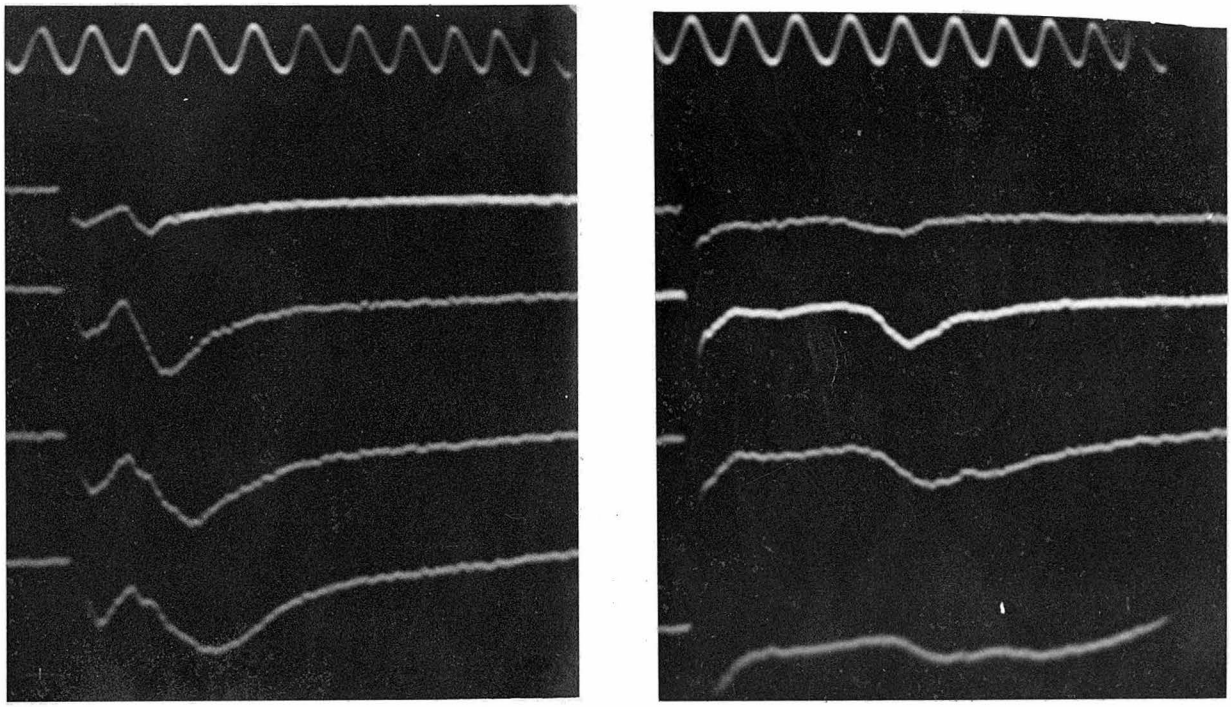
No significant variation in velocity was found except that it was greater when the fiber was surrounded by solution. This agrees with the finding of Hodgkin (1939), who worked with fibers from the shore crab, Carcinus. Larger fibers were also observed to conduct faster. However, the range of sizes of single fibers which were obtainable from one species of animal was not great enough for a significant correlation to be made. Pumphrey and Young (1938), working with cephalopod fibers of 30 to 718 μ diameter, found that in these unmyelinated fibers the conduction rate seemed to vary with the square root of the diameter. Table III lists velocity data from many of the present experiments.

The question as to whether there are fiber groups in crustacean nerves, especially groups corresponding to those found in frog and mammalian nerve has interested a number of investigators; see the introduction. Lullies and Bogue and Rosenberg thought there were at least three groups, which were even designated according to the nomenclature used for myelinated nerve, while Monnier and

Dubuisson and Bayliss, et al, found but two. Wiersma (1933) pointed out that Monnier and Dubuisson's results were very likely due to double discharge of the nerve fibers upon stimulation. Lullies' thresholds for his slow wave were very high, as were those of Bogue and Rosenberg. The latter authors considered the possibility of double discharge and indeed did not deny it.

In the present work the larger fibers have been treated as single axons and need not be discussed further. Fine bundles of sensory fibers of fifty fibers or less were prepared and their action potentials led off. The faster conducting, and hence the larger, of these fibers responded at almost the same threshold as the motor axons; their conduction rate was about 2 m. per sec. When the stimulating potential was raised by fifty per cent all the remaining fibers were probably stimulated, for the action potential was maximal and also rather smooth and prolonged. The velocity of the slowest impulses was about 1 m. per sec.

In order for a second wave to appear in the record it was necessary to stimulate with a much higher potential, up to three times the threshold stimulus. The second wave was never distinct if the leading off was near the stimulus. See Figure 14 for pictures of these action potentials led off at two distances and stimulated at various potentials. In view of the facts that a much greater stimulus is necessary to elicit the second wave and that crustacean fibers are extremely prone to double discharge



A B

Figure 14. Action potentials led off from a bundle of less than 50 sensory fibers. In A the leading off electrode was 2 mm. from the stimulus and in B, 7 mm. The duration of the stimulus was the same in all, but the strength as 2 : 3 : 6 : 10 in each case, reading down. Time, 1 msec.

under such conditions, I should certainly conclude that there are at most two fiber "groups" in crayfish nerve. One would consist of the large motor and inhibitory fibers having a diameter of 20 μ or more, and the other of the smaller, sensory fibers having a diameter of less than 8 μ (for anatomical data, see van Harreveld and Wiersma, 1937).

(6) Stretching experiments

In investigating optimal leading off conditions, the importance of the length to which the fiber was stretched was considered. Therefore a series of experiments were performed to determine the effect of altering the axon's length on its conduction rate and spike height.

When leading off took place in air or in the lidless trough, a marked increase in action current height took place if the fiber was gradually lengthened by screwing out the inner box. This effect, however, was spurious. When the amount of fluid, and hence the conductance, about the fiber was not allowed to vary, no significant increase occurred; in fact, if anything, the action potential tended to become smaller as the axon was lengthened. There was seen a possible, but momentary, increase in velocity in a few preparations. If the fibers were stretched to as much as 130 to 150% of their original length (in the animal), they soon began to lose their excitability, accompanied by a decline in the action potential. This decline was most easily brought about if the fiber was quickly restored to its original length. An increase in length

of 20% or less was quite reversible in its effect and not accompanied by any damage to the axon. See Table IV for data. If a small nerve from a frog or one of the dorsal spinal rootlets from a cat was stretched to an extent allowable for crustacean fibers, death of the bundle soon ensued.

Crustacean fibers are quite elastic and may be reversibly stretched up to 150 per cent of their natural length provided they are not stretched for too long a period. This property distinguishes them from myelinated fibers. Crustacean fibers are always under some tension in the animal, and it may be concluded that, for leading off purposes, it is best to stretch them to approximately their original length.

Table I. Conductance experiments

The results of experiments in which the effect of conductance was studied by varying the cross-section, A, of the trough. "Spike" is the action potential height in mv., a/d is the ratio of the duration of the ascending to that of the descending phase of the spike, duration refers to the duration of the spike.

Date	Preparation		A = .035 mm.^{2}	A = .084 mm.^{2}	A = .137 mm.^{2}	A = .209 mm.^{2}	A = .610 mm.^{2}
5/6/39	fast adductor 40 u	spike	0.63 mv.			0.24 mv.	0.17 mv.
		a/d	0.6			0.5	0.6
		duration	0.8 msec.			0.7 msec.	0.6 msec.
		velocity	5.5 m./sec.			5.2 m./sec.	6.0 m./sec.
5/9/39	abductor 24 u	spike	0.35 mv.		0.21 mv.	0.13 mv.	0.07 mv.
		a/d	0.7		0.6	0.6	0.6
		duration	1.0 msec.		0.9 msec.	1.0 msec.	0.9 msec.
		velocity	4.5 m./sec.		4.0 m./sec.	4.8 m./sec.	3.6 m./sec.
5/12/39	fast adductor 42 u	spike	0.61 mv.		0.41 mv.	0.33 mv.	0.18 mv.
		a/d	0.4		0.5	0.4	0.4
		duration	0.9 msec.		0.8 msec.	0.8 msec.	0.6 msec.
9/26/39	fast adductor 39 u	spike	0.70 mv.	0.54 mv.	0.49 mv.		0.21 mv.
		a/d	0.5	0.5	0.5		0.5
		duration	0.7 msec.	0.8 msec.	0.8 msec.		0.8 msec.
9/28/39	fast adductor 44 u	spike	0.75 mv.		0.51 mv.	0.46 mv.	0.25 mv.
		a/d	0.5		0.6	0.6	0.6
		duration	0.6 msec.		0.6 msec.	0.5 msec.	0.5 msec.

Table II. Conductance experiments

The results of experiments in which the effect of conductance was studied by changing the conductivity of the solution bathing the fiber in the trough. Under "Solution" is indicated whether it was the ordinary physiological one that was used, or the proportion with which the physiological solution was diluted with the designated isotonic, un-ionized solution. The spike heights are given in per cents of the initial one in each case.

Date	Preparation	Solution	Spike height, per cent
6/17/39	fast adductor	physiological	100
		1/2 glycine	108
		4/5 glycine	48
		physiological	40
		" (10 min. wait)	48
6/19/39	fast adductor	physiological	100
		3/4 glycine	33
		physiological	73
		1/2 glycine	88
6/21/39	slow adductor	physiological	100
		1/2 asparagine	120
		1/2 sucrose	77
6/21/39	fast adductor	physiological	100
		1/2 glycine	145
		1/2 " (10 min. wait)	140
		1/2 urea	65
		1/2 urea (10 min. wait)	45
6/24/39	slow adductor	physiological	100
		1/2 arabinose	120
		3/4 arabinose	35

Table III. Velocity measurements

The velocity obtained from different fibers when stretched to the length they possessed in the animal, under different conditions of leading off. "A" refers to the cross-sectional area of the trough.

Date	Preparation	Diameter μ	Velocity m./sec.	Excitation time msec.	Leading off conditions
3/8/39	Cambarus abductor	30	3.1	0.5	Moist air
3/11/39	Cambarus fast flexor	35	3.3	0.4	Moist air
4/13/39	Panulirus fast adductor	91	8.3	0.1	Trough, without lid
4/13/39	Panulirus slow adductor	56	10.0	0.2	Trough, without lid
5/1/39	Cambarus fast adductor		3.8	0.2	Trough, A = .209 $mm.^2$
5/6/39	Cambarus fast adductor	40	5.5	0.4	Trough, A = .035 $mm.^2$
			5.2		.209 $mm.^2$
			6.0		.610 $mm.^2$
5/7/39	Cambarus abductor	24	4.5	0.5	Trough, A = .035 $mm.^2$
			4.0		.137 $mm.^2$
			4.8		.209 $mm.^2$
			3.6		.610 $mm.^2$
5/12/39	Cambarus slow adductor	28	3.1	0.6	Trough, A = .035 $mm.^2$

Table III. Velocity measurements, continued

Note: The following measurements were all obtained from Cambarus, and the leading off was all carried out in the trough, with a fluid cross-section of 0.084 mm.^{2}

Date	Preparation	Diameter u	Velocity m./sec.	Excitation time msec.
7/18/39	slow adductor	30	3.9	0.5
9/6/39	slow adductor	24	4.0	0.3
9/7/39	slow adductor	28	4.2	0.6
9/9/39	fast adductor	40	4.2	0.2
9/12/39	fast adductor	—	8.0	0.2
9/16/39	fast adductor	45	8.2	0.2
9/18/39	sensory bundle of about 50 fibers			
	fastest	8 (?)	2.0	0.2
	slowest	1 (?)	1.0 approx.	—
9/29/39	sensory bundle of about 30 fibers			
	fastest	8 (?)	2.5	0.2
	slowest	1 (?)	1.5 approx.	—

Table IV. Stretch experiments

The results of experiments in which the effect of stretching single fibers was studied. "Length" refers to the length of the fiber in mm., and "spike" to the height of the action potential in mv.

Date	Preparation	Length mm.	Velocity m./sec.	Spike mv.
5/1/39	fast adductor	20		0.43
		21.5		0.43
		25		0.31
5/1/39	fast adductor	20	3.8	0.46
		27	4.1	0.40
		20	1.8	0.30
5/3/39	fast adductor 39 u	21		0.63
		10' wait		
		21		0.49
		23		0.60
		10' wait		
		23		0.60
		26		0.54
		10' wait		
		26		0.54
5/12/39	slow adductor 28 u	20	3.1	0.31
		22	3.6	0.31
		20	3.1	0.31

IV. Discussion

A. The polarized membrane theory of the action potentials

The most widely accepted view of the nature of action potentials, and for that matter, most bio-electric potentials, is that they arise from the presence of electrically polarized membranes. These membranes possess an active portion a few molecules thick, believed to consist of lipoid molecules oriented normally to the surface. This layer is, at rest, usually charged negatively on the internal side and positively on the external. Any action which will increase the electrical permeability sufficiently between these charges will cause excitation, during which there will be a current flow from the outside of the layer to the inside. This current flow will increase the permeability of adjacent regions and thus spread in a wave. The size of the wave or impulse at any point will depend only on the conditions which obtain there.

After the impulse has passed over a region, it gradually recovers from its depolarized condition by restoration of the electrical orientation of the layer. Energy is required both for the recovery and the maintenance of the membrane. This energy is obtained, at least in part, from materials contained in the axis cylinder of the fiber, in the case of nerve. Otherwise the function of the axis cylinder is structural and serves as an internal conducting medium. An external conducting medium would also seem to be essential, even if it be contained in a thin layer of connective tissue on the surface of the membrane.

Bio-electric potentials are most clearly manifested in nerve, muscle, in certain large single plant cells - algae such as Valonia, Nitella, Chara, etc., and in the sensitive plant, Mimosa. In all these objects action potentials can be excited. In most plants, in the skin from animals and in animal embryos, inherent or constant potentials can be obtained between certain points in or on the surface of the tissue; these potentials, however, are not of immediate interest in the present work.

There is at present much evidence supporting the "polarized membrane" theory of the action potential given above. Osterhout's work on large algae cells has definitely shown, by placing one micro-electrode into the protoplasm of the cell and another on the surface, that the surface membrane is the site of a potential difference. See his review (1936) for references to his extensive work in this field. Since the time of Bernstein, in 1871, the injury potential of nerve and muscle has been generally considered to be an expression of the potential difference existing across the surface membrane of the cell. Here, of course, the lead to the interior is by way of the injured part. This view is strongly supported by the fact that the potential is readily altered by treatment of the uninjured part with chemicals, heat and cold, while such treatment at the damaged region is of little effect.

Another important support of the membrane theory is the evidence that an external conducting medium is used for conduction of the action potential. In Nitella, Osterhout and Hill (1930)

found that the action potential could be conducted over a spot killed with chloroform by means of a salt bridge. Hodgkin's (1939) evidence that the nerve impulse is conducted at a faster rate in solution than in air also points to the importance of the external conducting medium. In the same paper this author states that the action current in Carcinus fibers is able to pass a narrow inactive region if this region is short-circuited out with metal.

The variations in the action potential with changes in the conductance outside the fiber reported in this thesis is again strong evidence that the action potential is a membrane mechanism.

In the iron wire model of nerve the wave of activity is conducted at a slower rate if the wire is confined within a narrow glass tube and thus exhibits a phenomenon similar to that described for nerve (see Lillie, 1936). The point of this is that this model also depends on surface activity. Another interesting effect shown by the iron wire occurs if short lengths of glass tubing are threaded along it, because then the wire is excited by currents eddying around the outside of the tubes and conduction is increased in rate. From this behavior, Gerard (1931) has suggested that the faster conduction rate observed in myelinated fibers depends on a similar mechanism, the re-excitation occurring from node to node. There is some evidence (see Erlanger and Blair, 1935, and Tasaki, 1939) from experiments on blocking the internodes, that conduction really proceeds in this way in myelinated fibers possessing nodes of Ranvier. However, the behavior of fibers in the central nervous system, where it is believed that nodes are absent, should also be examined.

How the membrane potential is set up and maintained is at present a theoretical matter. Some sort of diffusion potential is favored at present. It is well known that different ionic concentrations prevail in and outside of nerve and muscle fibers and plant cells which show bio-electric potentials. Inside, potassium is present in relatively high concentration while sodium is found in much lower strength. This is a common state of affairs in many other types of cells, but Osterhout believes that the differences in concentration of the highly mobile potassium ion partially explains his plant cell potentials. He has been able, for instance, to reverse the sign of the injury potential by applying solutions of electrolytes in certain concentrations. It has already been remarked that Cowan (1934) found liberation of potassium from crustacean nerve during activity.

The mere difference in ionic concentrations on two sides of a membrane is not enough, however, to explain bio-electric potentials. Osterhout has shown in cells of Valonia and Halicystis that when the same solution is present in the central vacuole and outside the cell a potential difference is maintained between the two. This means that the two membranes bounding the protoplasm of the cell must have different characteristics. These membranes are partly, at least, lipoid in composition; Beutner (1933), in particular, has studied a number of artificial lipoid membranes and has shown how potential differences may arise across them.

Furthermore, there is the problem of how the cell builds

up large concentrations of certain substances in its environment. This too must occur across the surface membrane. Finally, the changes in permeability which accompany an action potential are unexplained. How does excitation increase permeability, and how is it decreased again on recovery? Metabolic events attending the after potentials in nerve would indicate that the main cost of the impulse is paid during this recovery.

In the present discussion the bearing of some of the data presented in this thesis upon the nature and form of the action potential and its relation to the membrane theory will be considered. In particular the influence of the external medium on the leading off of the action potential will be discussed.

B. Explanation of the variation-in-conductance effect

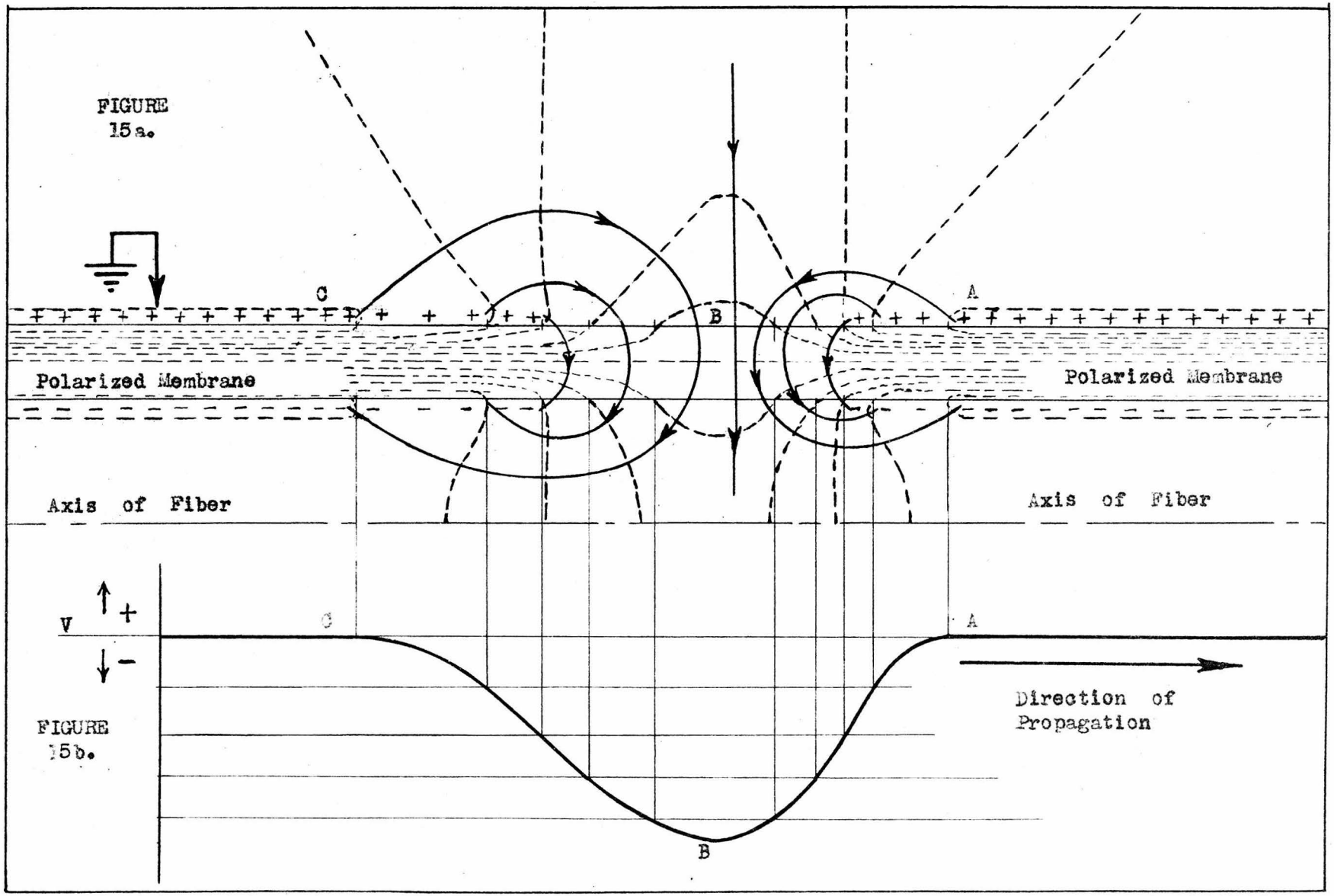
Experiments described in Section III have demonstrated that variations in the conductance of the fluid surrounding that section of the fiber between the two grid electrodes resulted in the generation of either positive or negative deflections in the record at points directly corresponding to the regions along the fiber at which the conductance varied.

As a matter of fact, that this variation could be responsible for the generation of the spurious response was suspected before some of the conductance experiments previously described were undertaken, so that the remaining were performed as verification of the analysis which will now be presented.

First, the mechanism by which the amplifier responds to an action potential starting from the grounded stimulating electrode and traveling down the axon with no external conductance variations present must be considered. It must be borne in mind that since the amplifier is differential in operation, the deflection produced at any instant will be proportional only to the difference in potential between the two grids, and is not in any sense a function of their absolute potential above or below ground. When the action potential is between the grounded stimulating electrode and the first lead off electrode, both grids will be affected equally, and therefore no deflection will be produced on the screen.

Before considering what happens when the impulse reaches the first grid electrode, the potential drop which moves along the fiber with the impulse should be made clear. The difference in potential between the two sides of the membrane at the regions preceding and following the instantaneous position of the action potential will produce a current flow from the outside to the inside of the polarized layer through the depolarized region. This current flow is diagrammed in Figure 15 A, in which equipotential lines from the depolarized region out into the surrounding medium are represented. Figure 15 B shows the accompanying instantaneous potential distribution along the fiber.

In this figure it will be clear that B also represents the shape of the crustacean action potential. Indeed, it was taken from one of the records. A of this figure was constructed from B by drawing vertical lines from a series of points on the potential



Current Flow and Potential Distribution in and around Propagating Nerve Impulse.

wave which represented a constant increment of potential. The points where these vertical lines intersected the boundaries of the membrane of A determined the points of emergence of the equi-potential lines of A. From these latter lines the lines of current flow were constructed. In this way it was possible to construct the approximate current flow shape from the action potential shape.

Therefore, when the depolarized region is under the first grid electrode, it will become negative in respect to the second, distal electrode, and the action potential will be represented on the cathode ray tube. When the impulse passes on to the region between the electrodes, both will again acquire the same potential but only if there is no variation in the action potential of the fiber in this region. When the impulse reaches the distal grid the latter will become negative with respect to the proximal one and a deflection in the positive direction will occur.

Experiments have been presented which show that the potential drop along the external medium surrounding the fiber, which corresponds to the led off action potential, becomes smaller as the conductance of that medium increases. In other words, the external action potential has been shown to decrease in high conductance media because, using a differential amplifier; it is the same potential as the led off signal.

From this experimentally determined fact it can be understood why variations in conductance of the external medium will produce spurious effects to the observer. Consider the case where the impulse encounters a region of suddenly decreased conductance. The external action potential will become larger as it travels into

this new environment. Figure 16 A represents the resulting potential distribution along the fiber as this change occurs. It will be seen that the distal electrode will become more positive with respect to the proximal one while the impulse is passing through the changing environment. This will present, therefore, a negative artifact on the oscillograph. After the impulse has completely passed into the region of uniform but decreased conductance, both grids will again acquire the same potential. Conversely, if the impulse encounters a region of increased conductance, a positive artifact will appear. see Figure 16 B.

It is not difficult to derive a mathematical expression for the artifact resulting in these cases. In Figure 17 A the action potential crosses a sharp boundary between region 1, where we shall denote the spike height by h_1, to region 2 where the spike height becomes h_2. Now let

$$e_1 = e_1(x - vt),$$

where e_1 is the external action potential, x and v are the position and velocity of the action potential, and t is the time. Then from experimental data, at least to a good approximation,

$$e_2 = \frac{h_2}{h_1} e_1(x - vt).$$

From the figure it is seen that

$$\Delta e = e_2 - e_1.$$

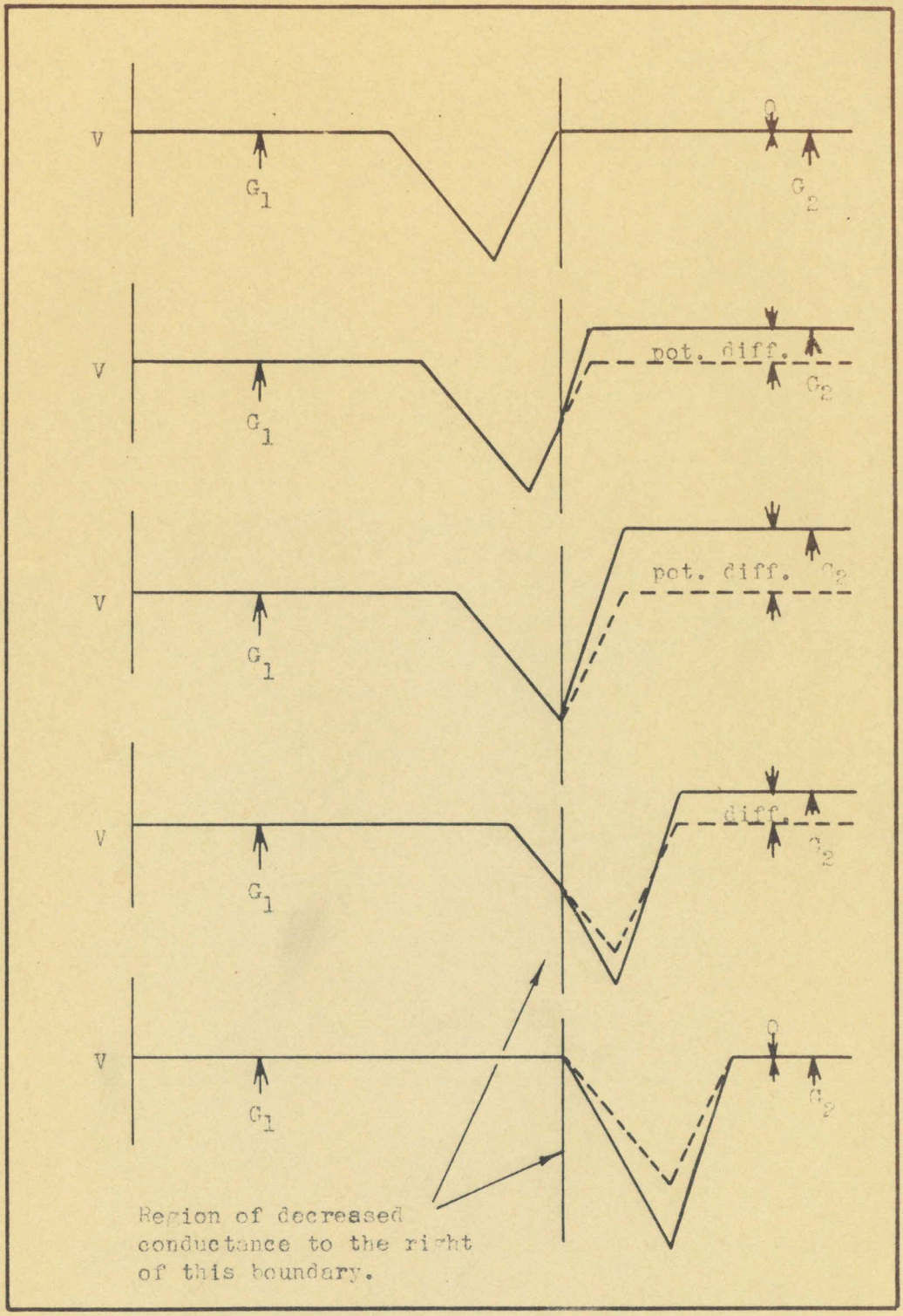


FIGURE 16 A

Action potential entering region of decreased conductance.

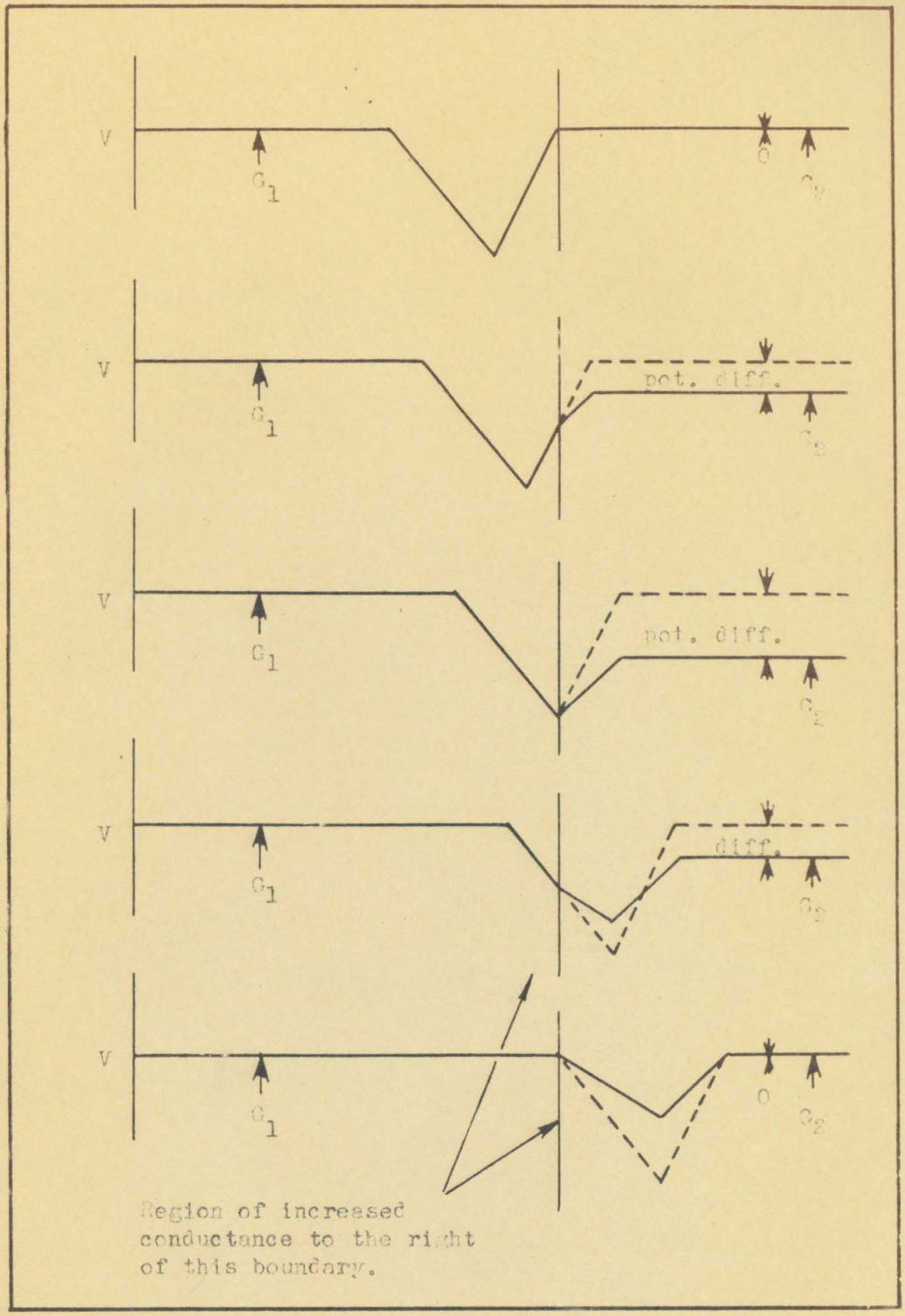


FIGURE 16 B

Action potential entering region of increased conductance.

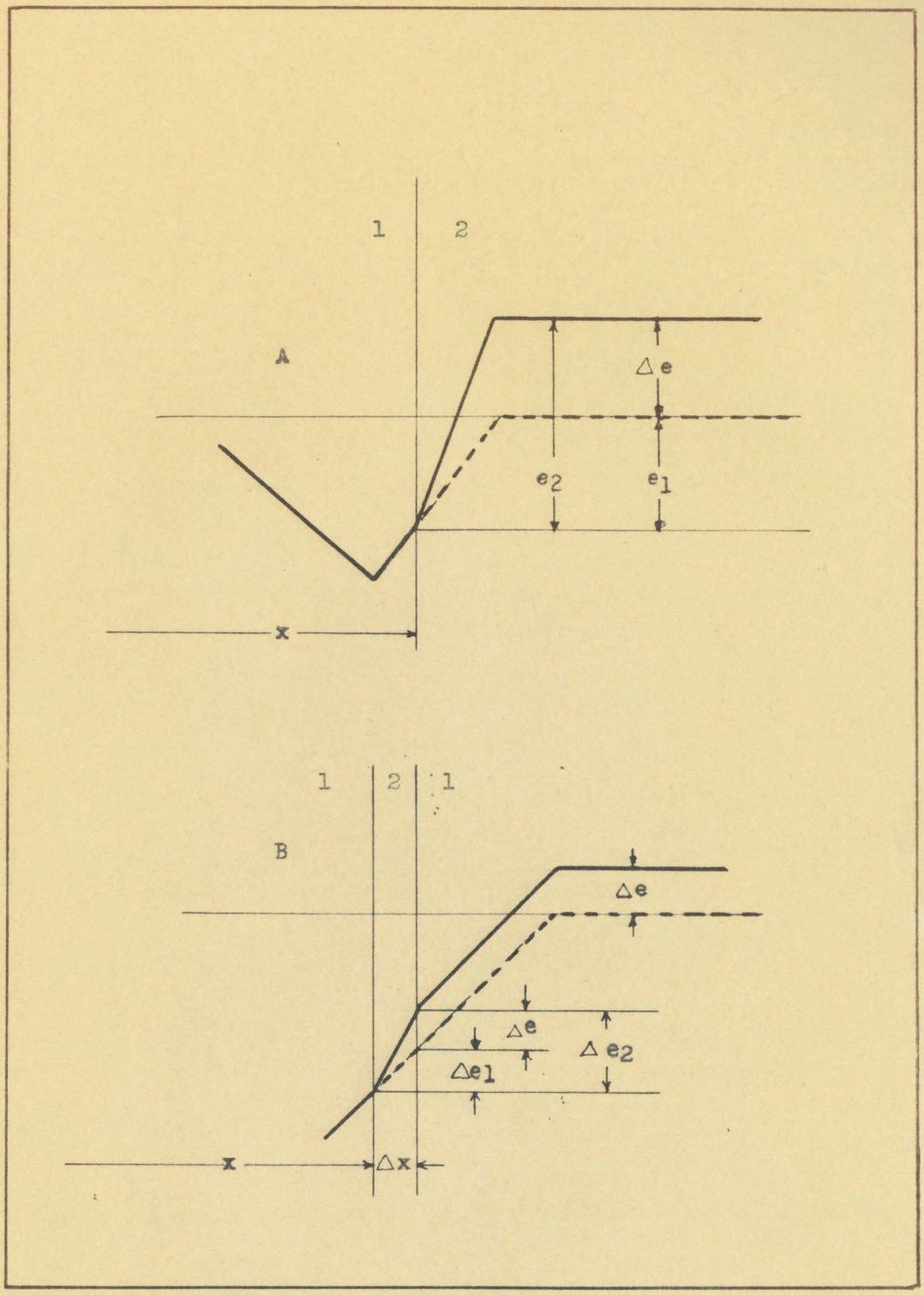


Figure 17. See text for explanation

Therefore:

$$\Delta e = \frac{h_2 - h_1}{h_1} e_1(x - vt),$$

or

$$\Delta e = \frac{h_2 - h_1}{h_1} e_1 = \frac{h_2 - h_1}{h_2} e_2.$$

Records of these artifacts have been shown in Figure 13, A and B. An expression for the artifacts shown in C and D of the latter figure can also be derived. Refer again to Figure 17. In B the action potential in region 1 suddenly encounters a narrow region 2 of different conductance. Using the same notation, it will be seen that

$$\Delta e_2 = \frac{h_2}{h_1} \Delta e_1,$$

and that

$$\Delta e = \frac{h_2 - h_1}{h_1} \Delta e_1.$$

By Taylor's series,

$$\Delta e_1 = \frac{\partial e_1}{\partial x} \Delta x + \frac{\partial^2 e_1}{\partial x^2} \frac{(\Delta x)^2}{2} + \ldots .$$

If Δx is sufficiently small we get:

$$\Delta e \triangleq \frac{h_2 - h_1}{h_1} \frac{\partial e_1}{\partial x} \Delta x$$

$$\triangleq \frac{h_2 - h_1}{h_2} \frac{\partial e_2}{\partial x} \Delta x$$

In the last expressions $\frac{\partial e_1}{\partial x}$ (and $\frac{\partial e_2}{\partial x}$) changes sign at the tip of the spike, accounting for the diphasic response already seen. If the ascending and descending arms of the spike were completely straight lines, the above derivatives would be constant during each phase and the last expression for Δe would be exact when Δx was small compared to the wave-length of the impulse. It should also be noted that as Δx decreases so does the artifact.

The expressions just derived for the two main types of spurious response do roughly agree, quantitatively, with experiment.

The artifacts led off from fibers in moist air should also receive a word. It will be remembered (see III, A (2)) that they too were both positive and negative. A positive-negative response should arise every time the impulse crossed an idle electrode or one of the beads of fluid that often collected on the fiber. The large positive or negative responses that were described are not as certain of explanation, but it will be recalled that constrictions in the fiber frequently arose which could very likely be the cause by altering the action potential as it passed.

It should be added that Bishop, Erlanger and Gasser (1926) also reported spurious electrical responses from myelinated nerve that were very similar to those described here. However their explanation of their artifacts does not fit in the present case.

C. The external action potential

The question now arises as to why the external action potential decreases with increasing conductance. Let e represent the difference in potential across the polarized membrane which causes a current, i, to flow from the outside surface through an external resistance, R_e, through the depolarized region and the axis cylinder, with an effective internal resistance, R_i, to the inside surface. Then

$$e = i(R_e + R_i).$$

Now iR_e is the external action potential*, and can be represented by the expression:

$$\text{external action potential} = e \frac{R_e}{R_e + R_i}.$$

Since e and R_i can be assumed to remain constant, and R_i to be large with respect to R_e, it appears that the action potential tends to vary almost proportionally with R_e.

R_e will be a function of the resistivity of the medium, and the geometry of the fiber and of its environment (here the trough). The relationship between R_e and the cross-sectional area of a triangular trough allows of no simple mathematical expression. Therefore the relationship expressed by the graph of Figure 11 cannot be reduced to an experimental relationship between R_e and the action potential, which would test the validity of the preceding equation.

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* I. e., its amplitude.

There are several consequences of the relationship expressed by the first equation of page 56. It is clear that if e and R_i are assumed constant, then an increase in R_e will imply that i decreases. This will mean that the rate of depolarization of the membrane will decrease, and hence the velocity of the impulse will be less. This, of course, is what has been observed experimentally: fibers led off in air have a slower conduction rate than those led off in solution.

If v represents the velocity and d the duration of the impulse at some point, then the wave length, λ, of the action potential at that place is given by:

$$\lambda = vd.$$

The wave-length will represent the electrically permeable region which accompanies the impulse down the fiber. It has already been pointed out that the permeability must increase as the impulse approaches a region of the fiber and decrease with recovery after the impulse has passed, following which the membrane begins to repolarize. This recovery process is probably an affair of the axis cylinder and the membrane and should be independent of the surrounding medium. Therefore variations in conductance should not influence it, and if the velocity increases with increasing external conductance then the duration should decrease. The present data, though insufficient on this point, suggest that this may occur, for the duration is about 1 msec. in air and 0.6 to 0.8 msec. in solution.

D. Leading off conditions: Conclusions

So far as the author is aware, this is the first time that leading off in solution has been adopted as a method and not just for a study of its effect. Craib (1928), who was interested in the electrical response of the heart, led off action potentials from strips of striated muscle bathed in solution. He used a string galvanometer, which has the same advantage as a differential amplifier in recording only the difference in potential between two points, but has the disadvantage of drawing current. However, his muscle was bathed in a large quantity of solution. This resulted in leading off triphasic action potentials from each point instead of monophasic ones, because of the bending back of the iso-potential lines in the surrounding medium (compare Figure 9, showing current flow in a sheet, and also Figure 15).

The quantity of solution which surrounds the preparation (in the present case a single axon) is an important consideration. It must be kept as small as possible, in order to obtain as accurate a picture as possible of the potential change along the surface of the fiber, but must be large enough so that variations in conductance along the fiber do not occur.

The reason that this method has not been used before is probably that, since most studies of nerve action potentials have been made on whole nerve bundles, the variations in conductances encountered have been minimized. These variations at any point, due to irregularities in the fluid clinging to the surface or the presence

of electrodes, would be small because of the relatively large cross-section of the bundle.

The method promises to be very satisfactory in this type of investigation. It should prove flexible enough to allow such experiments as the action of drugs upon the activity of single fibers to be carried out. And it may be possible to adapt it to allow experiments on the response at the exciting electrode, where the stimulus artifact is a complicating feature.

V. Summary

1. A review of the electrical phenomena found in myelinated and unmyelinated nerves and single nerve fibers appears in the introduction.

2. In this paper the action potentials of single unmyelinated motor fibers, mainly from the claw of the crayfish, Cambarus clarkii, were studied with a differential amplifier and cathode ray oscillograph.

3. Two types of leading off chamber are described: (1) for leading off action potentials in moist air, and (2) for leading off with the axon mounted in a trough and surrounded by a small quantity of physiological solution.

4. The electrical records obtained with the former method were usually complicated by spurious responses. The disadvantages of this method are described. The latter method was then adopted and its advantages pointed out.

5. However spurious electrical effects still appeared. The cause of these responses was found to be variations in conductance along the medium surrounding the fiber. They were eliminated by introducing a trough-lid which prevented these variations.

6. Experiments were then performed in which the dependence of size of the action potential on the conductance was shown. This was done by varying both the volume and the conductivity of the solution about the fiber. Spurious responses deliberately produced are described.

7. The shape and other properties of the action potential are given. Measurements of the conduction rates of single fibers showed that these ranged from 3 to 8 m. per sec. in Cambarus, being greater in solution than in air. The velocity in small sensory bundles ranged from 1 to 2 m. per sec. The question of fiber groups is discussed.

8. The effect upon the action potential of stretching the fiber is described.

9. The nature of bio-electric potentials is discussed. An analysis is made of how variations in conductance must produce spurious electrical effects. It is also shown how the height of the action potential is related to the resistance of the medium about the fiber.

10. Finally, the method of leading off in solution is shown to meet the requirements for obtaining undistorted action potentials from single fiber preparations.

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