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The effect of divalent cations upon the membrane potential of the crayfish giant axon

by

Richard Charles Hallgren

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Co-majors: Electrical Engineering
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Signature was redacted for privacy.

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Signature was redacted for privacy.

For the Major Departments

Signature was redacted for privacy.

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1975

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INTRODUCTION

Ever since Galvani (1791) first experimented upon a frog's leg, scientists have been attempting to explain the complex electrical phenomena that are a part of every human nervous system. With a better understanding of the human nervous system as their goal, scientists have devoted great amounts of time and energy to developing techniques to help understand the nervous system. The ability to add to existing knowledge concerning the human nervous system revolves around the accumulation of information about the system and the formulation of this knowledge into a workable theory. Unfortunately the obvious moral, technical and economic problems associated with using humans as subjects for experimental dissection have left the majority of investigators with no alternative but to use other animals. By using these animals the researcher can study such common events as accommodation of sensory fibers, relative and absolute refractory periods, subthreshold response, and many others without endangering the life of a human subject. In addition the technical problem of studying individual nerve fibers in a mammal are avoided by using the physically larger fibers found in the squid, lobster and crayfish. Of course since man is unique, there will be phenomena that can only be demonstrated and studied on a human subject.

The work that preceded the writing of this document was intended to add to the existing knowledge of the crayfish. It was found, while searching the literature, that the effects of variations in the sodium and potassium ions had been extensively studied but that the effects of variations in calcium ions received a disproportionately small amount of

attention. Firmly believing that all areas should be thoroughly examined, it was decided to investigate the effects that variations in the calcium ion concentration in the extracellular fluid had upon the membrane potential.

Because early investigators were hindered by very crude measuring instruments, their hypotheses were often based more upon intuition than upon fact. Bernstein in 1902 suggested that the potential measured across the membrane of a nerve cell was the result of the concentration of potassium ions inside and outside the nerve cell. Bernstein also hypothesized, incorrectly, that the amplitude of the action potential would be equal to the value of the transmembrane potential. With the development of the thermionic valve, which provided a means for measuring the very small voltages encountered in such work, and the development of the cathode ray oscilloscope, which allowed the investigator to conveniently and accurately observe a voltage as a function of time, information gathered from the studies of the electrical properties of nerve cells began to increase substantially. Hodgkin and Huxley in 1939 took advantage of vacuum tube technology to measure transmembrane potentials with longitudinal microelectrodes positioned in giant nerve fibers of the squid. They showed that the amplitude of the action potential was greater than the magnitude of the membrane potential and developed a new ionic theory which contributed to modification of Bernstein's hypothesis. For an excellent review of the work of Hodgkin and Huxley the reader is encouraged to obtain the lectures delivered by them in 1964 upon receiving the Nobel prize in physiology and medicine. The types of experiments performed by Hodgkin and Huxley are a good example of ways that various

characteristics of the human nervous system can be studied by using animals such as the squid. Lobsters, like the squid, have large nerve fibers which are surgically accessible and are also excellent for isolated nerve cell studies. Unfortunately unless the investigator lives by the sea, live squid are very hard to obtain, and lobsters because of their culinary qualities are very expensive.

The crayfish, found in fresh water streams throughout the world, has many characteristics which are common to the lobster. A very important characteristic, for the investigator desiring an isolated nerve preparation, is that both the lobster and the crayfish have a ventral nerve bundle which contains several giant nerve fibers, each having a diameter between fifty and one hundred microns. The crayfish is particularly easy to obtain, quite easy to maintain, and relatively inexpensive. While a great deal of research has been performed upon the crayfish, only a small part has been concerned with the effects that calcium ions have upon the electrical properties of the nerve cell. In fact the work that has been done has been concerned with gross changes of five millimoles or more of calcium ions. Since it is very desirable to have a complete understanding of an experimental animal it was decided to perform a series of experiments designed to discover the variation of the membrane potential, both at steady state and during an active response, as the calcium ion concentration in the bathing solution is varied through a series of relatively small increments. It was decided to isolate the axon by surgically removing the ventral nerve bundle containing the giant axons and to place the bundle in a chamber. The chamber would be constructed so that the concentrations of ionic substances making up the fluid bathing the nerve

bundle could be rapidly and predictably altered. A micropipette filled with 3 molar potassium chloride and inserted into the giant axon would be used as a microelectrode for recording the response of the membrane potential.

It was assumed that because of the similarities of the two animals, some of the responses of crayfish giant axons to an excess or deficiency of calcium ions would be similar to the response of the giant axons of the lobster. The membrane potential would be expected to remain constant at high concentrations of calcium ions and to gradually become more positive at low concentrations. Likewise the amplitude of the action potential would be expected to remain constant at concentrations higher than normal and to gradually decrease at concentrations lower than normal.

An interesting phenomena which had been reported by Brink et al. (1946) as occurring in the squid, and which would be watched for in the crayfish, is the occurrence of the spontaneous formation of an action potential. This would occur as a result of a decrease in the electrical stability of the nerve cell and would be expected to occur at concentrations less than one half of the normal calcium concentration.

This dissertation documents the observations made on the response of the giant nerve fibers of the crayfish to bathing solutions containing various concentrations of calcium ions. In addition, magnesium ions were tested to see if, as divalent cations, they could substitute for calcium ions. The main body of the dissertation deals with the procedure required to isolate the nerve fibers, the system that was constructed to control the concentration of calcium ions in the bathing solution, and the

equipment used to stimulate the nerve fibers and to record the resulting action potential. The section of results and discussion ties together existing theories with the experimental data obtained and conclusions developed.

LITERATURE SEARCH

One of the noteworthy characteristics of excitable membranes is their sensitivity to changes in their chemical environment. In the living system such changes in the environment can result in alterations in the threshold required for formation of an action potential which can serve to regulate the activity of the organism (Brink et al. 1946). An important reason for studying the effects that alterations in the chemical environment can have upon a living system is to see how the organism reacts in the hope of discovering the mechanism which regulates the particular animal's response. The same approach can be applied to a subsystem of the organism such as a single nerve cell. One of the most fruitful methods of obtaining information regarding the role of chemical components of the nerve structure and the mechanism involved in neural action has been to vary the concentration of a specific ion in the bathing solution of an isolated nerve preparation and to observe the changes that result (Brink et al., 1946). While a variety of chemicals could be used, the ones which would be of primary interest are those which normally exist in the extracellular fluid of the particular animal.

Many investigators have studied the effects of varying the concentrations of sodium and potassium ions bathing giant axon preparations from the squid, the lobster, and the crayfish. The squid has been extremely popular because of the giant axons which it has. Unfortunately the squid is seasonal and is difficult to obtain even in the coastal regions. The task of obtaining live squid and maintaining them away from the marine coast is almost impossible. Because of these problems, the lobster and

the crayfish have been examined to see if a giant axon preparation from these animals could be used in place of the giant axon preparation from the squid. The results reported by Dalton in 1958 and 1959 indicate that this is possible. Even though the squid, the lobster, and the crayfish are quite different in detail, the roles of sodium and potassium ions and the effects of varying these ionic concentrations are sufficiently similar to allow reviewing them together. The work that has been performed upon squid will be the primary source, by virtue of volume, and any deviations that the lobster and the crayfish might have will be indicated. For an excellent listing of some of the literature available in this area the reader is referred to an article written by A. M. Shanes in 1958.

Sodium and potassium ions are very important because they are the principle charge carriers in the membrane of a cell (Tasaki, 1968). Their charge carrying roles can be divided into membrane potential maintenance and action potential formation. Membrane potential maintenance is essential for electrical excitability, and the magnitude of the membrane potential at any time depends upon the distribution of the ions making up the intracellular and extracellular fluids and the permeability of the membrane to each of these ions (Ganong, 1969). An equation which describes the membrane potential at any particular point in time is the Goldman constant field equation:

$$V = \frac{RT}{F} \ln \frac{P_K[K_i] + P_{Na}[Na_i] + P_{Cl}[Cl_o]}{P_K[K_o] + P_{Na}[Na_o] + P_{Cl}[Cl_i]}$$

V = the membrane potential

R = gas constant

T = absolute temperature

F = the Faraday (number of coulombs per mole of charge)

P_K = permeability of the membrane to potassium

P_{Na} = permeability of the membrane to sodium

P_{Cl} = permeability of the membrane to chlorine

The brackets indicate concentration and the o and i subscripts refer to the outside and the inside of the cell. Since P_{Na} is low compared to P_K and P_{Cl} in resting cells, the concentration of sodium ions contributes little to the value of the membrane potential (Ganong, 1969). While the Goldman constant field equation is a valuable tool it is valid only under the following assumptions (Leibovic, 1972):

1. The only forces acting upon the system are concentration and voltage gradients within and close to the membrane.

2. Individual ions move through the membrane independently of each other.

3. Ionic fluxes due to diffusion and to the electric field are additive.

4. The steady state sum of the ionic currents is zero.

5. The membrane is homogeneous.

6. There exists a constant electric field within the membrane.

7. The ionic concentrations at the membrane interfaces are proportional to the ionic concentrations in the solution which is in contact with the membrane.

The formation of an action potential is due to a selective rise in the membrane permeability to sodium ions followed in time by a rise in the membrane permeability to potassium ions. Aidley (1971) reports that the termination of the response is due to a decrease in the membrane

permeability to sodium ions followed by a decrease in permeability to potassium ions. The variations in membrane permeability to sodium and potassium ions during an action potential are shown in Figure 1 after Hodgkin (1958). Since P_{Na} becomes large at the beginning of the active response, the concentration of sodium ions makes the largest contribution to the magnitude of the action potential. Dalton (1959) has extensively investigated the effects that varying the external sodium ion concentration can have upon the membrane potential of a crayfish giant axon. Since sodium is the predominant cation in the physiological fluid normal to the crayfish, an appreciable change in osmotic pressure can occur when a large percentage of the normal sodium is removed. Increasing the sodium ion concentration above normal presents such a problem in maintaining a normal osmolarity that it is not normally done. However, when the external sodium concentration is lowered below normal, dextrose is commonly used to substitute for the sodium removed and thus maintain the osmotic pressure. Dalton (1959) and Edwards et al. (1963) both reported that decreasing the external sodium concentration decreases the magnitude of the action potential but does not have any effect upon the resting potential. The magnitude of the action potential has been found to be a linear function of the logarithm of the external sodium concentration only for small changes in sodium concentration around the normal concentration. In addition to decreasing the magnitude of the action potential, decreasing the concentration of sodium ions in the external bathing solution also causes a decrease in the rate of rise of the action potential. Frumento and Mullins (1964) have found that decreasing the internal sodium concentration to approximately one half normal causes the sodium efflux during the action

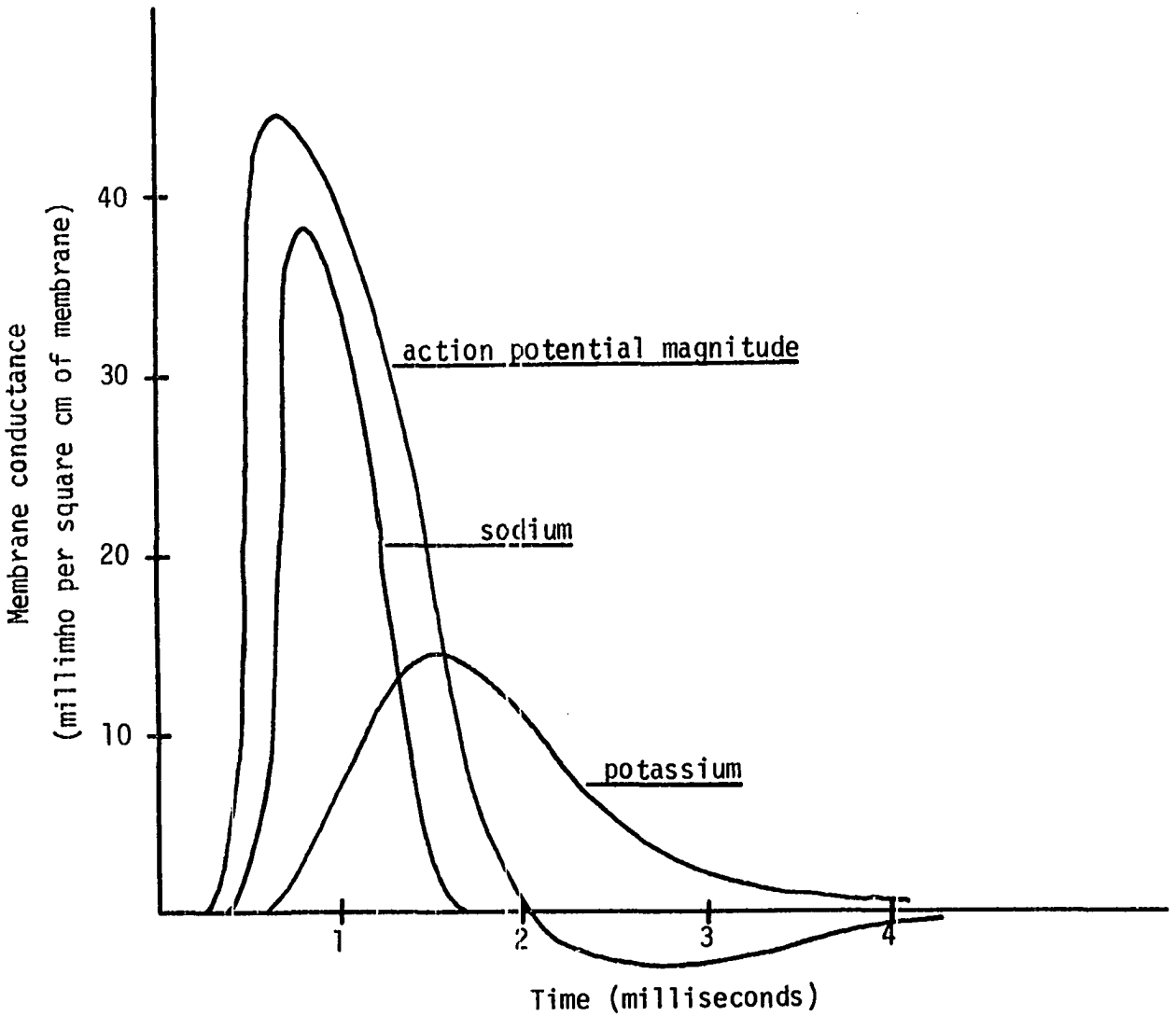

Figure 1. Changes in sodium and potassium conductance during the action potential in giant squid axon

potential to become insensitive to the presence of potassium ions in the external bathing solution.

Increasing the extracellular potassium ion concentration, though it may initially cause a lowering of threshold and in some cases actually result in the initiation of an all or none response, is known to depress nerve excitability after a steady state condition is reached (Wright et al., 1954). Tests have shown that axons placed in solutions containing excess potassium ions in the external solution show a significant decrease in latency of response at rheobase, a small decrease in conduction velocity, and a large decrease in the amplitude of the action potential. Zacharova and Zachar (1956) reported that axons became blocked to conduction at potassium concentrations greater than three times normal. They also found conduction velocity changes in potassium rich solutions and suggested that this might be due to a change in the normal potassium/calcium ionic balance. Both Tasaki (1959) and Moore (1959) have reported on the existence of a two stable state condition which exists when the axon is immersed in a bathing solution having an excess of potassium ions. Upon application of a hyperpolarizing current pulse the membrane developed a large hyperpolarizing response which could be abolished by a depolarizing current pulse after which followed a form of refractoriness.

Tomita et al. (1961) noticed that the voltage/current characteristics of an axon in the two stable state condition approximated the voltage/current characteristics of a semiconductor. They hypothesized that any tendency for an axon to form a repetitive response would be due to the negative resistance property of the membrane.

Decreasing the potassium ion concentration causes a decrease in the resting potential and an even larger decrease in the magnitude of the action potential (Dalton and Adelman, 1960). The decrease in the amplitude of the action potential is attributed to a direct decrease in the membrane potential rather than due to a decrease in the potassium ion concentration. The changes in resting potential approximate an ideal potassium electrode only at the higher potassium concentrations where the resting potential varies linearly with the logarithm of the potassium ion concentration (Tasaki, 1959).

The resting potential of the lobster giant axon is slightly more positive than the resting potential of the squid giant axon, and the resting potential of the crayfish giant axon is approximately 15 millivolts more positive than the resting potential of the lobster axon (Dalton, 1959). In addition, the characteristic shape of the action potential of a squid axon has a slight undershoot at the very end while the action potential of the lobster axon and the crayfish axon do not exhibit this undershoot (Dalton, 1958; Dalton, 1959). This could indicate a difference in the dynamics of the potassium carrying systems in the two species.

The amount of reported research that is concerned with the effects that varying the divalent cation concentration has upon neural tissue in the squid, the lobster, and the crayfish is small when compared to the amount that is concerned with the effects of univalent cations. While the effects of varying the concentration of sodium and potassium produced similar results in the squid, the lobster, and the crayfish, the effects

of varying the concentration of calcium and magnesium ions are different for these three species. The present theories of the roles of calcium and magnesium ions in neural processes are highly speculative rather than firmly based upon elementary physical processes which can be demonstrated in nerve cells. This is due in part to a lack of understanding of how the cell membrane is structured. Brink (1954) suggests that the calcium and magnesium ions react in some way with the surface structure of the cell membrane. Since so little is known about the structure of the cell membrane an analysis of the mechanism of divalent cation control is difficult to accomplish. The main facts that have been established concerning the action of calcium ions on excitable tissue are that increasing the calcium concentration in the external bathing solution raises the threshold for stimulation, increases the membrane resistance, and increases the ability of the nerve cell to accommodate (Brink, 1954; Lipicky and Bryant, 1962). Reducing the calcium concentration in the external bathing solution has effects opposite to those just listed. In addition to those items listed above, decreasing the external calcium ion concentration increases the fraction of the sodium transport system that is in an inactive condition (Frankenhaeuser and Hodgkin, 1957). This resulting increase in the membrane resistance to sodium ion flow could explain the decrease in the magnitude of the action potential caused by a decrease in the external calcium ion concentration. Tests conducted by Dalton and Adelman (1960) have shown that the change in the magnitude of the action potential per unit change in the magnitude of the resting potential is greater in solutions containing normal concentrations of potassium ions and low concen-

trations of calcium ions than in solutions containing normal concentrations of calcium ions and elevated concentrations of potassium ions. This would suggest that calcium ions have a direct effect upon the magnitude of both the resting potential and the action potential while potassium ions have a direct effect upon just magnitude of the resting potential.

When an axon from the squid is immersed in a calcium deficient solution, a condition of increased sensitivity to excitation is produced which is similar to the condition produced by a reduction in membrane potential (Frankenhaeuser and Hodgkin, 1957). Frankenhaeuser and Hodgkin suggested that the external calcium concentration determines the membrane potential at which specific changes in the permeability of the membrane to sodium and potassium occur allowing a propagated response to be formed. Decreasing the external concentration of calcium ions modifies the properties of the squid axon so that cyclic events, which have a frequency of occurrence that is a function of the characteristics of the specific fiber, develop in the fiber. Guttman and Barnhill (1970) and Brink et al. (1946) both found that if the calcium concentration is reduced far enough and if the fibers are in excellent condition the cyclic events will develop into propagated action potentials. Tasaki et al., (1969) reported that some of the axons they investigated, which were immersed in a calcium deficient solution, went into a completely depolarized state. In the squid axon a five-fold decrease in the calcium ion concentration produces a change in the sodium and potassium permeability similar to a depolarization of ten to fifteen millivolts (Huxley, 1959).

Experiments have shown that magnesium ions can substitude for calcium ions in the normal function of the squid nerve cell indicating that the magnitude of the divalent ion concentration is more important than whether the divalent ion is calcium or magnesium (Huxley, 1959; Dalton, 1958).

Dalton (1958) has investigated the effects that external ions have upon the membrane potential of the lobster giant axon. He has found that decreasing the calcium ion concentration of the fluid bathing a lobster giant axon causes a partial depolarization of the resting potential and a decrease in the amplitude of the action potential. The decrease in the amplitude of the action potential is greater than the decrease in the resting potential suggesting that the calcium ion concentration has an effect upon both the membrane potential and the amplitude of the action potential (Dalton and Adelman, 1960). Reducing the concentration of calcium ions in the external solution causes a marked decrease in the duration of the action potential. An undershoot also appears which is similar to the undershoot which is seen in the action potential of squid fibers in normal concentrations of calcium indicating a decrease in the damping properties of the membrane. Repetitive activity has been observed in solutions containing a deficiency of calcium ions, but this has been reported to be the exception rather than the rule (Dalton, 1958). Increasing the calcium concentration above normal does not increase either the membrane potential or the amplitude of the action potential above their normal values. It was concluded that the identity of the divalent ion in the external solution is important after noting that magnesium plays little or no role in the axon's ability to maintain a normal membrane potential and consequently a normal action potential (Dalton, 1958).

In the crayfish it was found that decreasing the external calcium ion concentration results in a partial depolarization of the membrane potential and a decrease in the amplitude of the action potential (Dalton, 1959). As in the lobster, the amplitude of the action potential is decreased more than the resting potential is decreased. Increasing the calcium concentration above normal had no effect upon either the resting potential or the amplitude of the action potential. It was observed that increasing the calcium concentration above normal caused the opposite of the undershoot seen in the squid axon suggesting that the increased calcium enhanced the stability of the membrane. While no spontaneous activity was seen with a 0 millimolar calcium concentration and a 2.6 millimolar magnesium concentration the investigator reported difficulty in obtaining a stable preparation (Dalton, 1959).

In summary, increasing the calcium ion concentration caused no change in either the membrane potential or the amplitude of the action potential in the squid, the lobster and the crayfish. Decreasing the calcium concentration caused a depolarization of the membrane potential and a decrease in the amplitude of the action potential in all three animals. Spontaneous activity at low concentration of calcium ions occurred predictably in the squid, sporadically in the lobster, and not at all in the crayfish.

METHODOLOGY AND INSTRUMENTATION

In attempting to describe the methodology of such a project it is tempting to consider certain areas as being obvious. Unfortunately experience has shown that a great many areas which seemed at first to be straightforward were in reality complicated by many subtleties. In the words of I. P. Pavlov, "Science progresses in steps depending upon the success of techniques. Each improvement in technique raises us a stage upward, from which a new horizon is uncovered containing phenomena not known before." (Bures et al., 1967). In electrophysiological work, technique is all important. For this reason the technique required to isolate the nerve bundle from the crayfish and to prepare the bundle so that an intracellular recording results will be described in detail.

General

Crayfish, as stated before, are excellent animals to use for single giant axon studies. They are inexpensive and readily available anywhere in the United States. They are hardy, require little care to maintain, and they are easy to work with. The surgical procedure required to expose a giant axon is straightforward, requiring more practice than skill. All of the experiments were performed on either Orconectes virilis obtained from Nasco[1] or on Procambarus clarkii obtained from Carolina Biological[2] (Crocker and Barr, 1968). The Procambarus clarkii specimens tended to be

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[1]Nasco. Ft. Atkinson, Wisconsin.

[2]Carolina Biological. Burlington, North Carolina.

physically larger and were easier to work with because the giant axons were easier to find. No problems were encountered in obtaining crayfish that were a minimum of 10 cm in length.

Everything necessary to maintain the specimens was obtained from a store which specialized in aquarium supplies. A seventy-five liter aquarium was found to be large enough to hold twenty-four crayfish. The water in the aquarium was completely changed once a month. For the sake of convenience the aquarium was located next to a source of water and a drain. Water temperature was not critical and was allowed to stabilize to room temperature. Because of the high concentration of chlorine in tap water the aquarium was filled with water and allowed to sit uncovered for two days before the crayfish were replaced. In between monthly cleanings the level of water was maintained by the addition of tap water. The water was kept reasonably clean by using an aquarium filter.[1] The crayfish were not troubled by murky water so no attempt was made to keep the water crystal clear. Changing the filtering material every other day and changing the charcoal every other week produced adequately clean water. It was very important to adequately aerate the water. While the filter provides aeration, it was found advisable to also have an air stone. The filter and air stone were run from independent air sources so that even if the filter ran out of water the air stone would continue to operate. The air pump[2] had two independent outputs of air and was used as described for two years without both the air stone and the filter quitting except during a

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[1]Bubble Up Aquarium Filter, Metaframe Inc. Maywood, New Jersey.

[2]Hush 2 Air Pump, Metaframe, Inc. Maywood, New Jersey.

power failure. The crayfish were fed every other day by sprinkling fish food on the water. Several brands, available in bulk quantities, were tried but the crayfish seemed not to favor any particular brand. When the crayfish were being overfed the tank quickly became very dirty. The aquariums were kept covered to keep the crayfish from crawling out and to provide a dark refuge.

The crayfish has a large ventral nerve bundle which is primarily involved with the innervation of the muscles of the abdomen. The abdomen is quite muscular and it is used in conjunction with the tail fan to propel the crayfish away from danger. The giant axons in the bundle although not myelinated are very large in diameter so that a nerve impulse having a high propagation velocity can be obtained (Furshpan and Potter, 1959; Robertson, 1961). There are a total of four giant axons in each animal. Figure 2, after Robertson (1961), shows a typical cross section through the bundle. Fortunately the giant axons lie close to the surface of the nerve bundle, thus there is no need to try to totally isolate the giant axon from the rest of the fibers. The two lateral fibers are usually about twice as large as the two medial fibers. It is therefore easier to insert a microelectrode into a lateral fiber. The lateral fibers are interrupted at regular intervals by septa which do not prevent conduction in either direction but can decrease the safety factor of conduction. The medial fibers have no septa. Experiments were run on both lateral and medial fibers and no apparent difference in response was observed.

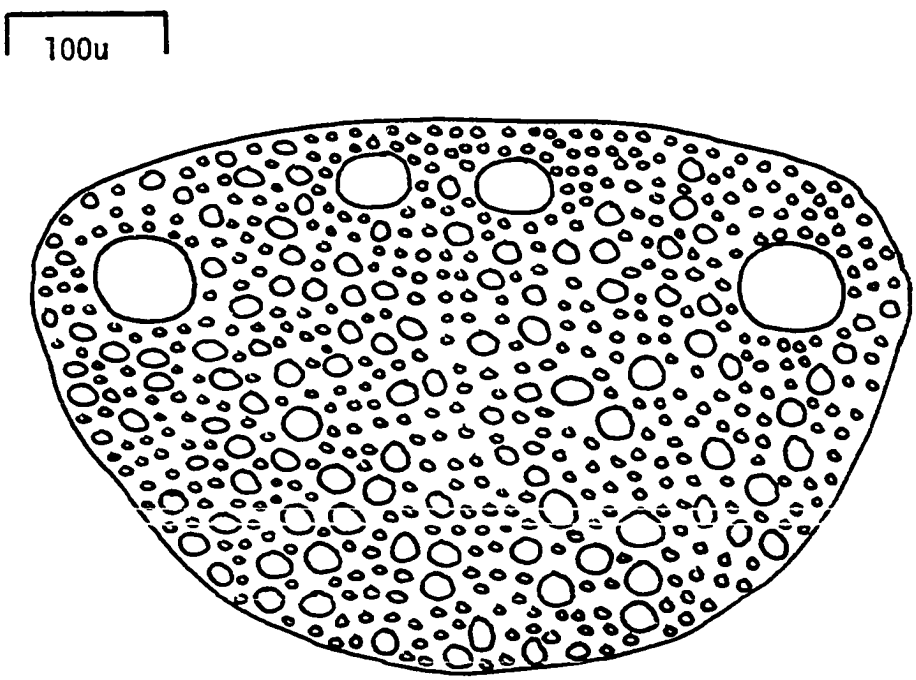

Figure 2. Cross section through the ventral nerve cord of the crayfish

Stability of the nerve fiber with respect to the microelectrode was found to be of utmost importance. Any small vibration or sudden movement would cause the microelectrode to damage the axon membrane. Because of this problem a special table was constructed from five cm angle iron. The iron angle was welded together to form the framework for a 1.27 cm thick piece of aluminum which served as the top of the table. Four large rubber stoppers, one for each leg, were placed between the legs and the floor to damp out any large high frequency vibrations. The table was electrically grounded.

To avoid the discomfort of being pinched by the fourth thoracic limb, the live crayfish were handled with a pair of double bend crucible tongs.[1] After removal from the aquarium a crayfish was cut into two pieces using a pair of post mortem shears[2] applied just behind the fifth thoracic limb (first pereiopod) (Bullough, 1951). The half including the head was placed in a small plastic bag containing just enough methyl alcohol to cover the head and was discarded. The half including the tail was placed ventral side up in a small container constructed from Plexiglas. This container was then filled with a modified Van Harreveld's solution (Van Harreveld, 1936). Van Harreveld's preparation is a physiological solution for crayfish. Refer to the section on solution preparation for details on modifications of the original solution. The Plexiglas container was then placed upon an aluminum bridge which was bolted to the

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[1]Catalog number A-2147, Clay-Adams, Inc. 141 East 25th St., New York, New York.

[2]Post mortem shears, forged steel, Arista Surgical Company. 67 Lexington Ave., New York, New York.

work bench. An adjustable 'C' clamp was used to simultaneously secure the tail fan of the crayfish and the Plexiglas container to the aluminum bridge. A piece of masking tape was attached to one side of the plexiglass container, placed over the thorax of the crayfish, and attached to the other side of the Plexiglas container, preventing the tail of the crayfish from curling.

The following surgery was performed under a stereo microscope.[1] Unless otherwise noted the microscope was fitted with the 10X eyepieces and set at a total magnification of 8X. A glass objective protector was used to prevent a buildup of salt crystals splattered from the Plexiglas container upon the objective lens. A high intensity illuminator[2] was used to provide the intense, oblique lighting required to see the giant axons. Some of the dissecting instruments used in this phase of experimentation are shown in Figure 3. A pair of straight tip extra fine microdissecting forceps[3] and a pair of Lempert Malleus cutters[4] were used to remove the first through fifth pleopods. The Lempert cutters were then used to cut through the segmental structures as close to the flanges, the pleurons, as was possible. Straight iris scissors[5] were used to cut the tissue in

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[1]Number SMZ-AU Stereo Zoom, Objectives: 10X, 20X. Type III stand, Objective glass protector, Frank Fryer Co. 60 E. Main St., Carpentersville, Illinois.

[2]Six volt, thirty watt Universal Microscope Illuminator, Frank Fryer Co. 60 E. Main St., Carpentersville, Illinois.

[3]Number C-911, extra fine microdissecting forceps, very fine sharp points, straight, Clay-Adams, Inc. 141 East 25th St., New York, New York.

[4]Number AU-11360, Lempert Malleus Cutter, V. Meuller. 6600 West Touhy Ave., Chicago, Illinois.

[5]Number C-926, extra fine dissecting scalpel, very delicate blade, Clay-Adams, Inc. 141 East 25th St., New York, New York.

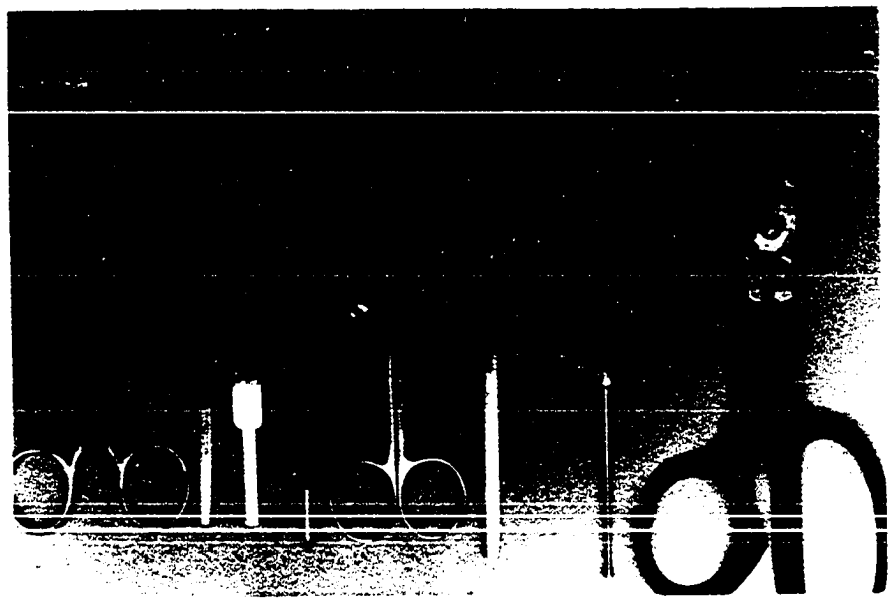

Figure 3. Surgical instruments showing from left to right Lempert Malleus cutters, extra fine dissecting scalpel, Trident 45° angle tip ultra-micro forceps, extra fine micro-dissecting forceps straight tip, straight iris scissors, iridocapsulotomy scissors, ultramicro needle 30° bend, post mortem shears

between the segmental structures on both sides of the nerve bundle. The cut extended from the first pleopod to the tail fan. Care was taken not to cut the nerve bundle. The microdissecting forceps were used to lift up the remaining tissue strip just below where it joined the first pleopod and the iris scissors were used to cut it free from the crayfish. The microdissecting forceps were held in the left hand and an extra fine dissecting scalpel[1] was held in the right hand and the strip of tissue was pulled towards the tail fan. The scalpel was used to cut free the abdominal muscles and other tissues that were attached to the strip of tissue. Care was taken not to stretch the nerve bundle or to cut it with the scalpel. The strip of tissue was separated from the surrounding tissue and completely detached from the crayfish. The nerve bundle was now seen lying in a groove formed by the abdominal muscles of the crayfish. Any remaining tissue covering the nerve bundle was carefully picked away with the microdissecting forceps. The nerve bundle was now transected by applying a pair of iridocapsulotomy scissors[2] midway between the last thoracic ganglion and the first abdominal ganglion. While grasping the severed end of the nerve bundle with the micro dissecting forceps and gently pulling on it towards the tail fan, the dorsal and ventral roots were cut using the iridocapsulotomy scissors. The dorsal roots were cut short but the ventral roots were cut long since they were to be used later in the procedure to manipulate the nerve bundle. After the roots from the

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[1]Number C-926, extra fine dissecting scalpel, very delicate blade, Clay-Adams, Inc. 141 East 25th St., New York, New York.

[2]Number OP-5570, iridocapsulotomy scissors, angular. V. Mueller. 6600 West Touhy Ave., Chicago, Illinois.

third abdominal ganglion were cut the nerve bundle was placed back on the abdominal muscles. While grasping the sixth abdominal ganglion with the microdissecting forceps the roots coming from the sixth abdominal ganglion were cut. The dorsal and ventral roots were then cut while gently pulling the sixth abdominal ganglion towards the thorax. After the section of nerve bundle was cut free from the crayfish it was positioned in the Plexiglas container so that it was completely covered by the modified Van Harreveld's solution. At this point in the procedure the Plexiglas container containing the crayfish and the nerve bundle was taken off of the aluminum bridge and placed aside on the work bench.

A special Plexiglas nerve chamber (see Figure 4) was built to provide a means of securing the nerve bundle and allowing a continuously flowing test solution to pass over the bundle. Figure 5 shows a photograph of the nerve chamber. The critical dimensions are shown on Figure 4 and the general size of the chamber can be taken from Figure 5. The slots between the test solution flow channel and chambers A and B are filled with dental wax.[1] The dental wax provides an excellent means of physically stabilizing the nerve bundle. The dental wax was first pressed into the slots using the micro scalpel. The sharp edge of the micro scalpel was used to eliminate any rough areas that might disturb the laminar flow of fluid through the test solution flow channel. After the wax had been

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[1]Surgident Periphery Wax, Lactona Corp. 2871 Grand View Blvd., Los Angeles, California.

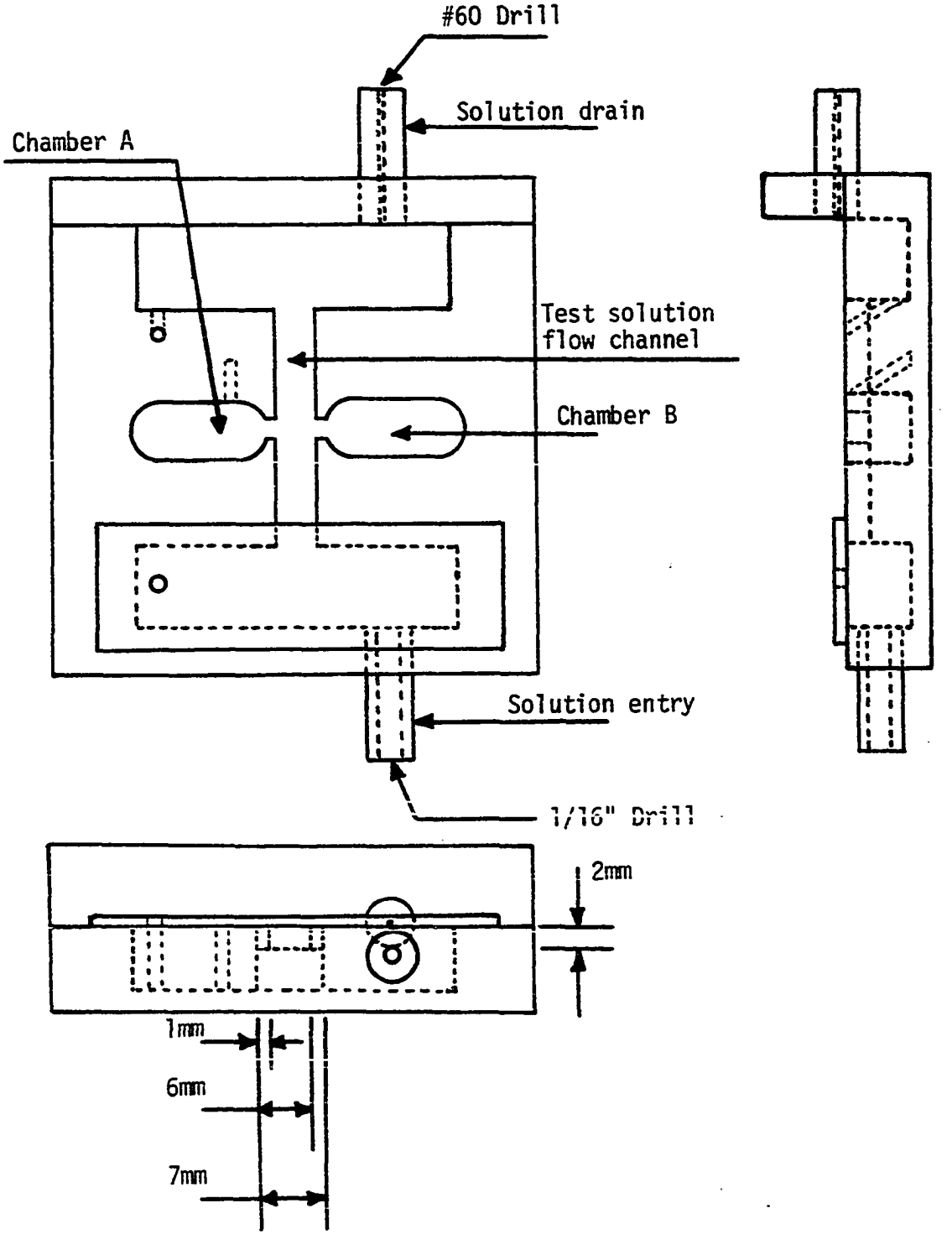

Figure 4. Plexiglas nerve chamber

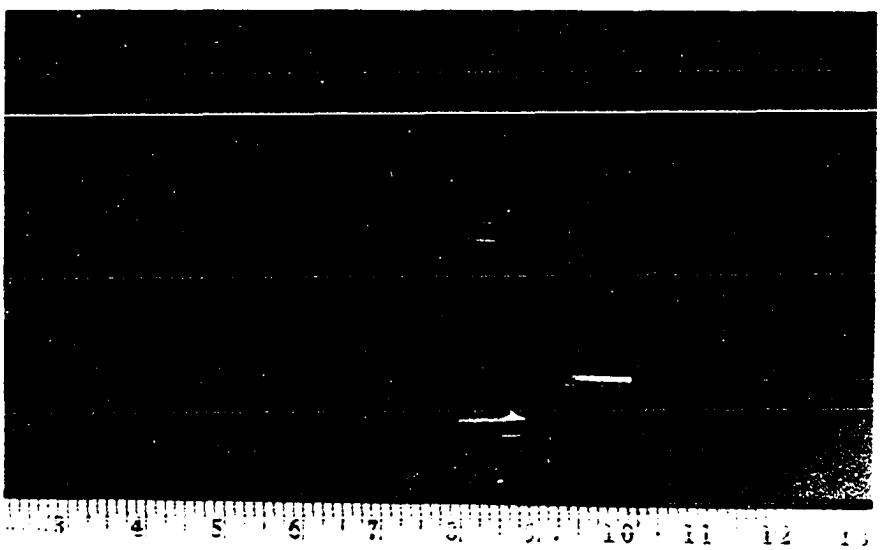

Figure 5. Plexiglas nerve chamber. Scale is in inches

packed into the slots an ultra micro needle was used to form a slot in the dental wax that was slightly wider than the diameter of the nerve bundle.

The nerve chamber was placed upon the aluminum bridge and secured to it with a 'C' clamp. The test solution source and drain connections, which will be discussed later, were attached and the solution was started. After the flow had stabilized both chambers A and B were filled with modified Van Harreveld's solution. The nerve bundle was then removed from the Plexiglas container with the microdissecting forceps and placed in the chamber nearest the investigator (chamber B). By using a pair of microdissecting forceps in each hand, the nerve bundle was manipulated so that the second abdominal ganglion was located in the flow path and the proximal end of the nerve bundle was in chamber A. The nerve bundle was positioned dorsal side up and very gently manipulated until it rested in the slots made in the dental wax. The position of the nerve bundle was adjusted until the second abdominal ganglion was against the wall of the flow channel nearest chamber A. The proximal end of the nerve bundle was held by a micro alligator clip[1] which was connected to a micromanipulator[2] through a brass adapting rod. The brass rod was bent so that the jaws of the micro alligator clip faced the end of the nerve bundle and were at a 45° angle to it. The clip was positioned over the cut end of the nerve

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[1]Number 34, Mueller micro-gator clip, distributed by Newark Electronics. 500 N. Pulaski Road, Chicago, Illinois.

[2]Model 5501, Brinkman Instruments. Westbury, New York.

bundle and lowered until the jaws of the clip just touched the modified Van Harreveld's solution. The clip was opened and, using the micro-dissecting forceps, the end of the nerve bundle was placed in the jaws of the clip. The height of the clip was adjusted so that the nerve bundle was completely covered by the modified Van Harreveld's solution. The position of the clip was adjusted to put a slight tension on the nerve bundle. The nerve bundle was positioned so that the dorsal side was facing directly upward. Using the iridocapsulotomy scissors the ventral roots that were in the test solution flow channel were cut from the nerve bundle.

The distal end of the nerve bundle was also held by a micro alligator clip which was connected to a micromanipulator[1] through a brass adapting rod. The brass rod was bent so that the jaws of the clip faced the nerve bundle and were at a 45° angle to it. The procedure required to grasp the distal end of the nerve bundle was identical to the procedure used to grasp the proximal end. After the end was grasped the clip was adjusted so that the nerve bundle was slightly in tension and completely covered by the modified Van Harreveld's solution. The test solution was still flowing smoothly and the portion of the nerve bundle in the test solution was completely bathed by the test solution. While looking at the portion of the nerve bundle in the test solution, under oblique illumination, it was possible to see the giant axons. The slots in the dental wax were now filled with more dental wax to provide a seal between chamber A and the

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[1]Model 305015, MH manipulator, Brinkman Instruments. Westbury, New York.

test solution flow channel and to stabilize the bundle where it passed from the test solution flow channel to chamber B. The micro scalpel was used to carefully pack and form the dental wax. Using a twenty gauge hypodermic needle connected to the drain line, the fluid was withdrawn from chamber A. Immediately the chamber was refilled with mineral oil. Enough oil was used to cover the nerve bundle but not enough to cause spillage over the top of the dental wax. The silver-silver chloride electrode which was used as a ground connection for the stimulator was placed into the hole drilled at the drain end of the nerve chamber. The ground connection for the preamplifier was connected to the test solution input end of the nerve chamber through a silver-silver chloride electrode and a three molar potassium chloride, agar bridge. The positive side of the stimulator was connected to a silver-silver chloride electrode placed in the hole drilled in the side of chamber A. The silver-silver chloride electrode was allowed to touch the nerve bundle.

Axon Exposure

The following procedure involves the opening of the sheath surrounding the nerve bundle and the insertion of a micropipette into a single giant axon. To aid in seeing the sheath and the axons, the 10X eyepieces on the microscope were replaced with the 20X eyepieces, and the microscope magnification was set for a total of 60X. The fibrous tissue comprising the sheath which surrounds the nerve bundle is dense enough to make it almost impossible to penetrate the sheath with a micropipette without causing excessive damage to the axons or to the micropipette. In addition, the sheath is elastic enough to be almost impossible to cut with a

sharp edge. The technique that finally proved successful was to use a pair of ultra micro forceps[1] much as a person would use a pair of scissors. The membrane was grasped with the tips of the forceps just below the second abdominal ganglion and the tips were allowed to slip past each other. The scissors action of the tips produced a hole in the membrane. If the opening was not large enough or if the desired axon was not exposed the procedure was repeated working from right to left across the nerve bundle. This procedure required a very steady hand and care had to be taken not to cut or stretch the giant axons. After the hole was made the individual giant axons were usually quite easy to distinguish.

The axons were disturbed as little as possible to avoid damaging the fibers. It was also observed that disturbing the position of the axons made the axons very difficult to see.

Micropipette Insertion

A micropipette filled with a three molar potassium chloride solution was selected and placed into the plexiglass holder that interfaced the micropipette with the micromanipulator.[2] The input of a preamplifier, which will be discussed in a later section, was connected to the micropipette by attaching the input to a silver-silver chloride electrode which was placed in the barrel of the micropipette. The preamplifier input was

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[1]Number 262-485, Trident forceps, Ultramicro, 45° angle tip, Curtin-Matheson. P.O. Box 1494, Marilyn Heights, Missouri.

[2]Prior, England, distributed in the USA as catalog number 930/T by Eric Sobatka. 110 Finn Court, Farmingdale, New York.

grounded during this part of the procedure. The micropipette was centered over the nerve bundle and lowered until the tip just touched the flowing stream of test solution. The ground was removed from the input to the preamplifier and the balance potentiometer was adjusted until the output of the preamplifier was zero volts. The micropipette was then slowly lowered until the tip just touched the surface of the giant axon that was to be impaled. While watching the meter connected to the ouput of the preamplifier, the micropipette was very slowly lowered. The meter would then indicate an abrupt change to approximately a minus 80 millivolts as the micropipette went through the membrane of the giant axon. Sometimes the meter would make an abrupt jump negative and then return to about a minus 20 millivolts. It was suspected when the meter did this that the micropipette had penetrated the axon membrane and had become lodged against the opposite surface of the membrane. This would cause a covering over the tip of the micropipette that would have a very high resistance and lower the magnitude of the membrane potential that would be indicated upon the meter. A very slight retraction of the micropipette usually caused the meter to jump from 20 millivolts to a normal reading approaching 80 millivolts. The photograph in Figure 6 shows the experimental setup with the microscope removed.

Test Solution Control System

The need to have a system where the extracellular calcium ion concentration could be accurately controlled led to the development of the test solution control system. It was desirable to be able to change the calcium concentration during the tests, and it was necessary that the change

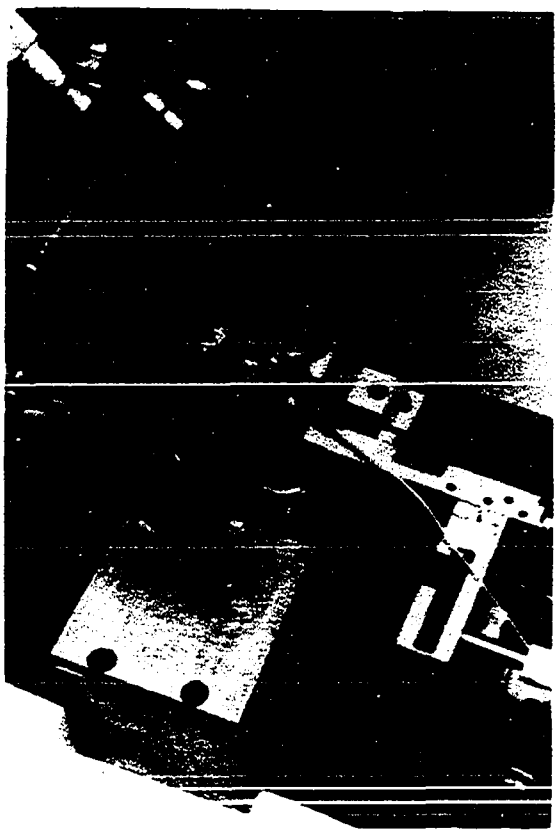

Figure 6. Experimental setup with the microscope removed

be accomplished without interrupting the flow to the nerve chamber, since any variation in the flow rate usually dislodged the micropipette. Figure 7 shows a diagram of the system that was developed. To aid in understanding the operation of the system it should be assumed that the rate of flow from reservoir 1 is equal to the rate of flow from reservoir 2. The solution in reservoir 1 was identical to the modified Van Harreveld's solution except that it contained no calcium chloride or sodium chloride. The solution in reservoir 2 was identical to the modified Van Harreveld's solution except that it had twice the normal amount of sodium chloride and no calcium chloride. A variable speed tubing pump[1], having a separate pumping head[2] for each reservoir, pumped fluid to a fitting at the center of the respective reservoir. If the pumps were operated at a high enough rate the fluid level would rise in the reservoir until it reached an overflow fitting placed at the top of each reservoir. The overflow fitting on each reservoir was connected through a piece of tubing to an individual four liter container for each reservoir. This overflow technique was used to ensure a constant driving force for the fluids going to the nerve chamber. The output of each reservoir came from the bottom of the container. The output from reservoir 1 flowed through a 52.5 centimeter piece of small diameter polyethylene tubing[3] to mixing chamber 1. The

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[1]Number 7545-15 Masterflex tubing pump, Cole-Parmer Instrument Co. 7425 North Oak Park Ave., Chicago, Illinois.

[2]Number 7015 Masterflex pump head, Cole-Parmer Instrument Co. 7425 North Oak Park Ave., Chicago, Illinois.

[3]Catalog Number PE 190, Intramedic polyethylene tubing, Clay-Adams Inc. 141 East 25th St., New York, New York.

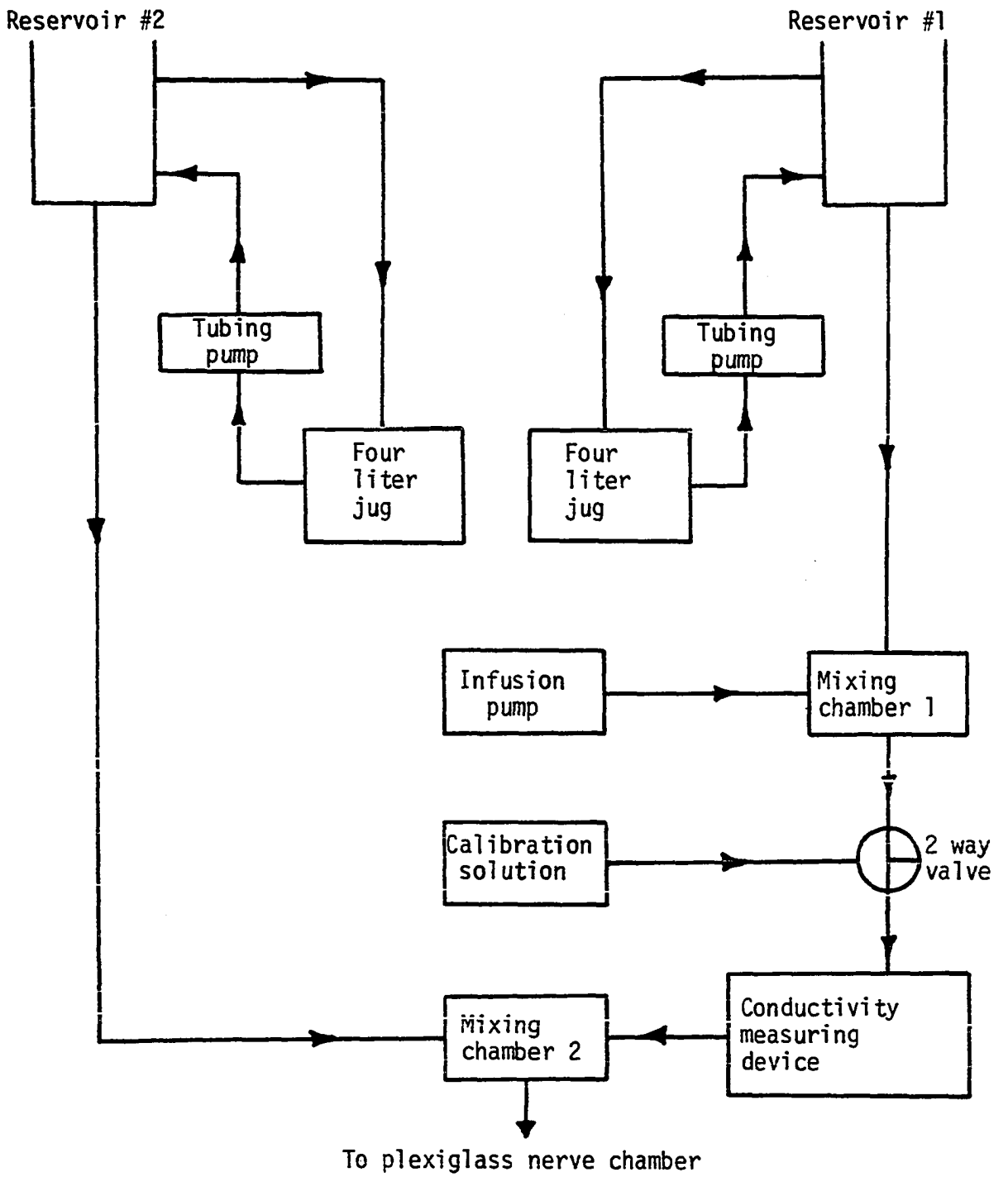

Figure 7. Flow system diagram

length of the tubing was adjusted to provide a flow rate of 8 milliliters per minute. The output of the infusion pump[1] also flowed to mixing chamber 1. Figure 8 shows a small D.C. motor[2] which has a teflon coated magnetic stirring bar glued to its shaft. An identical teflon coated stirring bar was sealed in the plexiglass container that forms mixing chamber 1. When the motor rotated the stirring bar on its shaft, the stirring bar sealed in the mixing chamber also rotated causing the fluids in the chamber to become thoroughly mixed. A 50 cc glass syringe was driven by the infusion pump and was used to introduce a 2 molar calcium chloride solution into mixing chamber 1. The infusion pump had a provision for controlling the infusion rate with a D.C. power supply[3] allowing the pump to be remotely controlled. The infusion pump was required to provide approximately .19 ml/min to obtain 13.5 millimoles calcium chloride in the test solution that reached the nerve chamber at a rate of 16 ml/min. The output of mixing chamber 1 went to the conductivity monitoring device.

Conductivity Monitor

The main component of the solution control was a conductivity monitoring device having a capacitively coupled probe. This probe allowed the device to be used with solutions that would cause conventional probes to

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[1]Dual infusion/withdrawal pump, Model 946, Harvard Apparatus Co., Inc. Mills, Massachusetts.

[2]Model RE-56 Mabuchi dc motor, distributed by Aristo Craft. Newark, New Jersey.

[3]Model IP-18, regulated power supply, Heath. Benton Harbor, Michigan.

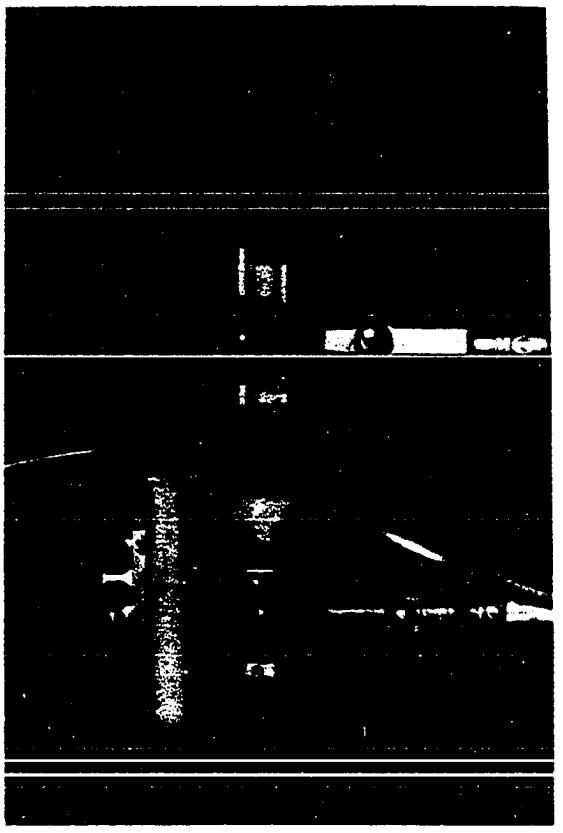

Figure 8. Mixing chamber, stirring magnets, and dc motor

polarize and to become contaminated thus causing the output to drift. Figure 9 shows a diagram illustrating the construction of the capacitively coupled electrode. Figure 10 shows a simplified electrical model of the A.C. portion of the capacitive conductivity monitor. V_s was an oscillator providing a square wave which was used to excite the system. Capacitors C_1 and C_2 were formed from two identical rectangular pieces of copper foil by wrapping the foil around the outside of the glass tube. The combination of the foil plates, the glass tube and the solution flowing through the inside of the glass tube formed the capacitors C_1 and C_2. The resistance R_f was the lumped resistance of the fluid from one copper plate to the other copper plate along the axis of the glass tube. Resistor R_L was the input impedance represented by some voltage measuring device.

Equation (1) describes the output voltage V_L as a function of the input voltage V_s.

$$|V_L| = \frac{R_L |V_s|}{[|Z_c|^2 + (R_F+R_L)^2]^{\frac{1}{2}}} \qquad (1)$$

where $|Z_c|$ was the magnitude of the impedance of the series combination of capacitors C_1 and C_2. The equation indicates that to make V_L more sensitive to changes in R_F and thus to changes in conductivity the values of R_L and Z_c should be small compared to R_F. Unfortunately decreasing R_L also decreases the magnitude of V_L. Therefore R_F was made as large as possible by using glass tubing having a small inside diameter and Z_c was made as small as possible by using foil plates having a large area and by using thin walled glass tubing.

Figure 11 shows the circuit diagram of the conductivity monitor and its connection to the capacitively coupled probe. The oscillator V_s was

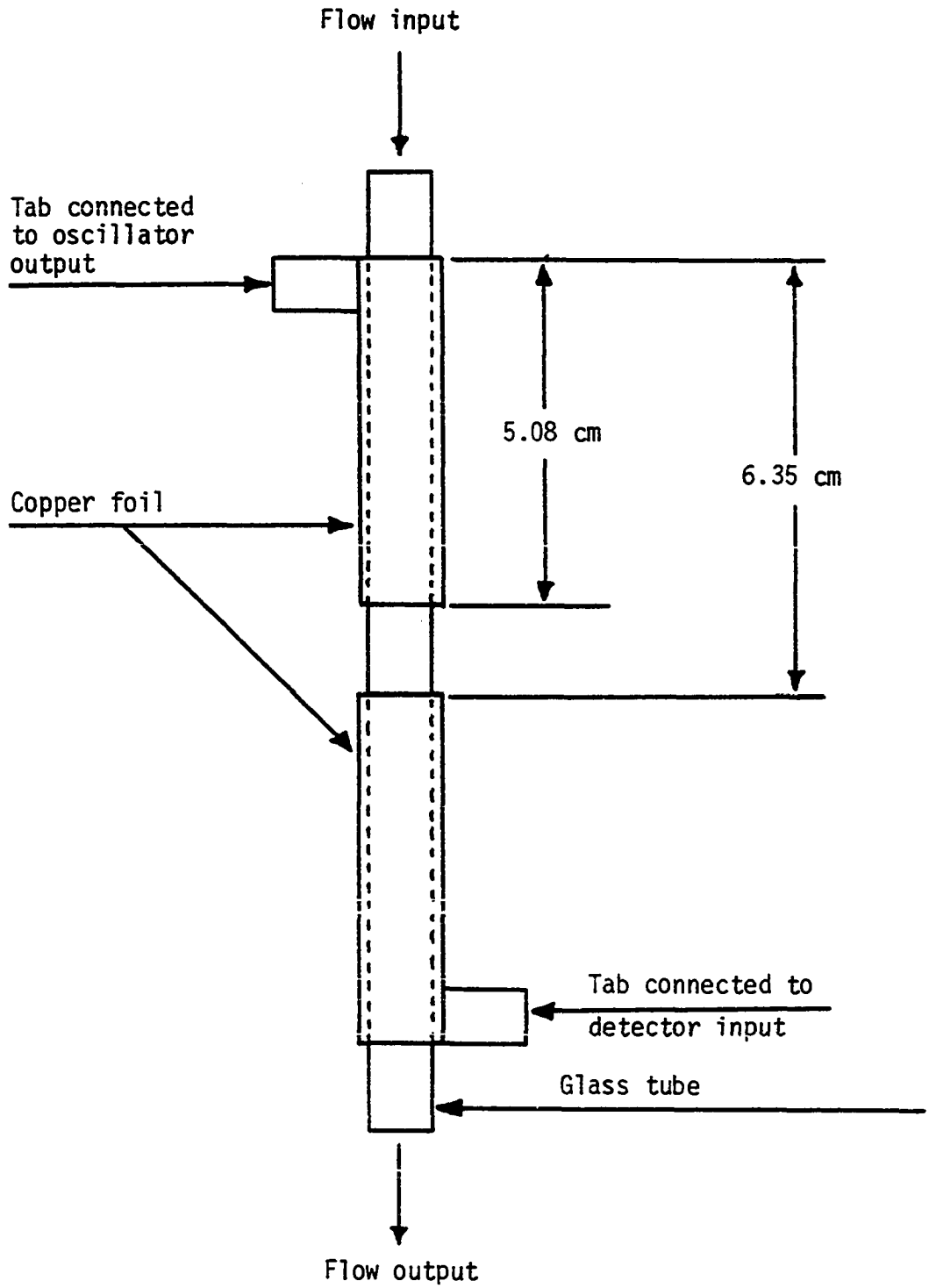

Figure 9. Electrode construction

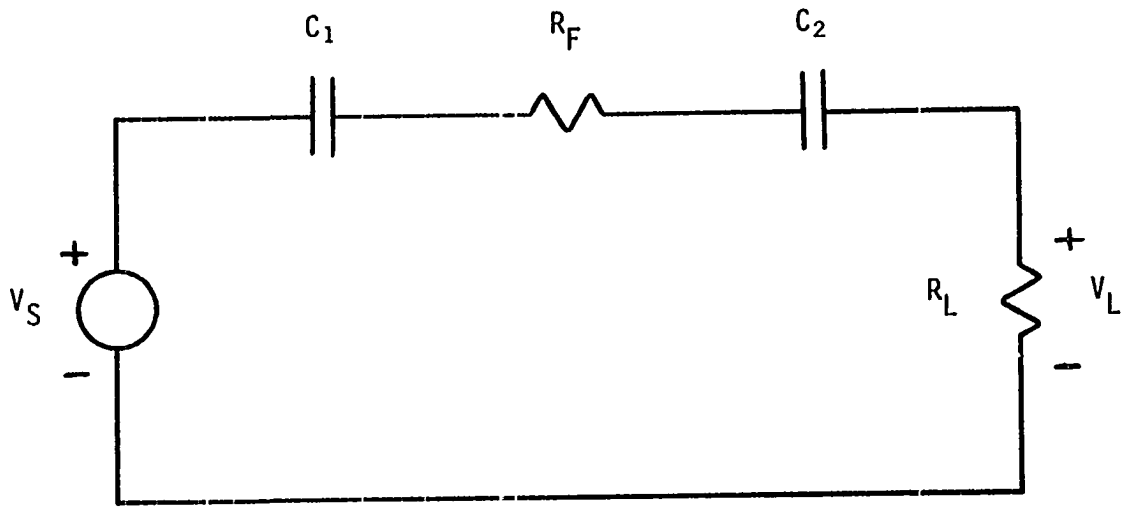

Figure 10. Electrical model of A.C. portion of the capacitive conductivity meter

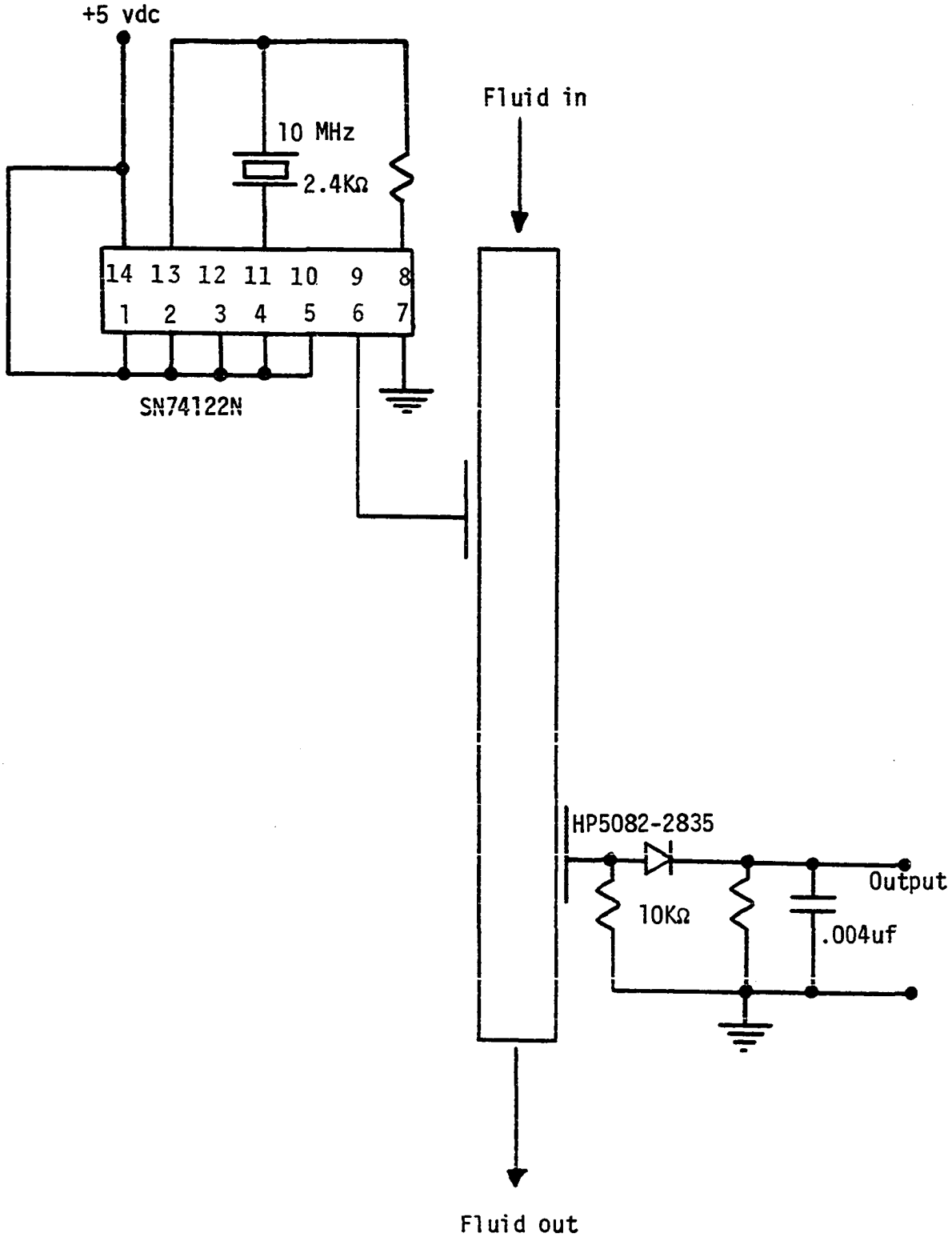

Figure 11. Capacitive conductivity monitor

constructed using a SN 74122N monostable multivibrator, a 2.4 x 10^3 ohm resistor and a 10^7 Hertz piezoelectric crystal. The output of V_s connected to the first foil plate consists of a 5 volt square wave having a frequency of 10^7 Hertz. The second foil plate was connected to a simple rectification circuit whose output was a D.C. voltage whose magnitude was a function of the conductivity of the fluid flowing through the glass tube. The magnitude of the output voltage was amplified by a circuit having a gain of 450 and its output was connected to a digital voltmeter.[1] The output of the conductivity meter was quite sensitive to changes in the temperature of the test solution. Figure 12 shows the voltage that was read on the digital voltmeter as a function of temperature for a 27 millimolar solution of calcium chloride. The temperature of the test solution was monitored with a thermistor[2] attached to the center of the glass tube of the capacitively coupled probe.

To calibrate the conductivity monitor, a solution of calcium chloride having twice the concentration of the desired calcium concentration for the test run was passed through the conductivity monitor. Twice the desired test solution concentration was used since the calcium concentration was divided by a factor of two in the second mixing chamber. For example if the test run was to pass twelve millimoles of calcium over the nerve bundle then a solution of twenty four millimoles of calcium ions was used for calibration. By noting the output voltage obtained from the calibra-

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[1]Model 330, digital multimeter, Darcey. Santa Monica, California.

[2]Number 44201, thermilinear component, Yellow Springs Instrument Co. Yellow Springs, Ohio.

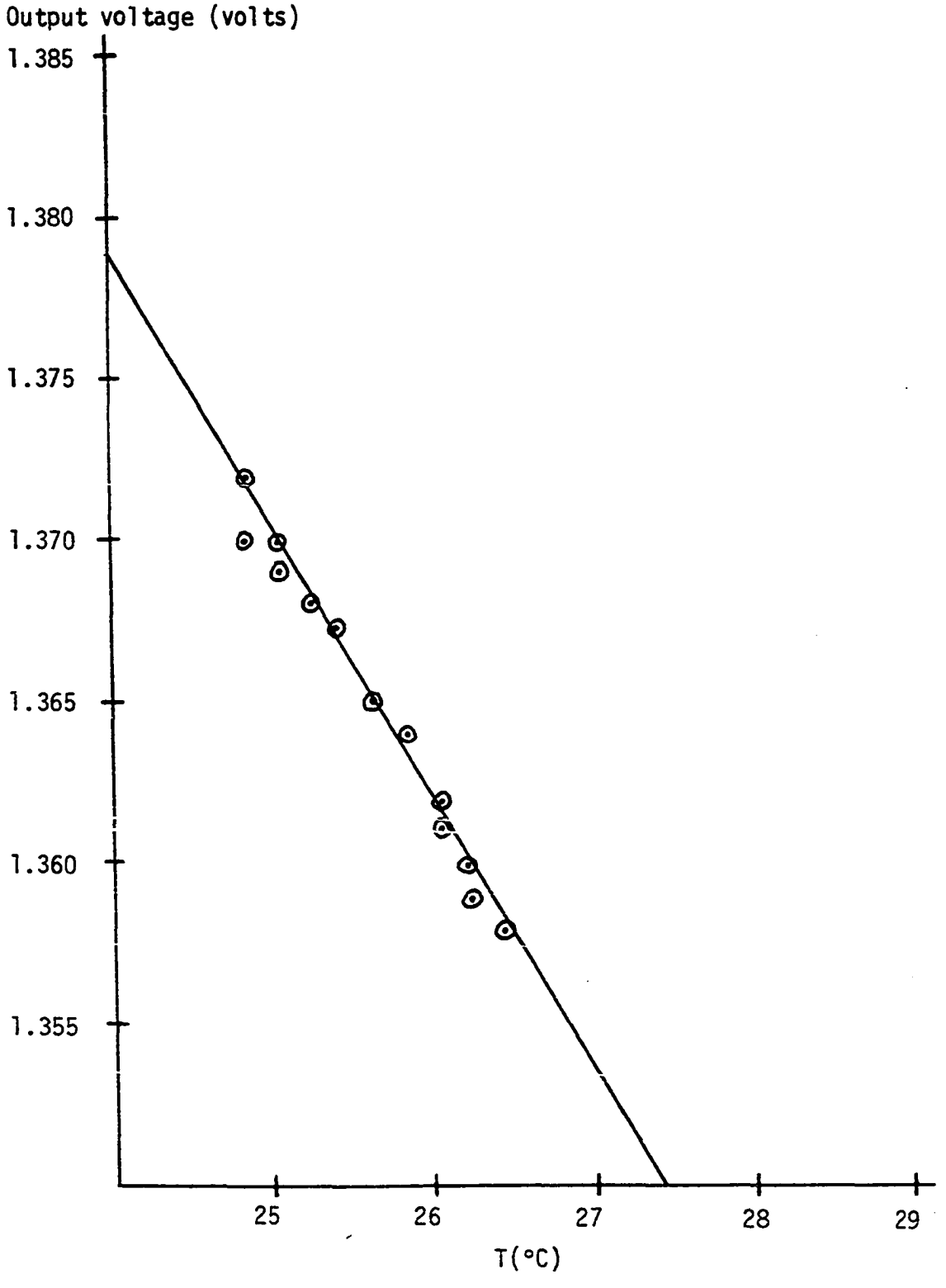

Figure 12. Output voltage as a function of temperature

tion solution and the temperature of the calibration solution a pair of standard values could be established. The calibration solution was then removed from the system and the test solution reconnected. The speed of the infusion pump was then adjusted to obtain a value of output voltage that was equal to the value of the output voltage obtained with the calibration solution. If the temperature of the test solution was not equal to the temperature of the calibration solution then equation 2 was used to obtain the corrected value for the output voltage. Equation 2 expresses the relationship of output voltage as a function of temperature obtained from Figure 12.

$$V_2 = V_1 - 1.656\ (T_2 - T_1) \qquad (2)$$

V_1 and T_1 are the output voltage and temperature respectively for the calibration solution. V_2 is the corrected output voltage that should be obtained by varying the infusion pump speed. T_2 is the test solution temperature. The output of the conductivity monitor and the output of reservoir 2 flowed into mixing chamber 2, which is identical in construction to chamber 1. The tubing that connected reservoir 2 and mixing chamber 2 is identical to the tubing that connected reservoir 1 and mixing chamber 1. The length of the tubing was adjusted so that the flow from reservoir 1 equaled the flow from reservoir 2. This was very important to insure that the sodium concentration was normal and the calcium concentration was one half that of the fluid flowing through the conductivity monitor. The output of mixing chamber 2 went to the input of the nerve chamber. The total rate of flow to the nerve chamber was 16 ml/min and the solution was the same as the modified Van Harreveld's solution except

for the concentration of calcium chloride which was controlled by the speed of the infusion pump.

The output flow of the nerve chamber was removed by the vacuum produced by an aspirator which was operated by the flow of tap water. A four liter jug was placed in the vacuum line and the waste test solution was collected in it. The jug was used to filter out any variations in the vacuum which were produced by variations in the flow rate of the tap water.

Solution Preparation

The distilled water which was available in the Biomedical Engineering Program at Iowa State University was obtained by condensing steam from the university heating system. It was discovered that morpholine, a secondary amine, had been found in the distilled water obtained in this manner. To avoid any question about the purity of the water used to prepare the solutions, it was decided to construct a small water still. The water collected from the still was passed through a water cartridge[1] to ensure even greater purity. The water cartridge had a multiple resin bed with selected cation and anion beds. It was used to eliminate any splash over that might have occurred from the boiler in the still.

At the very beginning of the project Van Harreveld's solution (43) was used as a physiological solution for the crayfish. After checking the pH of a batch of prepared solution several days apart and finding that the

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[1]Model D 8901 Barnstead standard water cartridge, distributed by Fisher Scientific, catalog # 9-035-20. 1458 N. Lamon Ave., Chicago, Illinois.

pH had changed by two units, it was decided to modify Van Harreveld's solution by replacing the $NaHCO_3$ with the standard biochemical buffer commonly called tris buffer. To prepare this buffer, Solution A was prepared by mixing 19.6 grams maleic anhydride and 24.2 grams tris (hydroxymethyl) aminomethane in 1000 ml distilled water, Solution B was a .02M NaOH and the final buffering solution was prepared by mixing 1036 ml of Solution A, 1121 ml of Solution B, and 1842 ml of distilled water together. To prepare 1000 ml of the modified Van Harreveld's solution, 12.6 grams NaCl, 1.5 grams $CaCl_2$, .4 grams KCl, .25 grams $MgCl_2$, and 100 ml of the buffering solution were mixed. Distilled water was then added to bring the total volume to 1000 ml. This resulted in a solution having a pH that was stable for several weeks of approximately 7.3. The pH was measured with a Beckman Expandomatic pH meter.[1]

Recording System

Electronics

The recording system used to monitor the steady state membrane potential and the transient action potential of a single giant nerve fiber was composed of a microelectrode, a low noise microelectrode headstage preamplifier, and a signal conditioner. Figure 13 shows the circuit diagram of the headstage preamplifier and the signal conditioner. In addition to the amplifiers, an electrode test oscillator used to measure the electrode tip resistance and an electrode balance voltage supply used to balance

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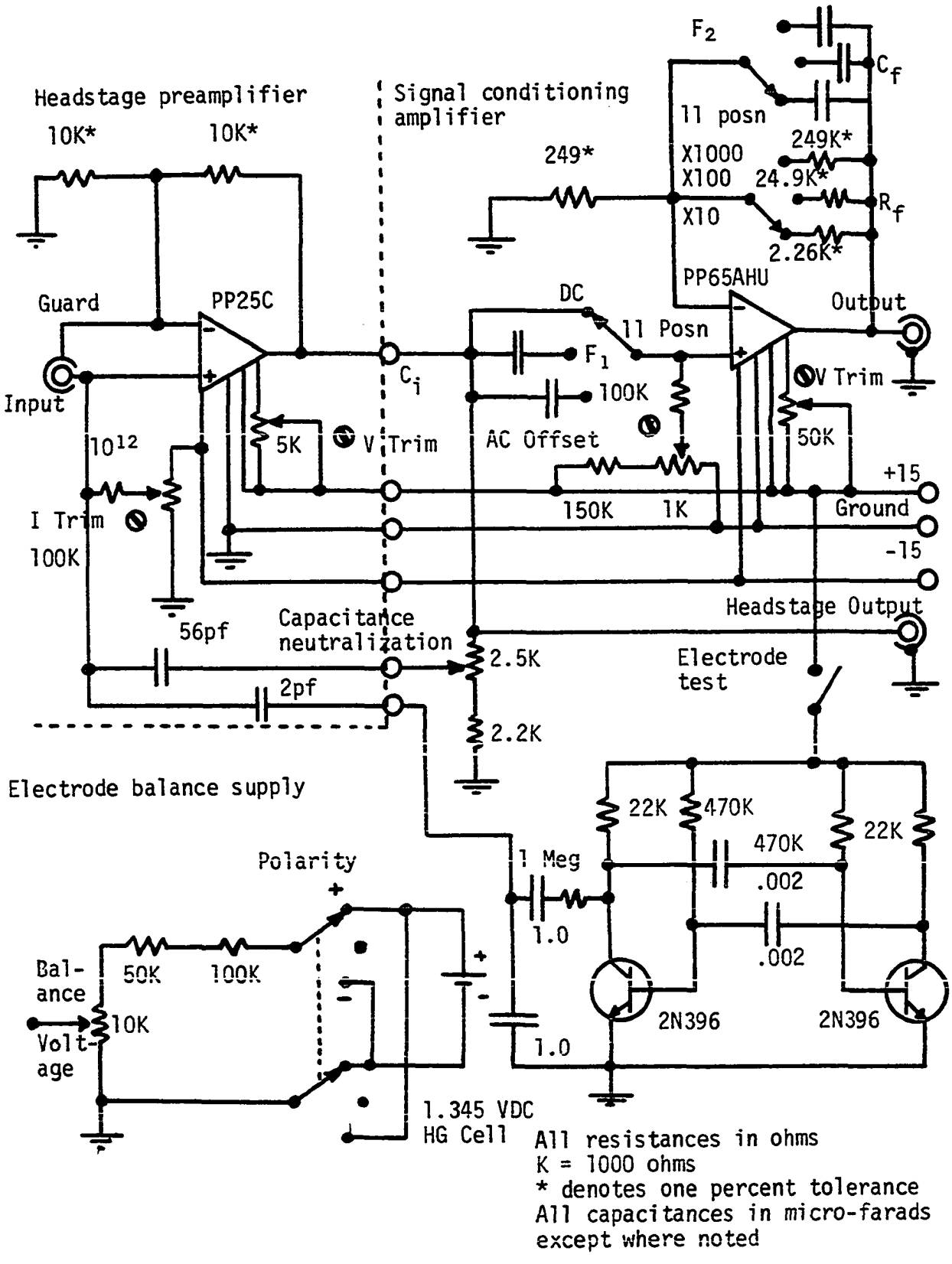
[1]Expandomatic pH meter, Beckman Instruments Inc. Fullerton, California.

Figure 13. Circuit diagram of the headstage preamplifier and signal conditioner


Headstage preamplifier
10K*
10K*
Signal conditioning amplifier
249*
F2
Cf
11 posn
249K*
X1000
X100
24.9K*
Rf
X10
2.26K*
PP65AHU
Output
Guard
PP25C
DC
11 Posn
F1
Ci
100K
V Trim
Input
10^12
5K
V Trim
AC Offset
50K
+15
150K
1K
Ground
I Trim
100K
-15
56pf
Capacitance neutralization
Headstage Output
2.5K
Electrode test
2pf
2.2K
Electrode balance supply
22K
470K
470K
22K
Polarity
1 Meg
.002
1.0
.002
Bal-ance
50K
100K
10K
Volt-age
2N396
2N396
1.0
1.345 VDC
HG Cell
All resistances in ohms
K = 1000 ohms
* denotes one percent tolerance
All capacitances in micro-farads
except where noted

electrode tip potentials were included in the system. The electronics that comprised the recording system were designed and constructed by W. H. Brockman, 1971. The headstage preamplifier was constructed using an operational amplifier[1] utilizing a field effect transistor input stage to maximize input resistance and minimize input current requirements (Graeme et al., 1968). The input resistance to the headstage preamplifier was approximately 10^{12} ohms. Because of the high input resistance the effective distributed capacitance at the tip of a microelectrode and at the input of the headstage amplifier had to be reduced if adequate high frequency response was to be realized. A positive-feedback (negative-capacitance) scheme was used to effectively reduce the tip and input capacitance. The amount of feedback was controlled by adjusting the capacitance neutralization potentiometer. A triangular wave was generated by the test oscillator and was coupled through a 2 picofarad capacitor to the input of the headstage preamplifier. A square wave resulted when the proper feedback was obtained because the resistive tip impedance of the micropipette in series with the 2 picofarad capacitor formed a differentiating circuit. The magnitude of the square wave obtained at optimum neutralization was used as an indication of the magnitude of the electrode resistance. Approximately one hundred microvolts at the output of the headstage preamplifier indicated an electrode resistance of 10^6 ohms. The electrode balance supply was used to eliminate the effects of constant liquid to liquid to metal junction potentials. With the micropipette tip immersed

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[1] Type PP25C operational amplifier, Philbrick Research, Inc. Dedham, Massachusetts.

in the test solution and the input ungrounded, the correct polarity was selected and the balance potentiometer was adjusted until the output of the headstage preamplifier returned to zero volts. The magnitude of the membrane potential was monitored by a meter placed at the output of the headstage amplifier. Since the headstage amplifier has a gain of two, the meter was adjusted to read two hundred millivolts from midscale to full-scale. The scale on the front of the meter was then changed to -100-0-+100 millivolts.

The output of the headstage preamplifier was connected to the signal conditioning amplifier either directly or through a coupling capacitor C_I. The lower -3db gain cutoff frequency was a function of C_I and was given by the following equation.

$$F_{-3db} = \frac{1}{2\pi C_I \, 10^5} \tag{3}$$

The signal conditioning amplifier had provision for varying the gain and the upper -3db gain cutoff frequency by adjustment of the impedance in the feedback path from the output of the operational amplifier[1] to its input. The upper -3db gain cutoff frequency was a function of both R_F and C_F and was given by the following equation.

$$F_{-3db} = \frac{1}{2\pi R_F \, C_F} \tag{4}$$

The output of the signal conditioning amplifier was connected directly to an oscilloscope.[2] This oscilloscope was used to set the optimum positive

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[1]Type PP65AHU operational amplifier, Philbrick Research, Inc. Deadham, Massachusetts.

[2]Type RM561A oscilloscope with type 3A6 dual trace amplifier and type 3B1 time base, Tektronix. Beaverton, Oregon.

feedback, balance the offset voltages, and to accurately measure the values of membrane potential and action potential height. The output of the signal conditioning amplifier was also connected to a high impedance probe[1] which was used in conjunction with a high performance ac preamplifier.[2] The output of the ac preamplifier was connected to the 'tape in' channel connector on the back of an oscilloscope[3] which had a variable persistance storage cathode ray tube for visual monitoring. The function of the ac preamplifier was to allow the vertical scale on the storage oscilloscope to be accurately defined. The high impedance probe was not specifically necessary but was used as a convenience because its output connector matched the input connector of the ac preamplifier. A permanent record of an action potential was obtained by photographing the trace stored on the storage monitor using an oscilloscope camera.[4]

To minimize electrical interference due to fluorescent lamps, pumps, motors, electrostatic discharges, and radio frequency sources, a screened cage was built. While a small cage would have been adequate, it was decided to build a screened cage in which it was possible for a person to work. The cage which was built for the experiments measured approximately

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[1]Grass model HIP511 DR B FET probe, Grass Medical Instruments. Quincy, Massachusetts.

[2]Grass model P511 DR ac preamplifier, Grass Medical Instruments. Quincy, Massachusetts.

[3]Model 1510A electromyograph storage monitor, Hewlett Packard Medical Instruments. Waltham, Massachusetts.

[4]Model HP 197A oscilloscope camera, Hewlett Packard Medical Instruments. Waltham, Massachusetts.

two meters high by two meters deep by two meters wide and was constructed using 5 cm by 5 cm pine for the frame. Angle brackets and wood screws were used to construct the framework and it was covered with aluminum screening which was stapled in place. The cage was built without a floor to prevent walking people from producing vibrations which would dislodge the recording electrode from the nerve fiber. A small 7.5 cm by 7.5 cm opening was made in the screening to allow cables to be brought into and out of the cage.

Electrodes

The recording electrodes which were used to monitor the steady state membrane potential and the transient action potential of a giant nerve fiber were formed from Kimax #34500 glass capillary tubes.[1] The glass tubes were first soaked in acetone for thirty minutes and then soaked in concentrated hydrochloric acid for thirty minutes. The tubes were then soaked in filtered, distilled water for thirty minutes and then dried in an oven at 100° C. The micropipettes were then formed by pulling them on a David Kopf #700B vertical pipette puller.[2] Some experimentation was required to determine heater and solenoid settings which will give the desired taper length and tip diameter. For the #700B, a heater current of twenty-one amperes and a solenoid setting of 50 produced micropipettes having a tip diameter of less than 10^{-6} meters and a taper length of 10^{-2} meters. These dimensions were measured using an optical microscope. Figure 14 shows two photographs of typical electrodes as observed with a

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[1] Kimble Products, Owens-Illinois. Toledo, Ohio.

[2] David Kopf Instruments. P. O. Box 636, Tujunga, California.

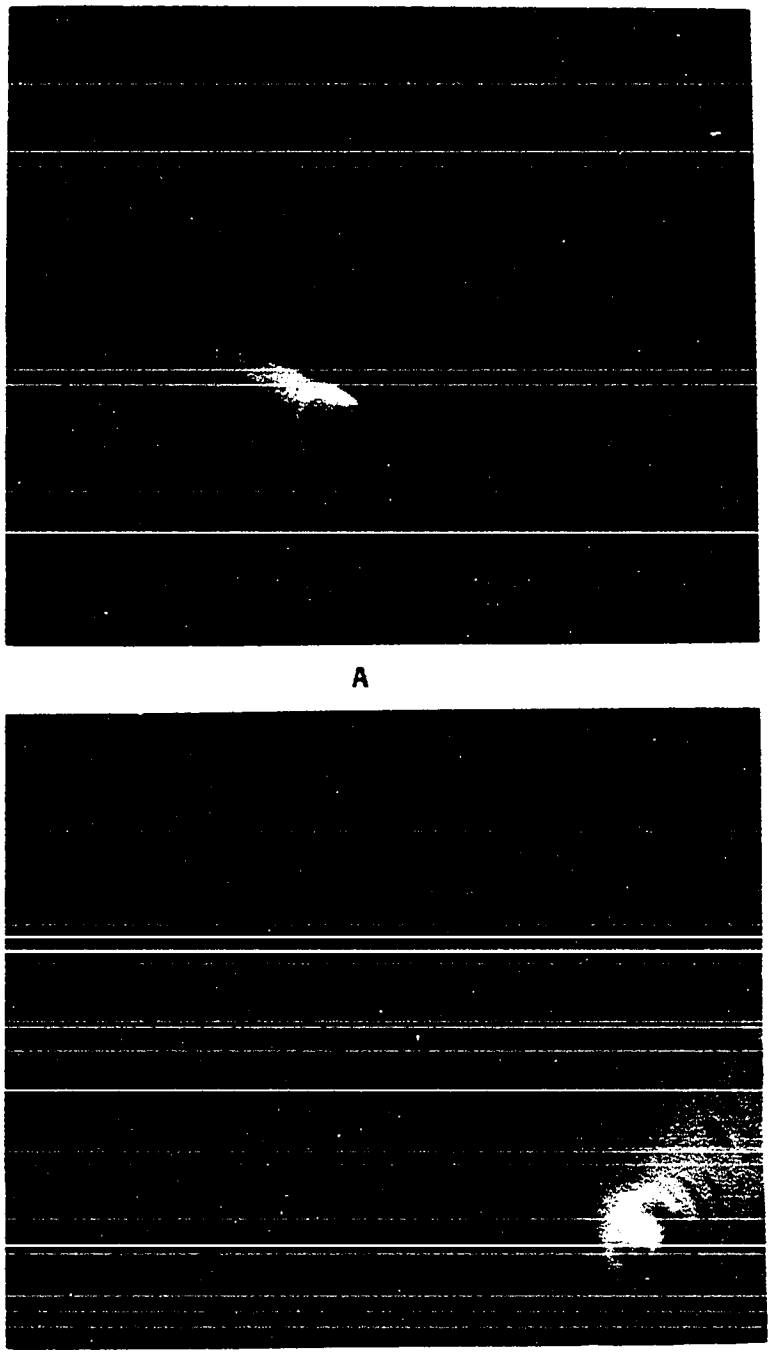


Figure 14. Scanning electronmicrograph of a micropipette tip (x2500)

A) Oblique view B) End-on view

scanning electron microscope. The tip can be clearly seen and has a diameter of approximately 1×10^{-6} meters. The resistance of this electrode was measured and found to be 11×10^{6} ohms.

Snell (1969) concluded that the tip diameter could be calculated from the following equation:

$$a(f) = \frac{\gamma l}{b\pi\Gamma}$$

where $a(t)$ = tip diameter in meters

l = taper length in meters

b = inside radius of glass capillary tubes in meters

γ = resistivity of the electrolyte in ohm · centimeters

Γ = tip resistance in ohms

Using this relationship a micropipette having a tip diameter of less than 10^{-6} meters and a taper length of 10^{-2} meters should have a tip resistance of greater than 10^{7} ohms. Measurements made on the scanning electron microscope support this conclusion.

After pulling, the micropipettes were placed in a beaker of filtered methyl alcohol. The alcohol in the beaker containing the micropipettes was boiled by placing the beaker in a desiccator and connecting the desiccator to an aspirator. The vacuum produced in the desiccator was just enough to pull air bubbles from the micropipettes at the approximate rate of one per second. The end of bubbling indicated that the micropipettes were filled. They were then placed in two rinses of filtered distilled water, each lasting for thirty minutes. The micropipettes were then removed from the distilled water and placed in a beaker containing a 3 M solution of potassium chloride. The micropipettes were allowed to

soak in the potassium chloride for at least six hours to allow the micropipettes to become completely filled. The micropipettes were now microelectrodes and could be stored in the potassium chloride for up to two days, Resistance tests indicated that approximately 20% of the microelectrodes had a tip diameter less than 10^{-6} meters.

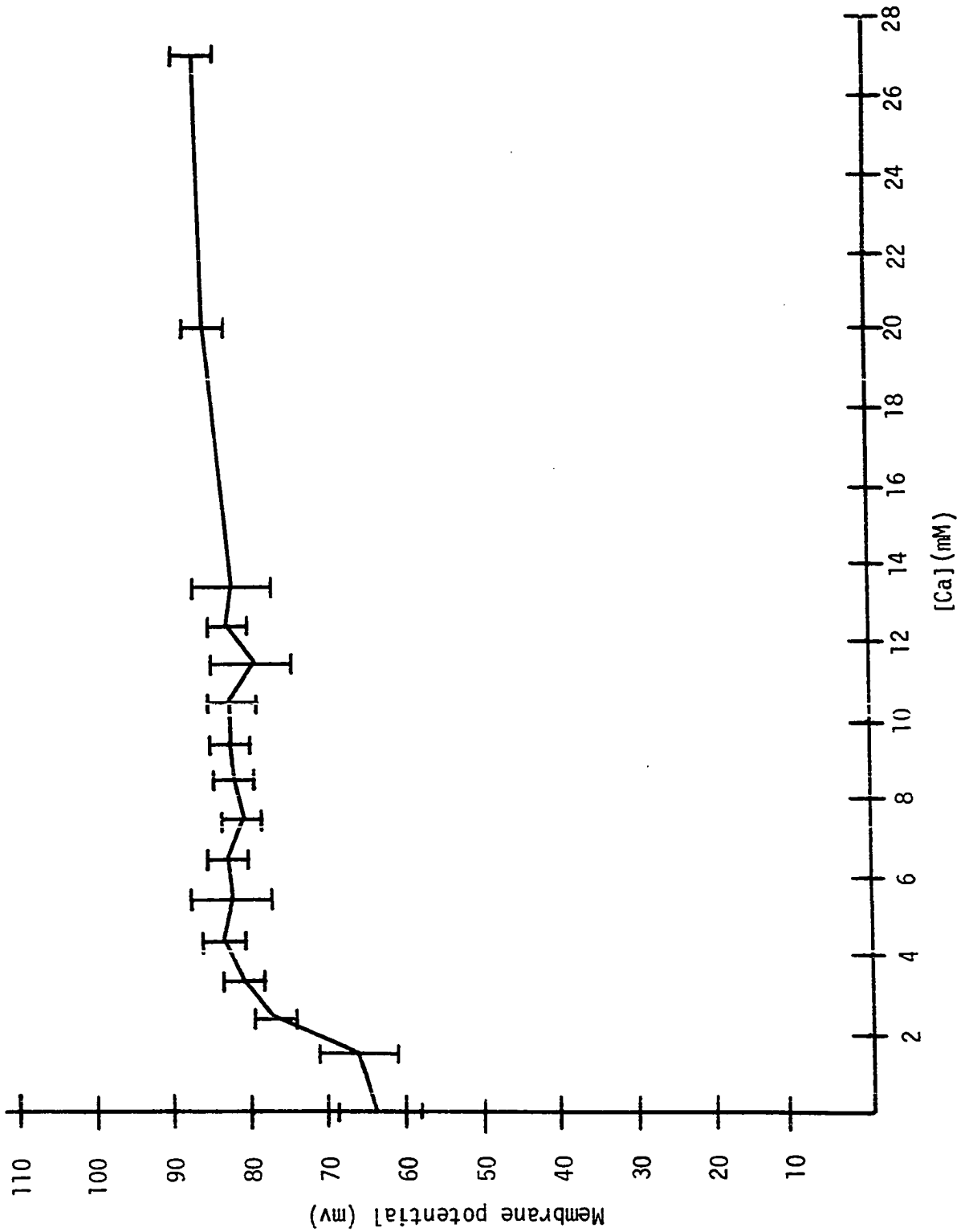
To combat the problems of a shifting D.C. baseline caused by polarization, a silver-silver chloride electrode was used to interface between the recording microelectrode and the amplifier. To reduce the tip offset potential difference a piece of eighteen gauge polyethylene tubing filled with a three molar potassium chloride solution and agar was used to connect the bathing fluid and the silver-silver chloride electrode connected to the low side of the amplifier input. Examination of the sum of the offset potentials shows that when the potential produced by inserting the silver-silver chloride electrode into the 3 M potassium chloride solution filling the micropipette, the potential produced at the tip of the micropipette when it is inserted into the nerve cell, the potential produced by inserting the KCl/agar filled piece of polyethylene tubing into the bathing solution, and the potential produced by inserting the other silver-silver chloride electrode into the end of the KCl/agar filled piece of polyethylene tubing are all added up they will theoretically sum to zero. In practice the offset potential difference was found to be less than one millivolt.

RESULTS AND DISCUSSION

An extensive series of experiments, as outlined in the section on methodology, were performed on the isolated ventral nerve cord of the crayfish. In order that a statistical average could be obtained and thus to allow for minor variations in animals and technique, the nerve cords from five crayfish were subjected to each change in calcium concentration. Repeatability and consistency of the experiments was excellent. Out of a total of eighty-five experiments only two were discarded and these were discarded because of an error made in mixing a particular test solution used in these experiments.

The concentration of calcium ions in the bathing solution was reduced in 1 millimole steps starting at the reported normal value of 13.5 millimoles (Van Harreveld, 1936). It was decided to limit the changes to 1 millimole because it was felt that a change that was 10% of the normal value would give adequate resolution without requiring a burdensome number of experiments. In all the tests the concentrations of sodium, potassium, and magnesium were maintained at values which have been reported as normal for the crayfish. Van Harreveld (1936) determined that a one liter solution containing 12 grams of sodium chloride, 1.5 grams of calcium chloride, .4 grams of potassium chloride, .25 grams of magnesium chloride, and .2 grams of sodium bicarbonate could be used as a physiological saline for crayfish. These values were used to prepare the physiological saline except for the changes made in the buffer described in the section on solution preparation. Figure 15 shows a plot of the average membrane potential as a function of calcium ion concentration. Each data point

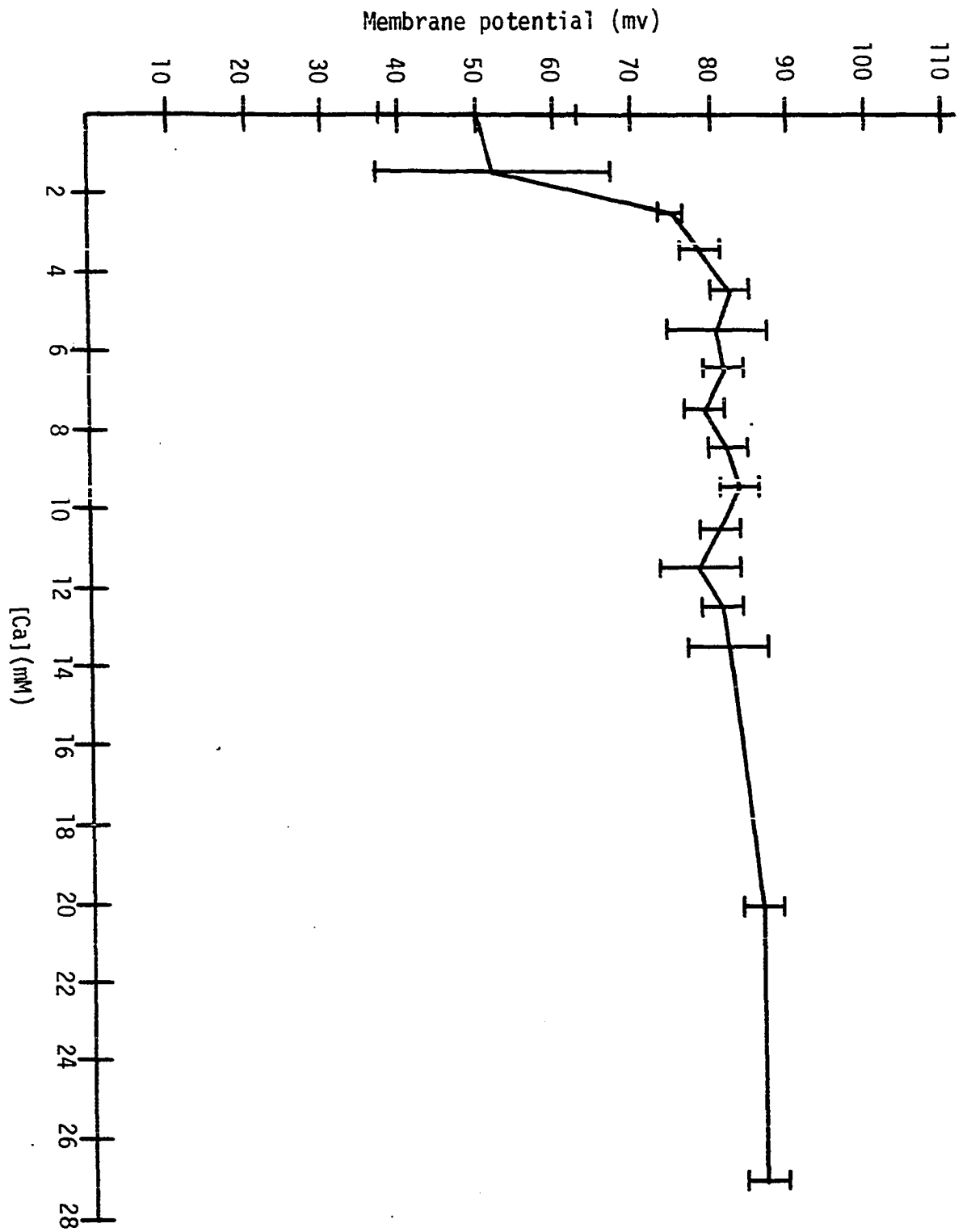
Figure 15. Membrane potential 15 minutes after insertion of the micropipette. Vertical bars at the data points are calculated standard deviations


Membrane potential (mv)
110
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
12
14
16
18
20
22
24
26
28
[Ca] (mM)

represents the average of the data obtained from a total of five crayfish. The data points were taken fifteen minutes after insertion of the microelectrode to allow the membrane potential to stabilize. Figure 15 also shows the standard deviation of the data taken for the average membrane potential as a function of calcium ion concentration. A vertical bar at each data point indicates the magnitude of the standard deviation of the data at that point.

Figure 16 shows a plot of the average membrane potential as a function of calcium ion concentration. Again, each data point represents the average of the data obtained from a total of five crayfish. The data points were taken from the same animals represented in Figure 15 but forty-five minutes after insertion of the microelectrode. The second set of data points were taken thirty minutes after the first set of data points to see if an equilibrium condition had been reached. In general the second set of data did not differ substantially from the first set of data. Figure 16 also shows the standard deviation of the data taken for the average membrane potential in Figure 16 as a function of calcium ion concentration. A comparison of Figure 15 with Figure 16 shows that for calcium ion concentrations above 3 millimoles the membrane potential reaches equilibrium within fifteen minutes. Below 3 millimoles there is an abrupt decrease in membrane potential with respect to calcium ion concentration that reaches a final value of 50 millivolts. It should be noted that the slope of the curve below a calcium ion concentration of 3 millimoles increases quite abruptly. This could indicate a dramatic change in the characteristics of the membrane caused by the decreased calcium ion concentration. A theory presented by Tasaki (1968), which

Figure 16. Membrane potential 45 minutes after insertion of the micropipette. Vertical bars at the data points are calculated standard deviations


Membrane potential (mv)
10
20
30
40
50
60
70
80
90
100
110
[Ca] (mM)
2
4
6
8
10
12
14
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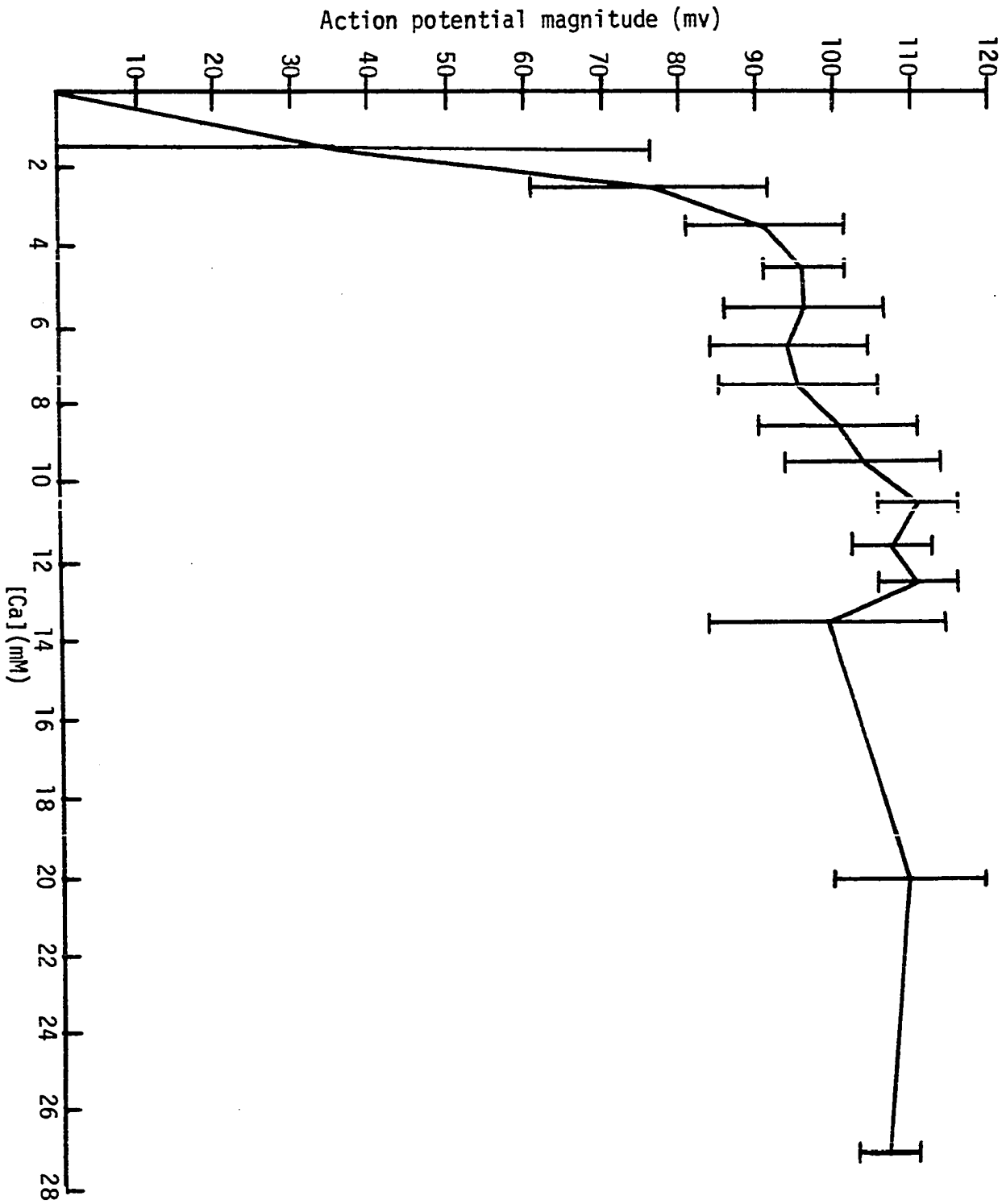
18
20
22
24
26
28

will be discussed further in the section, states that if calcium ions are displaced from the membrane they will be replaced by potassium ions. Tasaki also states that an excess of potassium ions in the membrane will cause depolarization and a lack of ability for the membrane to produce an active response. And in fact, as will be seen from the results of this research, the action potential failed to propagate at calcium ion concentrations below 3 millimoles. While the membrane potential reached equilibrium within fifteen minutes at calcium ion concentrations above 3 millimoles, such was not the case at concentrations below 3 millimoles. Notice that even after fifteen minutes the membrane potential continued to decrease to a final value of approximately fifty millivolts. This could indicate that as a result of the abrupt change in the membrane characteristics a quantity of calcium ions was freed from the bonding structures and proceeded to passively diffuse from the membrane. As the concentration of calcium ions within the membrane decreased and the potassium ion concentration increased the membrane potential decreased to a new steady state value.

Data points were also taken at calcium ion concentrations above the normal value of 13.5 millimoles. Dalton, in 1959, reported that increasing the calcium ion concentration of the extracellular fluid above the normal value does not have an effect upon the magnitude of the resting potential. The data points taken at concentrations of 15.5, 20 and 27 millimoles of calcium ions confirm the statements made by Dalton.

Figure 17 shows a plot of the average magnitude of the action potential, taken from five crayfish for each data point, as a function of calcium ion concentration. Again the measurements were taken fifteen

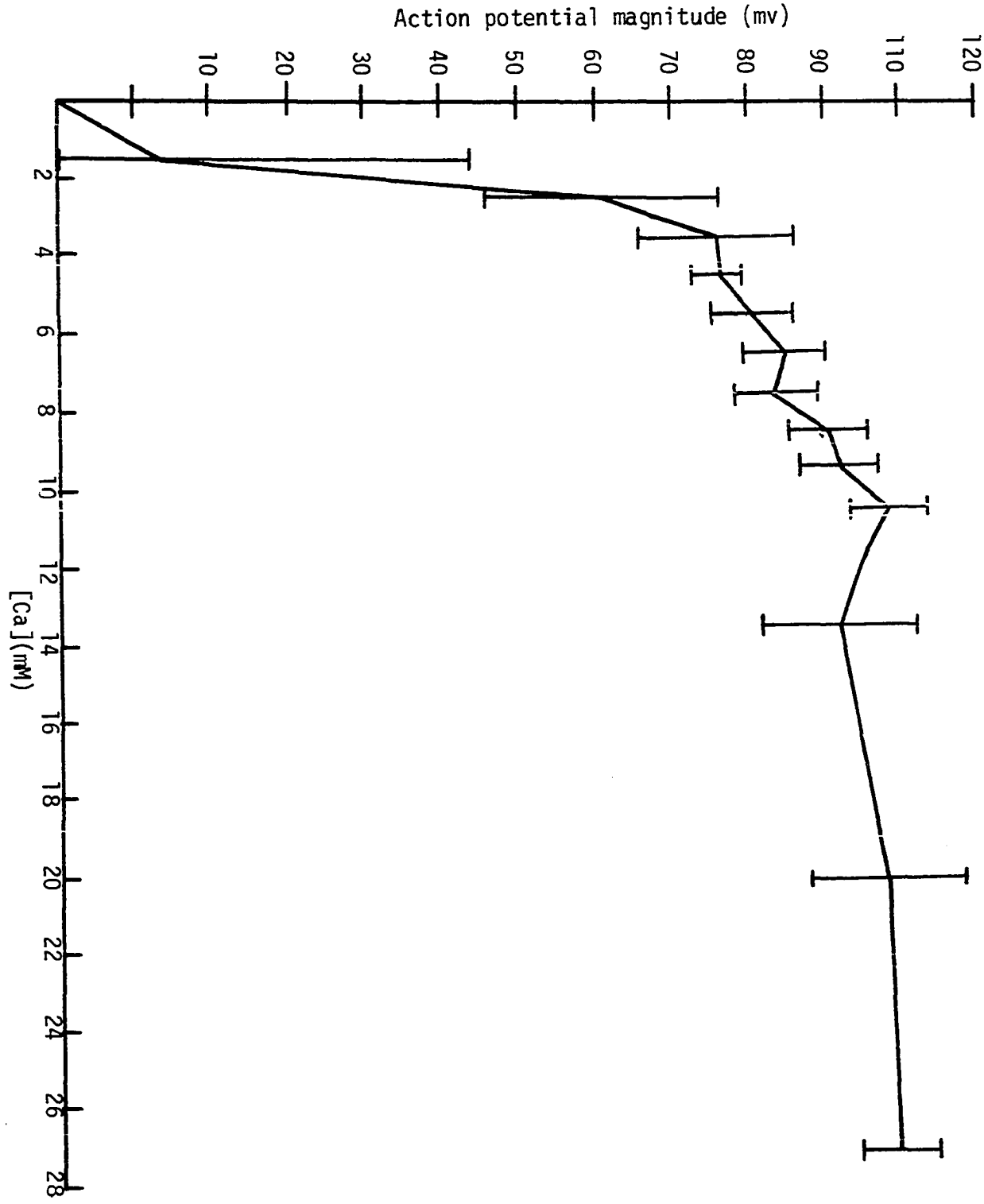
Figure 17. Action potential magnitude 15 minutes after insertion of the micropipette. Vertical bars at the data points are calculated standard deviations


Action potential magnitude (mv)
10
20
30
40
50
60
70
80
90
100
110
120
2
4
6
8
10
12
14
16
18
20
22
24
26
28
[Ca] (mM)

minutes after insertion of the microelectrode to allow the membrane to stabilize. Figure 17 also shows the standard deviation of the data taken for the average magnitude of the action potential as a function of the concentration of calcium ions. The vertical bar at each data point indicates the magnitude of the standard deviation of the data at that point. Figure 18 shows a plot of the average magnitude of the action potential, taken from five crayfish for each data point, as a function of calcium ion concentration. The data points were taken from the same animals represented in Figure 17 but forty five minutes after insertion of the microelectrode. Comparison of the data points from Figure 18 with the data points from Figure 17 indicates that for a given concentration of calcium ions the average magnitude of the action potential reaches a new state of equilibrium within fifteen minutes after changing the concentration of the solution.

Examination of Figure 18 shows that the average magnitude of the action potential reaches a peak at a calcium ion concentration of 10.5 millimoles. This seemed to be a curious place for such a phenomenon to occur especially when the normal concentration of calcium ions in the extracellular fluid of the crayfish had been reported to be 13.5 millimoles (Van Harreveld, 1936). It could be argued that the optimum concentration to have such a peak would be at the normal calcium ion concentration. This would allow the action potential to have its maximum possible value and thus reduce the possibility of failure of the active response to propagate. This would be especially advantageous for the crayfish since the blood calcium level fluctuates during the molt cycle (Passano, 1960), increasing just before molt and decreasing during the reformation of the

Figure 18. Action potential magnitude 45 minutes after insertion of the micropipette. Vertical bars at data points are calculated values of the standard deviation


Action potential magnitude (mv)
10
20
30
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110
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6
8
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14
16
18
20
22
24
26
28
[Ca](mM)

exoskeleton. It was decided to measure the calcium ion concentration of the extracellular fluid from a sampling of the crayfish which were being used in an attempt to understand why the peak occurred at a calcium ion concentration of 10.5 millimoles. Using a technique described by Van Harreveld (1936), serum was drawn from a total of five crayfish and analyzed for calcium ions with the help of a flame photometer.[1] The concentrations of the five samples were averaged and the average was found to be equal to 10.1 millimoles of calcium. This agrees very favorably with the value of 10.5 millimoles at which the peak was found. An obvious extension of this work would be to investigate the relationship of the extracellular calcium ion concentration in the crayfish as a function of the molt cycle. It would be interesting not only to find out how the calcium ion concentration varies but also to see if the point at which the maximum value of the action potential occurred varies along with the calcium ion concentration.

Further examination of Figure 18 reveals that the magnitude of the action potential decreases rapidly below a calcium ion concentration of 3 millimoles. The calcium ion concentration at which the magnitude of the action potential begins to decrease rapidly corresponds very closely to the calcium ion concentration at which the membrane potential begins to decrease rapidly. This would suggest that the reduced calcium ion concentration causes a change in the membrane characteristics that effects both sodium ion and potassium ion permeabilities.

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[1]Serum analysis performed by the Veterinary Diagnostic Laboratory at Iowa State University, Ames, Iowa.

Now that the experimental results have been examined it should be possible to explain how the changes in calcium ion concentration have caused the observed changes in the membrane characteristics. Unfortunately this endeavor is complicated by the fact that the structure and function of the membrane have never been completely described. Several hypotheses have been presented and a number of these will be reviewed to see if the data obtained in this dissertation support any particular theory.

Morphological studies performed by Robertson (1960) have shown that the axon membrane is a multilayered structure and that the excitable membrane is approximately 100 Å thick. The membrane is so thin that it is not only impossible to determine directly the concentration of various ions within the membrane but it is also extremely difficult to obtain the chemical composition of the membrane by direct chemical analysis. Treatment of the axon membrane with enzymes has indicated that the membrane is composed of proteins, lipids, and phospholipids (Tasaki, 1968). These experiments have shown that there are side-chain and terminal amino and carboxyl groups and phosphate groups in the membrane.

Baker (1966) presented a model for nerve excitation based upon the hypothesis that the excitable membrane contained two specific pores. The mechanism proposed that pores specific for potassium ions would open first, followed by the almost concurrent opening of pores specific for potassium ions and closing of the sodium pores. Mullins (1959) agreed with the pore model in that the distribution of the pore sizes strongly favored potassium ion transfer during the inactive state. He contended that it was possible that the charged membrane was deformed by the voltage

appearing across it causing the pore sizes to change. According to this hypothesis, depolarization of the membrane would cause the size of the pores to change to a configuration favoring sodium ions over potassium ions. This hypothesis hinges upon the assumption that the ions pass through the membrane in the hydrated state and that the hydrated ion radius of sodium is greater than the hydrated ion radius of potassium (Mullins, 1959). The pores would then revert to the inactive configuration which would favor potassium ions over sodium ions. This sequence of events could account for the rise and the fall in the membrane potential during excitation. However, it fails to account for the enormous increase in the interdiffusion of cations across the axon membrane during the action potential (Tasaki, 1963). According to Chapman et al. (1966), the phospholipids that are in biological membranes appear to be in a fluid state. This would suggest that it would be unlikely that small, rigid pores, as suggested by Baker, would exist in the phospholipid layer of the membrane. Tasaki (1968) suggested that it would seem reasonable to regard any pores that might exist in the membrane as dynamic, time dependent structures, rather than as rigid, static holes. Goldman (1964) also felt that it was highly unlikely that individual channels for the ions are established by any rigid structure. He felt that the phosphate groups of the membrane act as exchange sites through which the ions pass as they rush through the membrane. While conclusive evidence does not exist, Goldman assumed that the same sites are involved in the transfer of both potassium ions and sodium ions but that a different configuration exists for each ion.

Another model which was proposed by Danielli and Davson (1935) is the unit membrane model. It is visualized as having a central layer composed of lipids arranged so that their nonpolar heads face inward and outward. A sandwich effect is produced by a thin coating of nonlipid material which is though to be mostly protein. While this model is supported by X-ray diffraction studies (Robertson, 1960) it has a number of deficiencies, the most serious being that it does not provide for the dynamics of the membrane structure. Similarly, the equivalent circuit model developed by Hodgkin and Huxley (1952b) lacks a descriptive appreciation of anything other than steady state conditions.

To provide a model which more fully explains the mechanism of membrane function in excitable cells, Tasaki (1968) has taken a macromolecular approach to the problem. Tasaki took the Danielli model and expanded upon it by suggesting that protein filaments extend through the membrane and that fixed charges exist within the membrane giving it certain characteristics analagous to a cation exchange resin. He further suggested that the phosphate groups in the membrane form negatively charged exchange sites which are occupied by calcium ions during the inactive phase. This is most likely due to the preference of a cation-exchanger for divalent cations over univalent cations (Helfferich, 1962). Tasaki considered the membrane to be asymmetrical because the effects of ionic and electrical influences differ when applied to one side of the membrane versus the other side. For example, normally the concentration of potassium ions at the internal surface of the cell membrane is much higher than the concentration of potassium ions at the external surface of the cell membrane. The cell then exists in a normal, excitable state. If the concentration

of potassium ions at the external surface of the cell membrane is increased above normal the membrane potential will decrease to approximately 50 millivolts and the membrane fails to propagate an active response (Strickholm and Wallin, 1967). Using these results, Tasaki (1968) developed an argument in which he concluded that the ions bound to the negatively charged sites in the membrane must be sodium and calcium since potassium would cause inactivation. He suggested that initiation of an active response occurred when the ratio of divalent ions to univalent ions in the cell membrane was reduced to a critical value. Goldman (1964) theorized that the calcium ions could combine with the exchange sites and form an area having the characteristic of low conductance for specific ions. He felt that changes in the electric field and thermal agitation could modify the binding properties of the phosphate groups at the exchange sites causing a change in the characteristics of the membrane.

Tasaki (1968) suggested that an abrupt inward current applied to an excitable membrane would drive the external sodium and calcium ions into the membrane. Since calcium ions tend to combine with the exchange sites in preference to sodium ions, the ratio of divalent ions to univalent ions increases (Tasaki, 1968). The result is that potassium ions are driven out of the membrane, the membrane hyperpolarizes, and an active response is not formed. When an abrupt outward current is applied to the same membrane the internal potassium ions are driven into the membrane. These large cations have the ability to disrupt and displace the calcium ions that are present in the membrane. The result is that some of the calcium ions are driven out of the membrane lowering the ratio of divalent ions to univalent ions. When the ratio has been lowered sufficiently an active

response is initiated. In contrast to an abrupt current, if the magnitude of the outward current increases slowly then the potassium concentration in the membrane also increases slowly. Because the time rate of change of the current is small it is possible that fewer calcium ions are displaced from the exchange sites. The ion exchange process in the membrane sees this as analogous to the slow increase in the external potassium concentration. The final result is that the membrane is depolarized but an active response is not initiated (Tasaki, 1968).

One of the main stumbling blocks that has existed in the attempt to explain the mechanism responsible for the formation of the action potential has been the abrupt permeability change that occurs at the beginning of the action potential. Tasaki (1968) suggested that calcium ions are driven out of the membrane causing a reduction in the stability of the membrane. Goldman (1964) theorized that the binding properties of the phosphate groups at the ion exchange sites are modified by a change in the electric field across the membrane. Both agreed that the calcium ions play an essential role in the excitation process. Waterman (1928) has reported on a physical occurrence that shares some similarities with the initiation of an action potential. He started by measuring the resistance of an emulsion of .1 M sodium hydroxide and olive oil. He then proceeded to add small amounts of a calcium chloride solution to the emulsion. He found that there was a gradual decrease in the resistance until the concentration of calcium chloride reached a critical level. At that point the resistance was found to increase suddently and profoundly. Waterman also reported that along with the change in resistance that there was also a concomitant rise in the viscosity and a visible change in the opacity of

the mixture. He attributed the changes to a transition of the soap emulsion from an oil-in-water state to a water-in-oil state. Barrer and Falconer demonstrated in 1956 another type of phase transition in which potassium and sodium ions were the principal elements. The transition mechanism proposed by Barrer is thought to occur because occupancy of neighboring sites by the same kind of ions is energetically more favorable than occupancy of adjacent sites by different kinds of ions.

At this point it becomes enlightening to compare the data obtained from varying the calcium ion concentration in the extracellular fluid bathing crayfish nerve fibers with the theories and observations presented in the preceding review. As a result of the experiments performed for this dissertation it was found that at concentrations of calcium ions below 3 millimoles the magnitude of the membrane potential and the action potential decreased quite rapidly. In fact the rate of decrease was so great that if the increment by which the calcium ion concentration had been reduced had been made smaller the decrease might have been seen to approach a step function. This type of response would indicate an abrupt change in the characteristics of the membrane. This would suggest some type of phase transition that occurs as a result of a reduction in the concentration of calcium ions in the membrane. The idea that potassium ions bind to the phosphate groups in preference to calcium ions is suggested by the fact that the membrane potential fell to 50 millivolts and the active response was suppressed at calcium ion concentrations below 3 millimoles. This is analogous to the situation where Strickholm and Wallin (1967) increased the concentration of external potassium ions and reported the same results as this investigator observed with decreased

external calcium ion concentration. Tasaki (1968) also reports that an excess of potassium ions in the membrane produces depolarization. An interesting quality of the membrane system is that a slow increase in the concentration of potassium ions in the membrane produces depolarization and inactivation while a rapid increase in the concentration of potassium ions in the membrane produces depolarization and activation of a propagated response. While Tasaki (1968) did not consider the possibility of fixed pores to be practical, he felt that the concept of a change in the binding of certain ions could create various patterns of permeabilities. Consider the excitation process as resulting from a phase transition caused by calcium ions being broken away from the phosphate groups at the ion exchange sites by an outward directed current. The outward directed current drives potassium ions into the membrane which displace the calcium ions already bonded to the phosphate groups. Depolarization occurs but the important result is that a localized phase transition occurs. The characteristics of phase transition are a dramatic decrease in the resistivity and a visible change in the opacity. It is commonly assumed that the high influx of sodium ions is due to a decrease in the resistivity. But it is also interesting to note that changes in the optical characteristics of the nerve fiber during an action potential caused by changes in the refractive index of the nerve fibers has also been reported (Carnay and Tasaki, 1971). So it is quite possible that a phase transition does occur. As a result of the phase transition there is an inward directed current that flows through the excited area and back out through the membrane at a resting area. Not only does this tend to move the resting area toward the excited state but also tends to move the excited

area to the resting state by driving calcium ions back into the membrane. Also helping to bring the membrane back from an excited state to a resting state is the fact that interdiffusion of cations across the axon membrane is enormously enhanced during excitation. This enhanced interdiffusion tends to raise the concentration of potassium ions near the external surface of the membrane and to raise the concentration of sodium ions near the internal surface of the membrane. Both of these occurrences tend to lower the amplitude of the action potential. Therefore the return of the membrane from the excited state to the resting state may be the result of calcium ions being driven back into the membrane and the accumulation of interdiffusing cations near the axon membrane.

CONCLUSIONS AND RECOMMENDATIONS

A series of eighty-five crayfish were used in an extensive series of experiments to determine the effects that reduced external concentration of calcium ions would have upon the membrane potential and the magnitude of the action potential of the isolated nerve fiber of the crayfish. It was hoped that this information might help future investigators explain the mechanism through which calcium ions work to control various characteristics of the nerve fiber.

It was found that for calcium ion concentrations between 3 millimoles and 27 millimoles the membrane potential remained essentially constant. However, for extracellular concentrations of calcium ions below 3 millimoles the membrane potential was found to decrease rapidly to a value of 50 millivolts at a concentration of zero millimoles.

As the calcium ion concentration was varied between 3 millimoles and 27 millimoles the magnitude of the action potential was found to reach a maximum at a concentration of 10.5 millimoles. Serum from five animals was collected and by using flame photometric analysis the average calcium concentration was found to be 10.1 millimoles. Previous workers have reported the normal calcium ion concentration to be 13.5 millimoles. It is possible that this discrepancy is a result of the variations that occur in the serum calcium ion concentration during the molt cycle of the crayfish. It is also possible that this discrepancy is due to variations in the environment from which the specimens were collected. An additional possibility is that the peak that occurred in the magnitude of the action potential at 10.5 millimoles is not a function of the calcium ion concen-

tration of the serum. At present one can only speculate that the crayfish has the ability to maintain a peak action potential at a calcium ion concentration equal to the blood calcium concentration. It would be interesting not only to know how the calcium ion concentration varies as a function of the molt cycle but also how the magnitude of the action potential varies as a function of the molt cycle and the in vivo extracellular calcium ion concentration. The data obtained from these experiments would suggest that the magnitude of the action potential is at its maximum value at a calcium ion concentration equal to the serum calcium ion concentration. At calcium ion concentrations below 3 millimoles the magnitude of the action potential decreases rapidly to 0 millivolts at a concentration of 0 millimoles.

The fact that both the membrane potential and the magnitude of the action potential decrease rapidly below a calcium ion concentration of 3 millimoles indicates an alteration in the membrane characteristics which affects both systems. The fact that the change in the membrane characteristics occurs suddenly, that it occurs at decreased concentrations of calcium ions, and that the membrane characteristics indicate an increase in the permeability all suggest some type of phase transition. Such a phase transition could occur as a result of displacing the calcium ion's bond to phosphate groups by an outwardly directed flow of potassium ions.

In an effort to determine if magnesium ions could substitute for calcium and thus maintain an active response, the concentration of calcium ions was reduced to 0 millimoles and the concentration of magnesium ions was increased to 13.5 millimoles. It was found that the magnitude of the action potential and the membrane potential did not differ significantly

from the test run where the calcium ion concentration was 0 millimoles and the magnesium ion concentration was at its normal value of 2.5 millimoles. While this would indicate that magnesium does not substitute for calcium it does not rule out substitution by other divalent cations. An obvious extension of this work would be to examine other divalent cations to see if they substitute for calcium ions. This information might help to further reveal the function of calcium ions.

It became increasingly obvious as the data was taken and the dissertation was written that there are certain areas and topics which would be worthwhile to pursue further. The 1 millimole concentration steps were chosen to allow a large concentration range to be covered in a reasonable period of time. After the data was assembled it became obvious that the points of greatest interest lay between a calcium ion concentration of 0 and 5 millimoles. An obvious extention of the work would be to reduce the concentration steps from 1 to ½ millimoles or less. This would allow the investigator to have a more accurate knowledge of the rate at which the magnitude of the membrane potential and the magnitude of the action potential decrease as a function of calcium ion concentration.

At the beginning of the experiments, it was hoped that spontaneous formation of action potentials could be observed occurring at decreased calcium ion concentrations. It was hoped that studying this phenomena would clarify some of the stabilizing effects that calcium has upon the excitable membrane. Unfortunately this spontaneous activity was never observed. Of course the possibility always exists that it is not valid to compare the response as a function of calcium ion concentration in a salt water animal with that of a fresh water animal. Or it could be possible

that by varying one of the other ionic species in the extracellular fluid the spontaneous activity could be made to occur. An extension of the research would be to vary either the sodium ion or the potassium ion concentrations and then to reduce the calcium ion concentration to see if the spontaneous activity would occur.

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