THE ROLE OF DEPOLARIZING AGENTS IN THE CONDUCTION AND TRANSMISSION OF ACTION POTENTIALS IN NERVES AND MUSCLES

BY

AARON JOSEPH AVERBUCH
B.S.E.E., Illinois Institute of Technology, 1962

THESIS

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Table of Contents

Acknowledgments iii

I. Statement of the problem 1

II. Background 2

III. Conduction and transmission of action potentials 8

IV. Experimental design 12

V. Description of apparatus 16

VI. Experimental procedure 23

VII. Results. 28

VIII. Discussion 36

IX. Conclusions 42

I. Statement of the Problem

The understanding of the propogation of electric potentials along nervous pathways is fundamental to the study of electro-physiology. The propogated electric potentials play the vital role as the trigger mechanism in muscular contraction. Although these potentials have been extensively investigated, the current theories of how polarized membranes break down and recover rapidly to produce and action potential do not have much direct experimental verification. The problem to be discussed in this thesis is the role of depolarizing substances in conduction and transmission of action potentials in nerves and muscles. Also, some experimental evidence is presented on the effect of a depolarizing substance applied to a muscle externally and is discussed in relation to the current theories of conduction and transmission.

II. Background

It has been known since the time of Galvani that electricity plays an important role in the functioning nerves and muscles. Over 180 years ago, Galvani demonstrated the similarity of nerve and muscle excitation by connecting two nerve-muscle preparations in series (Giese, p. 445). He wrapped the cut end of one nerve around the muscle fibers of the second muscle. When the second nerve was stimulated by pinching with a fine tweezers, the second muscle contracted as in the normal fashion, and the first muscle contracted after a short time delay.

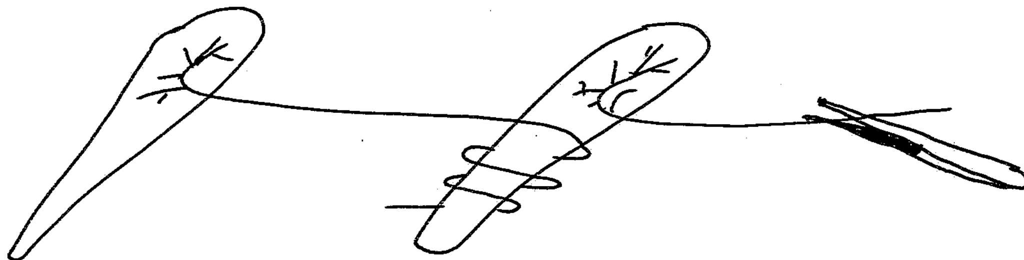

Fig. 1. Diagram of Galvani's experiment

With the development of modern electronic instrument atom, the measurement of biological potentials has become a somewhat straight forward procedure and reasonable precision can be obtained. The use of glass tubing drawn to a tip diameter of less than one micron and filled with a 3 molar potassium chloride solution has permitted the measurement of the static and dynamic potentials appearing between the inside and outside of nerve and muscle fibers (Ling and Gerard, 1949). A skeletal muscle fiber has a potential difference of about one-tenth of a volt across

the fiber membrane with the inside negative with respect to the outside. When the membrane is depolarized beyond a threshold level of about 50 millivolts, the membrane "breaks down" and depolarization proceeds regeneratively. The membrane actually reverses its polarity and becomes about 30 millivolts positive inside with respect to the outside. The membrane recovers spontaneously to its resting level. The time required for a frog skeletal muscle fiber to depolarize is about one millisecond and the time required for recovery is about one and one half milliseconds. Figure 2 shows a typical action potential.

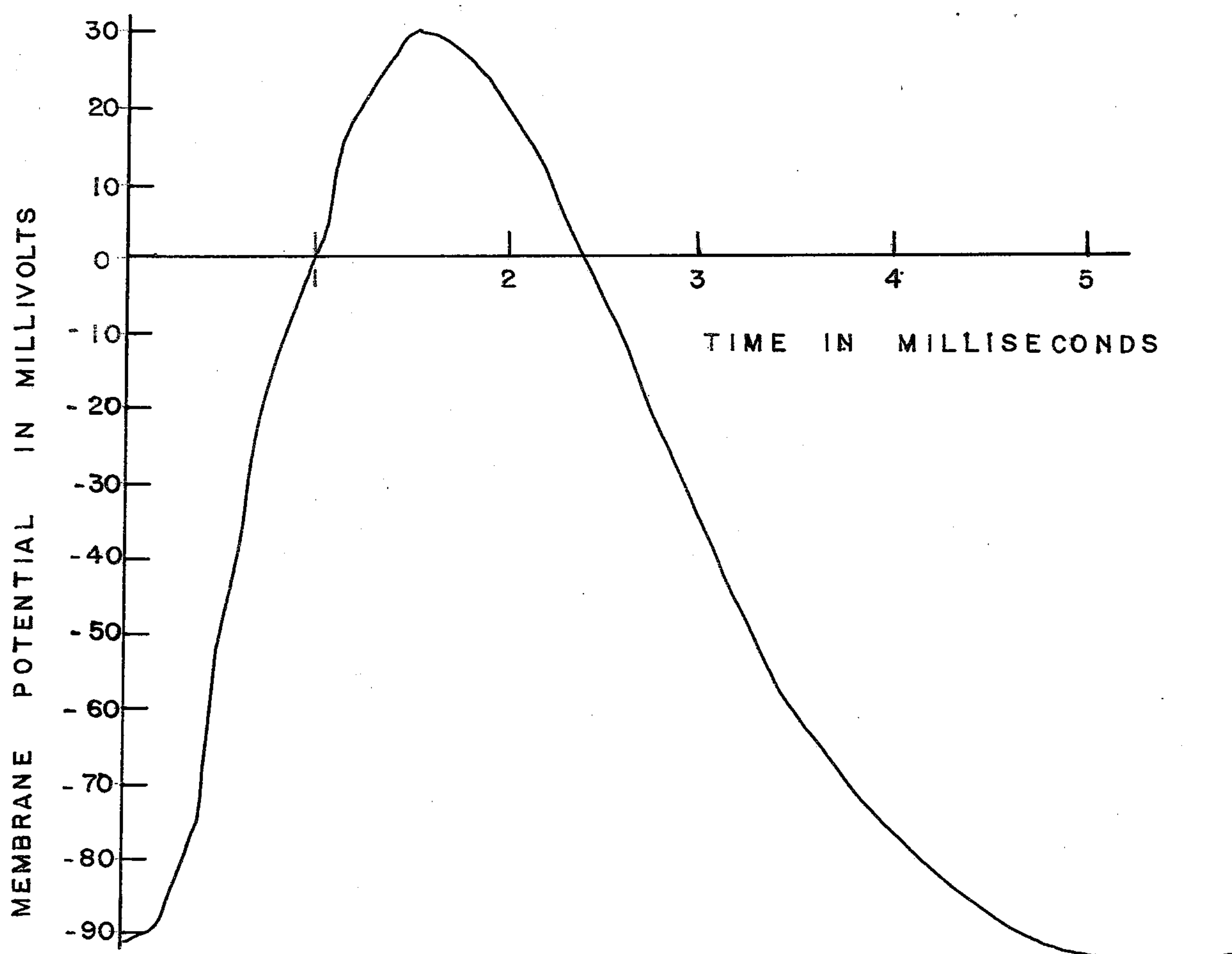

Fig. 2. Typical form of the action potential found in frog skeletal muscle fiber.

The fibers of the sciatic nerve of the frog have a lower resting potential, a lower threshold and a faster time course for depolarization and recovery than frog skeletal muscle. The entire process is completed in less than one millisecond. The time relations vary considerably in the animal kingdom. For example, in human cardiac muscle, the muscle remains depolarized for about one second. These transient variations in the electric potential of the interior of the fiber relative to its exterior are called action potentials.

The resting potential described above is presumably maintained by the nerve and muscle fibers as long as the fibers are alive and active and it has been shown to be produced by sustained differences in the cation and anion concentrations between the inside and the outside of the fibers. Hodgkin and Katz (1949) derived the following equation for the resting potential in terms of only the three ions present in highest concentration in the cell and bathing medium, viz., sodium, potassium, and chlorine.

$$E = \frac{RT}{F} \ln \left(\frac{P_{K^+}[K^+]_{in} + P_{Na^+}[Na^+]_{in} + P_{Cl^-}[Cl^-]_{out}}{P_{K^+}[K^+]_{out} + P_{Na^+}[Na^+]_{out} + P_{Cl^-}[Cl^-]_{in}} \right) \qquad 2.1$$

where E is the membrane potential in volts,
R is the universal gas constant,
F is the faraday,
T is the temperature,
P_{K^+}, P_{Na^+}, and P_{Cl^-} are the permeability coefficients of the K , Na and Cl ions, respectively, and in and out refer to the inside and outside of fiber, respectively.

The three ions play a major role in the process which generates action potentials. There are other ions present in the liquids that bath the nerve and muscle fibers, but they do not

effect the membrane potential to an appreciable extent. Hodgkin and Huxley (1952 a, b, c, d) showed how the conductances of the three ions varied as the voltage across the membrane was varied. They were also able to derive a differential equation, which could be solved using numerical methods, from which the shape of an action potential could be calculated. The resultant curve was found to be in good agreement with the experimentally observed action potential. However, their results are based entirely on experiments performed with the giant axon of the squid "Loligo" which has a diameter of about 1 mm. The same type of investigation has not been done on smaller nerve fibers, or on muscle fibers, due to the difficulties in performing the conductance measurements.

The theory of the resting and action potentials, which appear across nerve and muscle fiber membranes raise several important questions. What causes the membrane conductances to vary rapidly once the threshold potential has been reached? How does the depolarized membrane repolarize spontaneously? How are the observed concentration differences between the inside and the outside of the fiber maintained? The last question has been investigated to some degree and it is postulated that living cells are capable of actively transporting (or pumping) ions across cell membranes against even very large concentration gradients. The details of this mechanism are still quite obscure. The answers to the other two questions are the subject of this thesis.

The repolarization of a depolarized membrane can be considered to occur by virtue of the cells internal pumping mechanism

which drives the membrane toward its resting condition. The experiments of Hodgkin and Huxley (1952 a, b, c, d) show that sodium ions enter the squid axon during depolarization and repolarization occurs when the flow of sodium inward is blocked and potassium ions flow outward to reestablish the original distribution of charge. Diffusion then allows the resting condition of the membrane to be reestablished. The permeabilities of the various ions through the fiber membrane change drastically as a function of the potential across the membrane. Also, there must be considerable hysteresis effects. However, it is not unreasonable to consider that the complex moleculer structure which comprises the fiber membrane may have the necessary properties.

An alternate explanation for the depolarization and repolarization of membranes has been suggested by Nachmansohn (1961). Any stimulus reaching the membrane releases an ester, acetylcholine, once the threshold potential has been reached. Acetylcholine then reacts with a "receptor protein" which is altered. The receptor protein is postulated to be located either on the inside surface of the fiber membrane or within the membrane itself. The altered protein is responsible for the increase in sodium permeability. An important part of this theory is that the acetylcholine-receptor complex is in dynamic equilibrium with free acetylcholine and receptor protein. This means that the concentration of acetylcholine near the membrane is the major factor which determines the membrane's sodium permeability. If the free acetylcholine is removed (in this case hydrolyzed by acetylcholinesterase into acetate and choline, both of which are

relatively inert in depolarization reactions), the sodium conductance is blocked since the proportion of altered receptor proteins falls sharply. Potassium crosses the membrane down a concentration gradient to reestablish the membrane potential and thus permits the membrane to return to its resting condition. It is interesting to note that the hydrolysis of acetylcholine by acetylcholinesterase is one of the fastest enzyme reactions known. The experimental evidence for this point of view is discussed in section III.

III. Conduction and Transmission of Action Potentials

Once an action potential is produced, it is capable of depolarizing below threshold the membrane area adjacent to and contiguous with that already depolarized. Thus, the action potential will be propogated along a muscle or nerve fiber without decrement. For a frog skeletal muscle fiber, the propogation velocity is about two meters per second. For the purposes of this thesis, propogation of an action potential along a fiber is called conduction. The propogation of an action potential from one fiber or cell to another fiber or cell is called transmission.

Two types of nerve fibers are distinguished, i.e., axons, which conduct action potentials away from the nerve cell body (the enlarged portion of a nerve cell containing the nucleus) and dendrites, which conduct action potentials toward the cell body. Nerve fibers are typically connected in a network such that impulses may be transmitted throughout the nervous system. The junction between the axon of one neuron and the dendrite of a connecting neuron in the network is called a synapse. At the synapse, the axon and the dendrite in most cases do not actually touch, i.e., there is a gap of 200 to 400 Å between the two. In some cases the endings do touch and, in fact, the membrane of one is partially joined to the membrane of the other. This last type of connection is found in smooth muscles and in cardiac muscle. To distinguish this type of junction from the synapse, it is termed a nexus.

One might assume that a propogated action potential reaching a nerve ending would be able to depolarize the post synaptic

membrane and thereby produce a propogated action potential to occur in the dendrite. This type of propogation has actually been observed. For example, electrical transmission has been observed across the synaptic regions between the lateral giant fibers and motor giant neurons of crayfish. A characteristic of this type of transmission is a lack of delay across the junction (Prossor and Brown, 1961 p. 599 ff). However, a different type of transmission is more commonly found. The junction of a motor nerve fiber ending and a muscle fiber is called the end plate region. Considerable indirect evidence indicates that a chemical agent is released at the nerve ending when a propogated action potential arrives. It is considered that the agent then depolarizes the post synaptic membrane of the dendrite or, in the case of muscles, the agent depolarizes the muscle fiber membrane. If sufficient quantity of the agent is released, the resulting depolarization is sufficient to produce a propogated action potential in the dendrite, or muscle, membranes. An enzyme, believed to be located just inside the post synaptic membrane, splits the chemical agent into inert fragments allowing the membrane to repolarize. Since the agent must diffuse across a gap, this type of transmission is characterized by a time delay. In frog skeletal muscle end plates, the delay is about one half millisecond.

In the motor nerves and skeletal muscles of some amphibians and mammals, the chemical agent acetylcholine has been found which is capable of depolarizing the end plate membrane of muscle fibers. Further, chemical staining techniques have shown that

acetylcholinesterase is present in the post synaptic regions of muscles and nerves. As pointed out earlier, the splitting of acetylcholine by acetylcholinesterase is one of the fastest enzyme reactions known.

A possible dual role of the acetylcholine system has been the subject of much controversy. Experiments on the frog sartorius muscle show that external application of acetylcholine to the end plate region results in depolarization. The continued application would result in blocking the transmission of action potentials from the nerve to the muscle (del Castillo and Katz, 1957b). However, external application of acetylcholine a few millimeters from the end plate region has no effect. This result seems to contradict the view that the acetylcholine system is involved in conduction along a fiber. The most widely accepted view at present is that the surface membrane of the muscle fiber contains specific chemo-receptor molecules which are concentrated in the immediate vicinity of the motor nerve endings, and the surface density of which diminishes rapidly with distance away from the end plate region (Miledi 1960b).

There are, however, several instances in which the chemoreceptors do not appear to be localized. One is the chronically denervated muscle in which the surface area depolarized by acetylcholine increases with time in an outwardly direction from the end plate region (Miledi 1960a). Nerve conduction can be blocked by a chemical which blocks the action of acetylcholinesterase (Dettbarn, 1960). In some muscle experiments, acetylcholine, injected intravenously, results in a reduction in sensitivity to

electrical depolarization (Irwin and Wells, 1959). Some recent experiments, described in the next section, have been performed with contradicting results. An examination of these experiments may help to resolve the controversy over the possible dual role of the acetylcholine system in conduction and transmission of action potentials.

IV. Experimental Design

A number of attempts have been made to pretreat a muscle of nerve fiber in such a way as to allow externally applied depolarizing agents to decrease the resting potential. However, these treatments involve such chemical agents as detergents and snake venoms (reviewed by Nachmansohn, 1961). It is suggested that these agents remove a barrier to external depolarizing agents so that the applied agents may then enter the cell. They may however, also damage the cell. To produce depolarization by externally applied acetylcholine on the frog sartorius muscle, the chronic denervation, mentioned before, is another method commonly used. However, the muscle atrophies when this procedure is used. There is a need to find a method of causing a conduction block in nerve and muscle without resorting to such drastic pretreatment when acetylcholine is applied externally to muscle and nerve fibers.

The experiments of Ochs and Mukherjee (1959) showed that acetylcholine can depolarize the membrane all along the fiber of the frog sartorius muscle. Katz and Miledi (1961) repeated the experiments of Ochs and Mukherjee with the result that acetylcholine appears to affect only the end-plate region of the fiber. Here is an instance of two different results under similar conditions using the frog sartorius muscle. It must be mentioned that, prior to adding acetylcholine to the solution bathing the muscle and measuring the resting potential levels, eserine, a chemical agent that blocks the action of acetylcholinesterase, was added to the bathing solution. Since eserine alone may block conduction

(Nachmansohn, 1961) in nerve fibers, a decisive experiment on the action of ACh on muscle without the use of eserine is needed.

Ochs, Annis and Mukherjee (1960) showed that succinylcholine (SCh) has a general depolarizing action similar to acetylcholine (ACh). They also showed that the mechanical response to direct electrical stimulation, i.e., stimulation of the muscle fiber directly, is reduced. Irwin and Wells (1959, 1961) have shown that SCh does not affect the contractile material in the muscle of the rat gastrocnemius directly. They conclude that the reduction in mechanical response is due to the altered membrane properties.

A relationship exists between depolarization of a muscle membrane and the muscle's mechanical response. When an action potential is conducted along a muscle fiber, the fiber contracts. The contraction of the fiber produces shortening of the muscle or, if not allowed to shorten, produces tension. A single contraction produced by one conducted action potential is called a twitch. A maintained contraction produced by a long series of conducted action potentials is called a tetanus. If a depolarizing substance such as ACh or SCh blocks conduction along a muscle fiber, the mechanical response should decrease sharply. Hence, the effectiveness of a depolarizing substance in blocking conduction can be measured conveniently on muscle fibers by observing the muscle's mechanical response to direct electrical stimulation. The use of electrical stimulation assures that the fiber membrane will be depolarized beyond the threshold level required to initiate an action potential.

Katz and Miledi (1961) observed the twitch tension of a muscle as a function of time of presence of SCh and found a

transient dip, the depth of which was related to the amount of SCh added to the solution bathing the muscle. However, the muscle always recovered to the initial twitch tension value. Their results on mechanical response with SCh paralleled their results on membrane potentials with ACh in the presence of eserine. Thus, a determination of which factor, or combination of factors, produced a difference between Katz and Miledi's results and those of Ochs, et.al. can be made by measuring the mechanical response of the frog sartorius muscle and noting whether or not a maintained reduction in mechanical response occurs when SCh is added to the solution bathing the muscle. However, a number of differences in procedure employed by the two groups of investigators are to be noted (See Table I).

Ochs and Mukherjee (1959) Ochs, Annis and Mukherjee (1960)	Katz and Miledi (1961)
1. Stimulated at 15 sec. intervals	1. Stimulated at 1 min. intervals
2. Used a $NaHCO_3$ buffered solution	2. Used an unbuffered solution
3. pH not mentioned	3. pH not mentioned
4. Measured twitch response isotonically	4. Measured twitch response isometrically
5. Used frog but did not specify species	5. Used frog _Rana_ _Temporaria_
6. Used 5 ms. stimulus pulse	6. Used 0.2 ms. stimulus pulse
7. Stimulated on one end of muscle	7. Stimulated over entire muscle with multiple electrode assembly

Table I. A comparison of the methods used by the two groups of investigators

It is the purpose of this thesis to investigate experimentally the conflicting results reported by Ochs, et.al. (1959 and 1960) and Katz and Miledi (1961). Because of the availability of equipment and for reasons of convenience, the experimental conditions used in the first experiments were chosen similar to those of Katz and Miledi (1961). Two major differences, however, are the use of the frog _Rana Pipiens_ and the use of the so-called massive electrodes to stimulate the entire muscle. It is expected that a change in one or more of the items listed in Table I will result in a change from the result of Katz and Miledi's to that of Ochs _et al._ The change from a transient dip in muscle twitch tension as a function of time to a permanent reduction is easy to observe. The following sections describe in detail the experimental apparatus, the experimental procedures, and the results obtained. The final section discusses these results in relation to the current theories of conduction and transmission of action potentials.

V. Description of Apparatus

The muscle was supported in a stainless steel holder consisting of a fixed frame and a movable platform. A lead screw mechanism was used to adjust the position of the platform relative to the frame. (see Fig. 3).

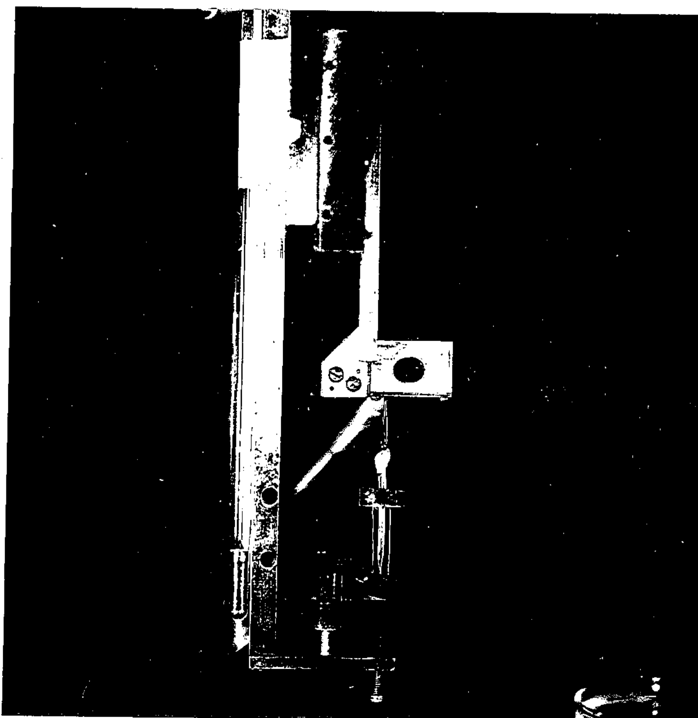

Fig. 3. The Stainless steel muscle holder.

In operation, the excised muscle (with bone and tendons) was attached to the hooks of the holder by means of stainless steel rings approximately 2 mm in diameter. Holes were drilled in small sections of bone to which the muscle tendons were attached. Nichrome wire, 0.004" in diameter, was threaded through the bone and the ring and tied securely. The attachments were inspected under a low power microscope to insure that a strong

connection was obtained. The lower hook was adjustable to provide a means of positioning properly the muscle between the plates of the mass electrodes. The upper hook was an integral part of the photoelectric strain gauge. The strain gauge was mounted on the movable platform enabling adjustment of the static tension of the muscle to be made.

Two different types of electrodes were used to stimulate the muscle. The most often type used was the short mass electrode, which consisted of two rectangular gold plates mounted in a plastic block with their large (relative to the muscle dimensions) parallel faces and separated by a distance sufficient to allow free movement of the muscle. The mass electrode shown in Fig. 4 covered only two-thirds of the muscle. The mass electrode shown in Fig. 5 covered the entire length of the muscle and was used to check the efficiency of the other electrode. However, the results of twitch tension observations were the same for both types of mass electrodes.

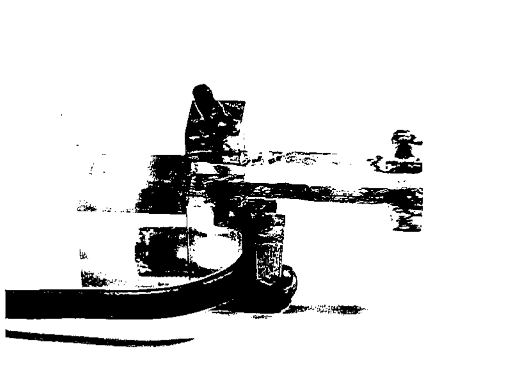

Fig. 4. Short mass electrode about 27 x 5 x 0.25 mm with a separation between plates of 7 mm.

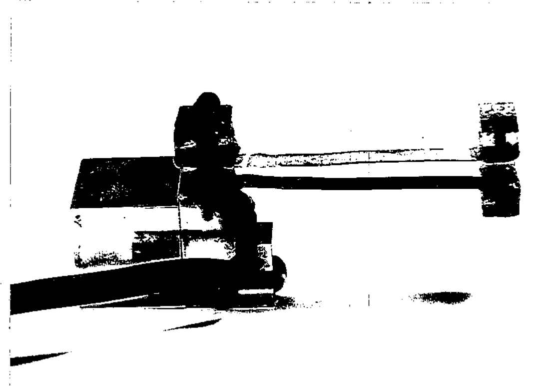

Fig. 5. Long mass electrode about 40 x 5 x 0.25 mm with a separation between plates of 5 mm.

Three stimulators, designed at the Biophysical Research Laboratory of the University of Illinois by Dr. G. Leichner and constructed in the laboratory, were employed to supply the electrodes with sufficient current and voltage to stimulate the muscle maximally. One stimulator is a constant current source with switch selected currents ranging from 0.01 amp to 1.99 amp in steps of 0.01 amp. Constant current is assured as long as the load voltage does not exceed 50 volts. The second stimulator is also a constant current source, but provides a continuous range of currents from 100 microamps to 50 milliamps at voltages up to 10 volts. The third stimulator is a constant voltage source with a continuous output from 0 to 50 volts at currents up to 3 amps. The block diagram of the high current constant current stimulator is shown in Fig. 6.

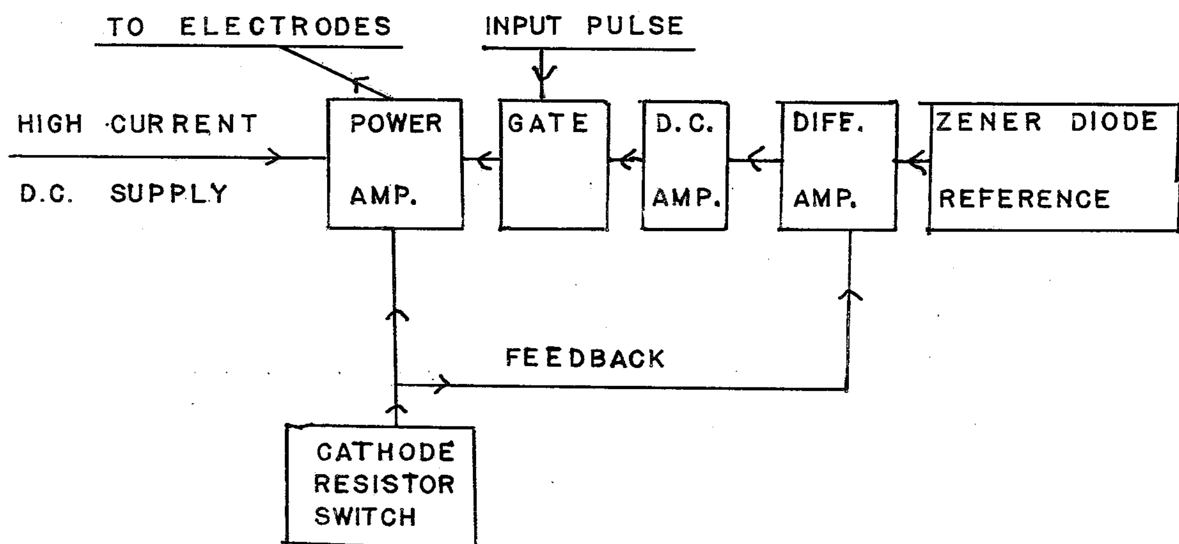

Fig. 6. Block diagram of high current stimulator (HCS).

The power amplifier consists of 10 6DQ5 power pentodes. The number of operating tubes in parallel depends upon the current output desired. The switching was arranged so that as increased

current is desired, additional tubes were switched into the circuit. Simultaneously, resistors in parallel are switched in the cathode circuit. No single 6DQ5 ever carried more than 200 ma. The cathode resistor switching circuit is arranged so that the desired load current produces 10 volts across the cathode resistance. This voltage is compared against a zener diode reference and the error was fed back in opposite polarity by a two stage d.c. amplifier and applied to the grids of the 6DQ5's.

The gate functions to bias the power tubes to cut-off until a pulse of -50 volts amplitude is applied at which time it opens and allows the d.c. voltage from the d.c. error amplifier to be applied to the 6DQ5 grids. The gate remains open as long as the input voltage remains at -50 volts. Thus, the duration of the input pulse determines the duration of the output pulse. The amplitude of the output depends only upon the cathode resistor value.

The smaller constant current stimulator operates in a similar manner (See Fig. 7).

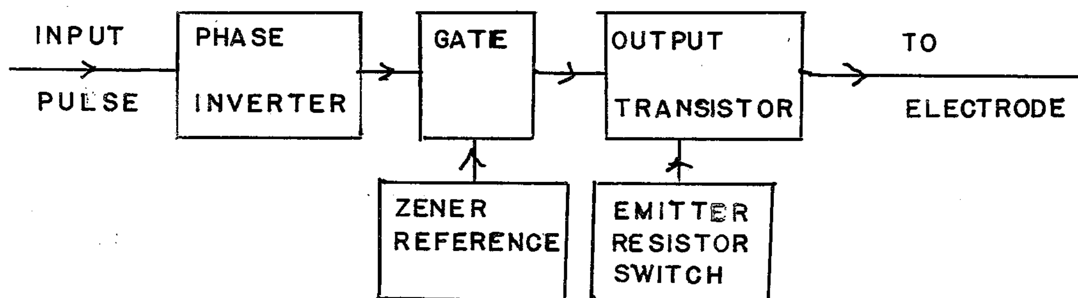

Fig. 7. Block diagram of low current stimulator (LCS).

Transistors were used throughout since low currents and voltages were required (a special Motorola transistor SM-1530 a silicon

computor type). The output transistors are biased to cut-off such that when a pulse is applied to the input, it is inverted and applied to the gate. The gate closes and allows the zener reference to set the bias level. Current in the load increases until the voltage across the emitter resistor reaches the reference voltage. The output stage consists of two transistors arranged in a Darlington connection to give high current gain and linear output.

The constant voltage stimulator operates in a different manner. The basic curcuit consists of a linear amplifier stage and a cathode follower output (See Fig. 8). Feedback from the output to the input decreases the output impedance. The cathode follower stage consists of 16 6AS7 power.pentodes in parallel to

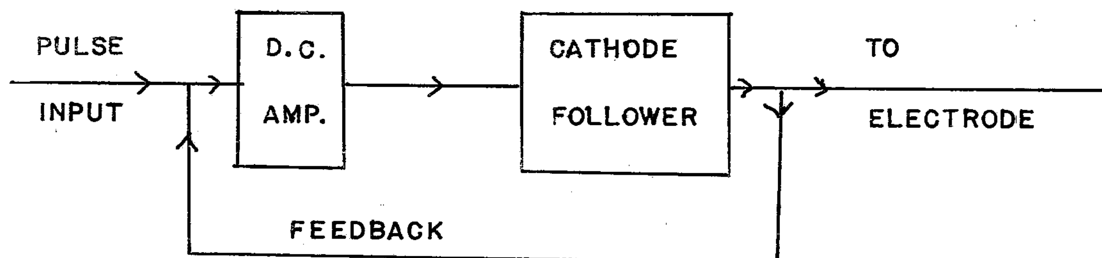

Fig. 8. Block diagram of constant voltage stimulator (CVS).

achieve a high current capacity and low output impedance. When the long mass electrodes are used the load is approximately 9 ohms. The output impedance of the stimulator is less then 1 ohm and the overall voltage gain is about unity.

The trigger and synchronizing pulses are produced by combining a Tektronix type 161 pulse generator and two Tektronix type 162 wave form generators. The type 161 pulse generator is triggered by a negative sawtooth from the type 162. The output

pulse to drive the stimulator can be adjusted to occur at any point on the sawtooth. A pulse available from the 162 generator at the beginning of the sawtooth triggers the oscilloscope sweep. The second type 162 generator allows the generation of two or more pulses in a train. (See Fig. 9).

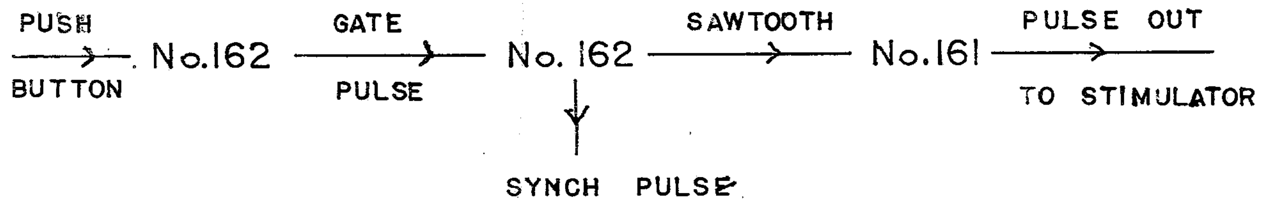

Fig. 9. Connections used to obtain triggering and synchronizing pulses for stimulation and recording.

The tension developed by the muscle is measured by a modified SA-78 photoelectric strain gauge manufactured by C. H. Stoelting and Company of Chicago, Illinois. A force applied against an internal spring can be calibrated accurately. A beam of light is used to energize a pair of photo-cells mounted side by side and connected electrically to oppose each other. (See Fig. 10) A light gate mounted on the internal spring adds light to one cell as it subtracts light from the other cell when the spring is displaced by an applied force. The light source is a miniature incandescent lamp (No. 323-Chicago Miniature Lamp Works). The bulb is supplied with direct current from a regulated current source to reduce drift during an experiment. The mechanical self reasonant frequency of the strain gauge is about 250 cps. The maximum tension the transducer can handle is 225 grams. For the

range of twitch tensions encountered with the frog sartorius muscle, the electrical output is around 30 mv. This is high enough to drive the oscillosipe directly without the need for preamplification. The spring constant for the strain gauge is about .006 mm/g.

A Hewlett-Packard type 130-BR oscilloscope is used to observe the electrical and mechanical data since high sensitivity is needed lowest range (1.0 mv/cm). All oscilloscope traces are photographed on 35 mm film to provide a permanent record of the data.

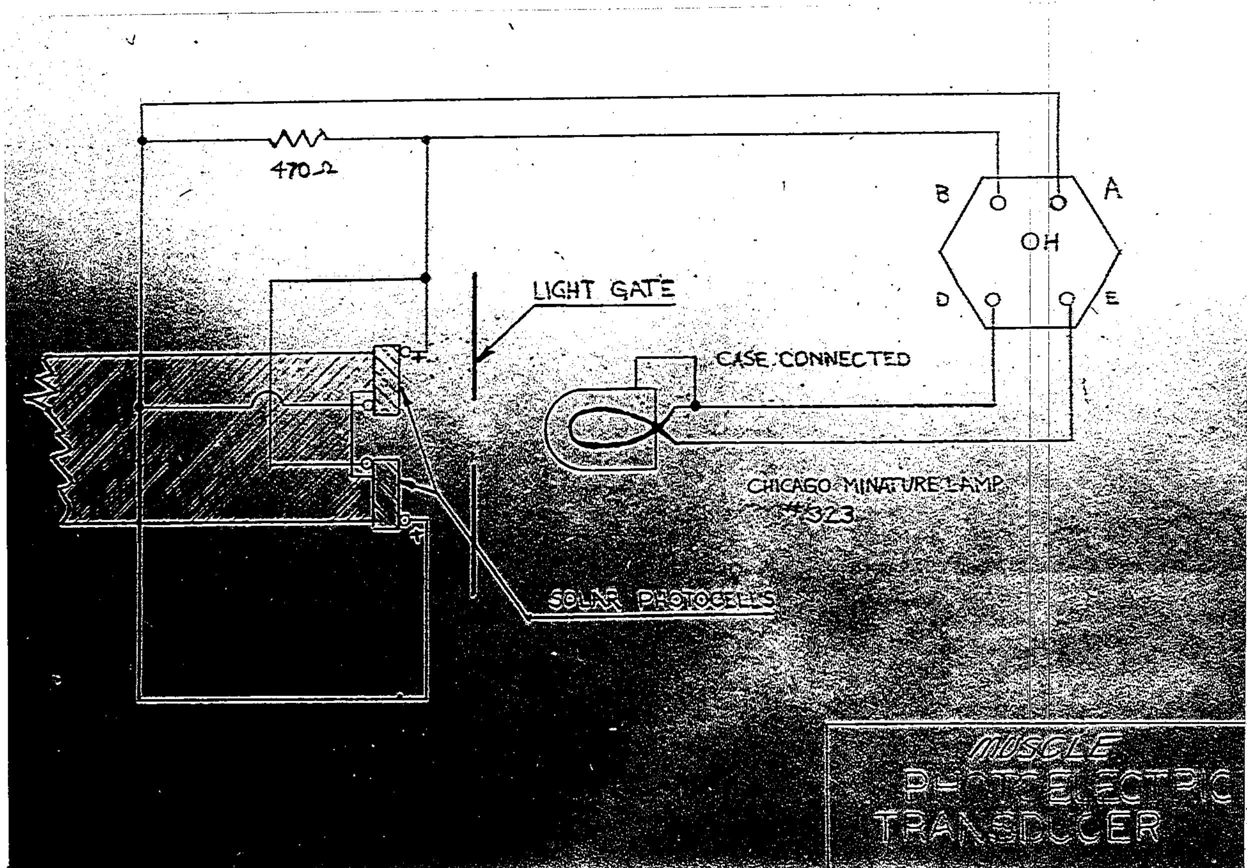

Fig. 10. A schematic drawing of the photoelectric strain gauge.

VI. Experimental Procedure

The experimental animal chosen for this work is the frog, _Rana Pipiens_ and the particular muscle used is the sartorius, a long flat muscle of a relatively constant cross section lying on the ventral (front) side of the thigh. It is attached at one end to the inside and back of the proximal end of the tibial bone (just below the knee joint). The other end is attached to the ventral side of the pelvis. The reason for choosing this muscle is that a comparison of the results can be made with the work of other investigators.

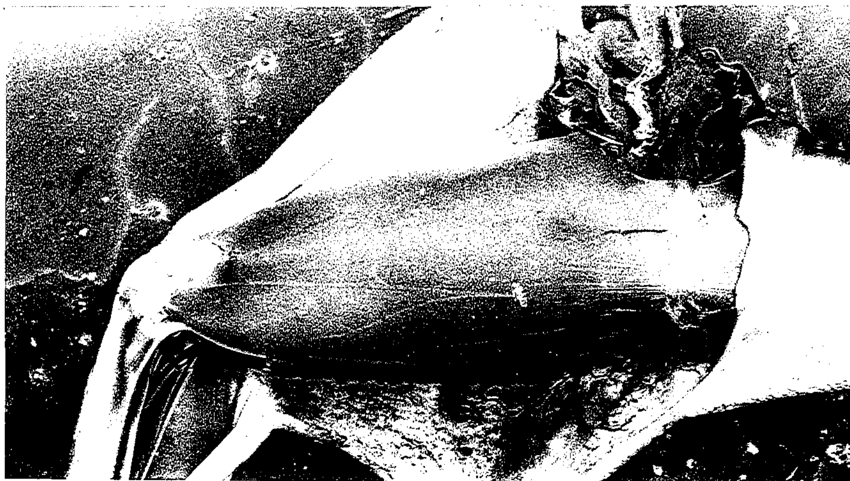

Fig. 11. _Rana Pipiens_ with sartorius exposed just prior to dissection.

The sartorius muscle has a single nerve bundle enervating it which is a branch of the ischiadicus nerve which terminates in the lower half of the muscle. The attachment of this nerve (a motor nerve) to the muscle is popularly called the motor end plate.

The preparation of the muscle for an experiment includes excising the muscle from the frog and attaching stainless steel rings. The dissection is accomplished using small eye scissors.

Care was taken not to injure the muscle in any way and handling of the muscle was kept to a minimum. The frog is initially decapitated and the abdomen is then cut to remove the legs from the body. The legs are then immediately put in 2°C frog Ringer's solution and kept cold during the entire dissection procedure.

The bones to which the muscle tendons are attached are removed with the muscle (See Fig. 12). The bones are trimmed to reduce size and weight, but sufficient material is left remaining to attach to the mounting hardware. This mounting method is that used by Kelly (1964) and has the advantage of employing the natural attachments of the muscle eliminating injuries to the tendons or the muscle fibers which can occur with other procedures. Holes are drilled into the bone and .004" nichrome wire inserted through the holes to tie a 2 mm. diameter stainless steel ring to the bone. The rings provide a very convenient method for attaching the muscle to the mounting hooks.

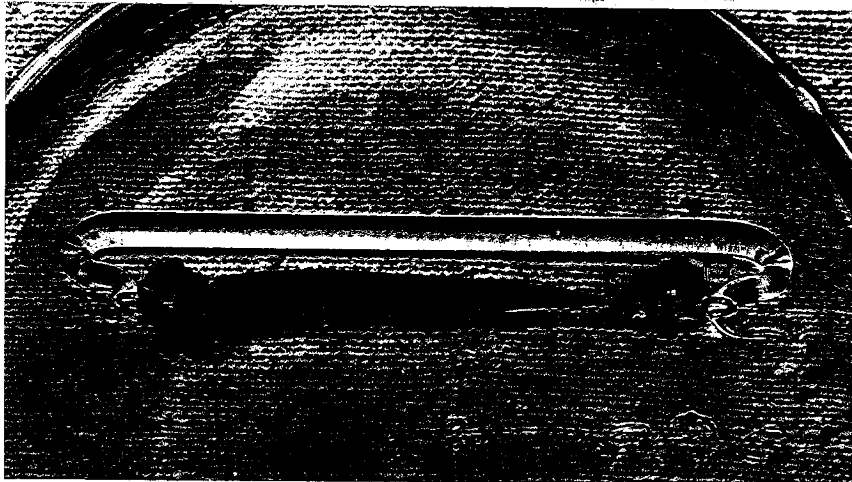

Fig. 12. The completed muscle preparation.

The stimulation of the muscle is accomplished by passing a high current density through it and, with the mass electrodes, the muscle is rather uniformly stimulated. During the experiment

the stimulus is increased in a step-wise fashion until the developed tension shows no further increase with increase in stimulus. A stimulus whose value is higher than that required for maximum developed tension is called supermaximal.

At room temperature, the time duration of the stimulus has a great bearing on the maximum developed tension. It is found that 0.2 ms and 10 ms pulses result in a large difference in maximum developed tension. Kelly (1964) describes this characteristic in detail as well as its relation to temperature. Since the action potential of the muscle membrane is about one and a half milliseconds at 20°C, a pulse whose time duration is short compared to one and a half milliseconds will be termed a short pulse, etc. All experiments were run at 18°-22°C.

Three bathing solutions were used during the experiments to bath the muscle. During the excision procedure, the muscle is kept wet by the bathing solution chosen for that experiment. The three solutions used were variations of frog Ringer's solution. This solution is a balanced salt solution that is isotonic with the frog's blood. Table 2 shows the variations in composition.

The pH of the bathing solutions was checked by a Beckman pH meter. When it was necessary to change the pH of a solution, hydrochloric acid or sodium hydroxide was diluted with some of the solution to 0.1 molar and sufficient quantity added to give the desired pH.

The depolarizing substance used in the following experiments was succinylcholine chloride (SCh). SChCl powder was dissolved in

Kelly (1964)
Ringer's #1

Na Cl	6.7 gm
K Cl	0.2 gm
$CaCl_2 \cdot 2H_2O$	0.26 gm
$Na H_2 PO_4 \cdot H_2O$	0.01 gm

This solution was buffered to pH 7.2 by adding a sufficient quantity of 0.15 Molar $Na_2HPO_4 \cdot 7H_2O$.

Ochs and Mukherjee (1959)
Ringer's #2

Na Cl	6.5 gm
K Cl	0.177 gm
$CaCl_2$	0.144 gm
$NaHCO_3$	0.24 gm

Katz and Miledi (1961)
Ringer's #3

Na Cl	6.76 gm
K Cl	0.15 gm
$CaCl_2 \cdot 2H_2O$	0.265 gm

Table 2. The chemicals added to one liter of distilled water for the bathing solutions used.

a small quantity of bathing solution to make a concentration of 0.1g/ml. This solution was always prepared fresh prior to the beginning of an experiment. Sufficient quantity of this latter solution was added to the bathing solution to obtain a final concentration of 60 micrograms SCh per milliliter of bathing solution.

Ochs, et.al. (1960) also found that under the conditions of their experiment, the depolarizing action of SCh could be blocked

by pretreatment of the muscle with d-Tubocurerine Chloride (DTC). For the experiments reported here, DTC was obtained as a solution (3 mg DTC/cc). This solution, when used in the experiment, was added directly to the solution bathing the muscle. Sufficient quantity was used to make a final concentration of 20 micrograms of DTC per millilitar.

VII. Results

The first experiments were designed to duplicate the results of Katz and Miledi (1961). The main features are now listed for comparison with Table 1. The mechanical response of the muscle was measured isometrically. The muscle was stimulated at 1 minute intervals. The excised muscle was bathed in an unbuffered Ringer's solution (#3), the pH of which was measured and found to be 7.0. The short mass electrodes were used for electrical stimulation and the stimulus pulse duration was 0.2 ms. The species of frog used was Rana Pipiens.

In the initial phases of the experiment, the stimulus current was increased in 10 ma. steps starting from zero current. As expected, the peak tension climbed rapidly at first then leveled off rather sharply. Although the peak tension could be increased slowly by raising the current pulse to larger and larger levels, the increase did not amount to more than 10% within the range of currents that the stimulator could deliver. A current pulse of 50 ma. amplitude was sufficient to raise the peak twitch tension past the knee of the peak tension vs. stimulus current curve for all fresh muscles tested. To obtain a stimulus sufficient to stimulate the muscle adequately after SCh was added to the bathing solution, Katz and Miledi (1961 p. 404) stated they used a stimulus "with a ten times greater shock intensity." A 500 ma. pulse was used in all cases presented here. This amplitude is not critical and was chosen for convenience in comparing results with those of Katz and Miledi.

In order to intercompare the results of muscles used in the various experiments, the ratio of twitch to tension to tetanus tension was determined. The tetanus tension of a fresh muscle was measured only at the beginning of an experiment. The muscle was stimulated with a series of pulses of 0.2 ms. duration each and 50 ma. amplitude. The pulses were applied at a rate of 100 per second for a fourth of a second. Since only about 25 pulses were needed to obtain a good measurement, the muscles were not fatigued.

The twitch to tetanus tension ratio for a fresh muscle using a 300 ms. stimulus pulse is approximately 0.3. When SCh was added to the bath, there was some spontaneous twitching of the muscle, a dip in the peak twitch tension ratio and a recovery to a value of about 0.35. The recovery was always found using the above experimental arrangement and is similar to the results shown in Katz and Miledi's Fig. 6-B.

A quantity of Ringer's solution was made up according to the data of Ochs and Mukherjee (1959). This solution (Ringer's #2), which had an initial pH of 7.8, was allowed to stand for two days prior to an experiment. The pH was then found to be 8.0, a value which remained stable for long periods of time. The present experiment was repeated exactly as the previous one except that the #2 Ringer's solution was used instead of the #3 Ringer's solution. The twitch to tetanus ratio for a fresh muscle was approximately 0.5. When SCh was added to the bathing solution, the twitch response decreased. The peak twitch tension vs. time curve for both sets of experiments is shown in Fig. 13.

Not only did the muscle not recover its tension capability in the presence of SCh, but repeated attempts to wash the SCh from the muscle in a fresh Ringer's solution for an hour and a half did not produce any significant recovery. Fig. 14 compares the responses given in Fig. 13 with the results given by both groups of investigators.

A change in the effect of SCh on twitch tension was found by changing simultaneously two items in the list given in Table 1. However, the necessity of changing two parameters has yet to be established. In order to keep the solution as close to their original composition as possible, the pH was varied by means of appropriate quantities of sodium hydroxide or hydrochloric acid (both 0.1 molar).

If sufficient HCl was added to Ringer's solution #2, it was possible to reduce the pH level from 8.0 to 6.5 At this pH, recovery of the muscle occurred. This result, shown in Fig. 5, corresponds to Katz and Miledi's result except for a longer latent period and a deeper depression of the twitch tension. Interestingly enough, the twitch to tetanus ratio for a fresh muscle in this modified solution was approximately .36, similar to the value for solution #3.

The reverse experiment was also performed, i.e., NaOH was added to Ringer's solution #3 until the pH was 7.6 Under these conditions, the muscle was still able to recover when SCh was added to the solution. Also, the twitch to tetanus ratio was approximately 0.3 for a fresh muscle. Apparently, the muscle does not recover when both a bicarbonate buffered Ringer's

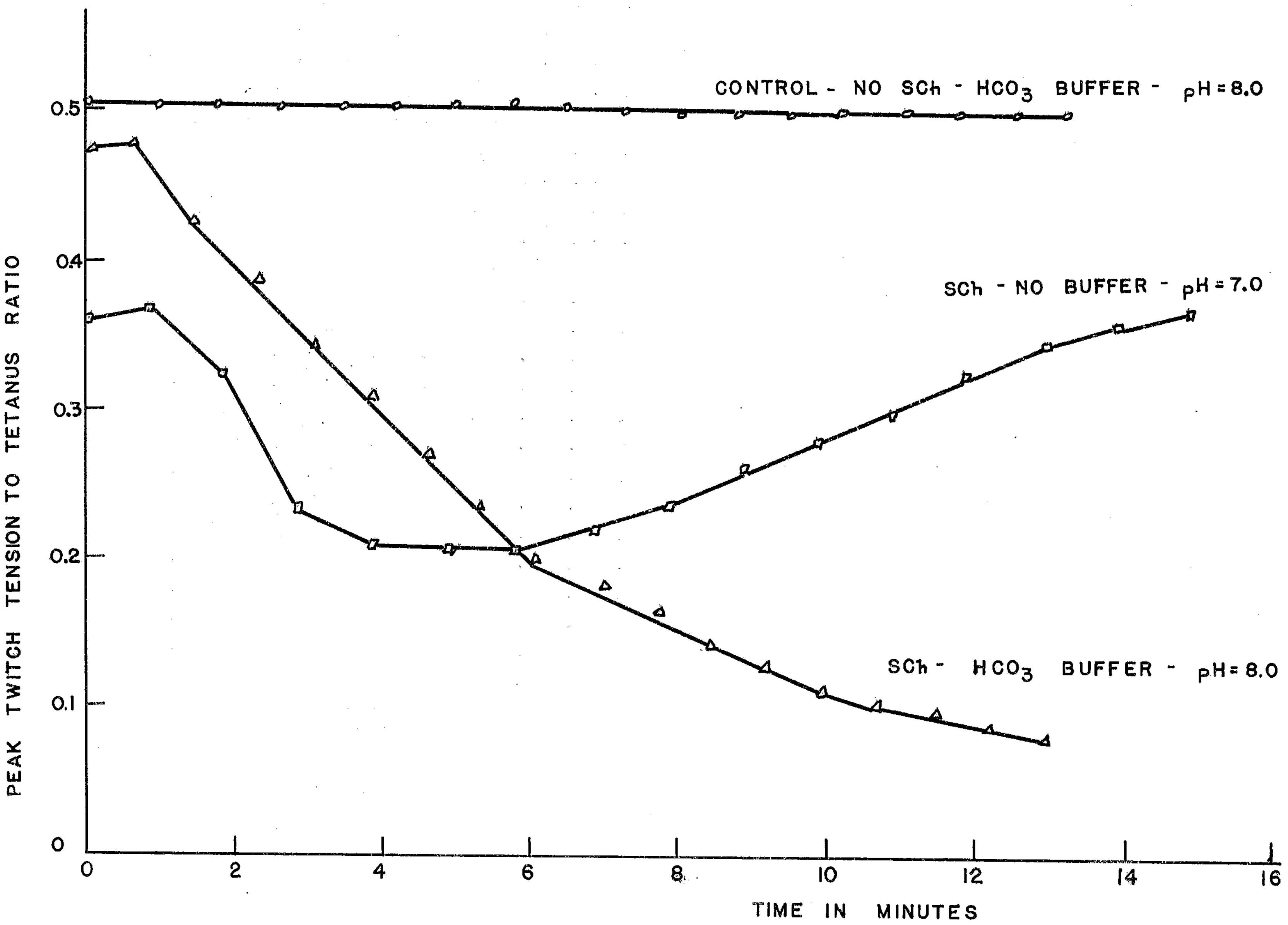

Fig. 13. The effect of SCh and bathing solution on twitch tension.

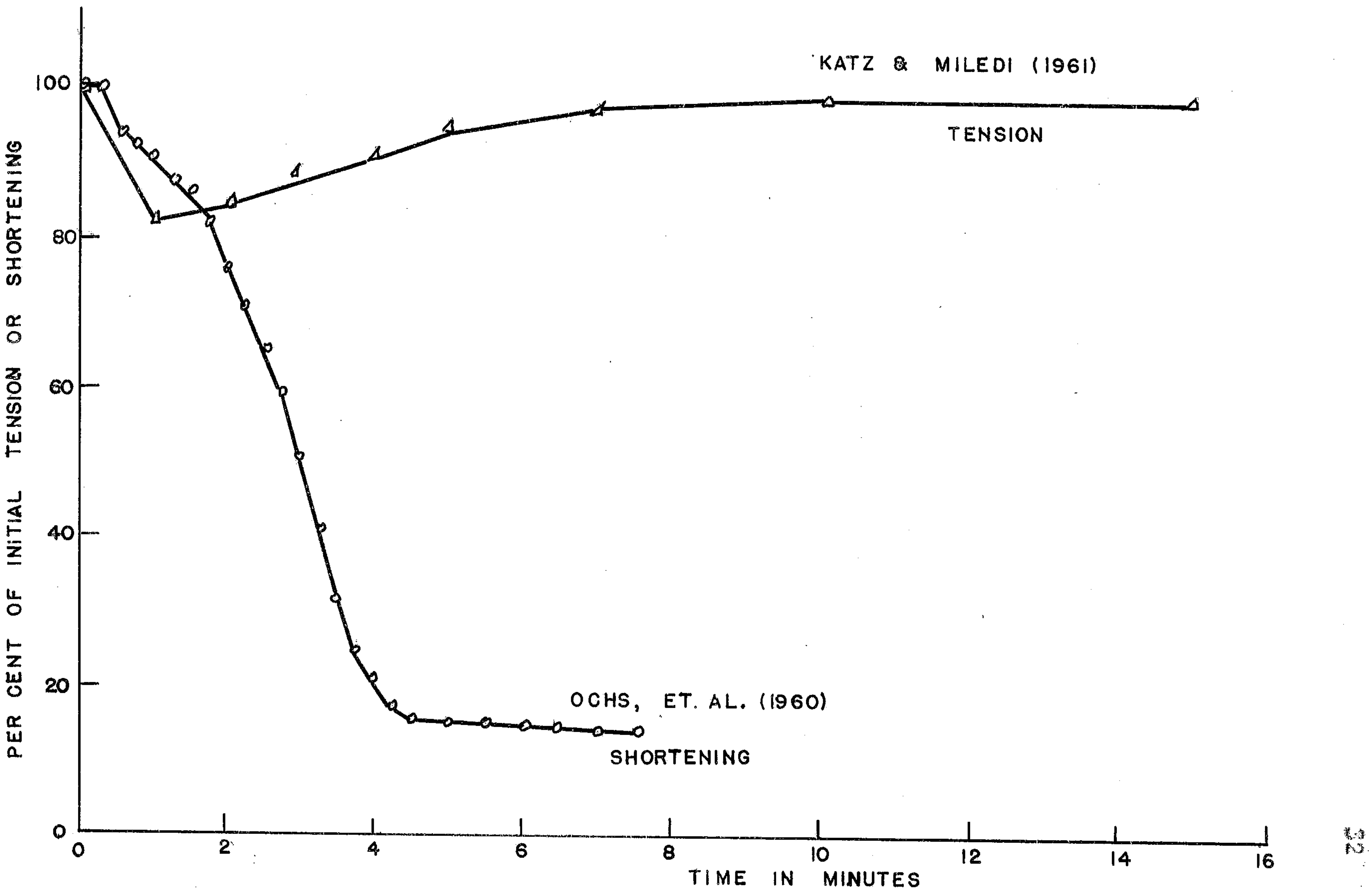

Fig. 14. Comparison of results of the two referenced authors.

solution is used and the pH is approximately 8.0. The twitch to tetanus ratio is also higher under these circumstances.

One set of experiments remains to be performed. What will happen to the peak twitch tension if a muscle bathed in Ringer's #3 and SCh is switched to a bath containing Ringer's #2 and SCh and if sufficient time is allowed for the muscle to recover in Ringer's #3 before changing solutions? Fig. 16 shows the result. The muscle tension did not decrease to those levels observed when only Ringer's #2 with SCh was used in a comparable time interval. The tension was not observed for a very long period after the solutions were switched since it was desired to determine whether the tension would decrease in a comparable time interval. However, an increase in tension was observed just after the solutions were switched. After this relatively small increase, the tension gradually decreased during the period of observation.

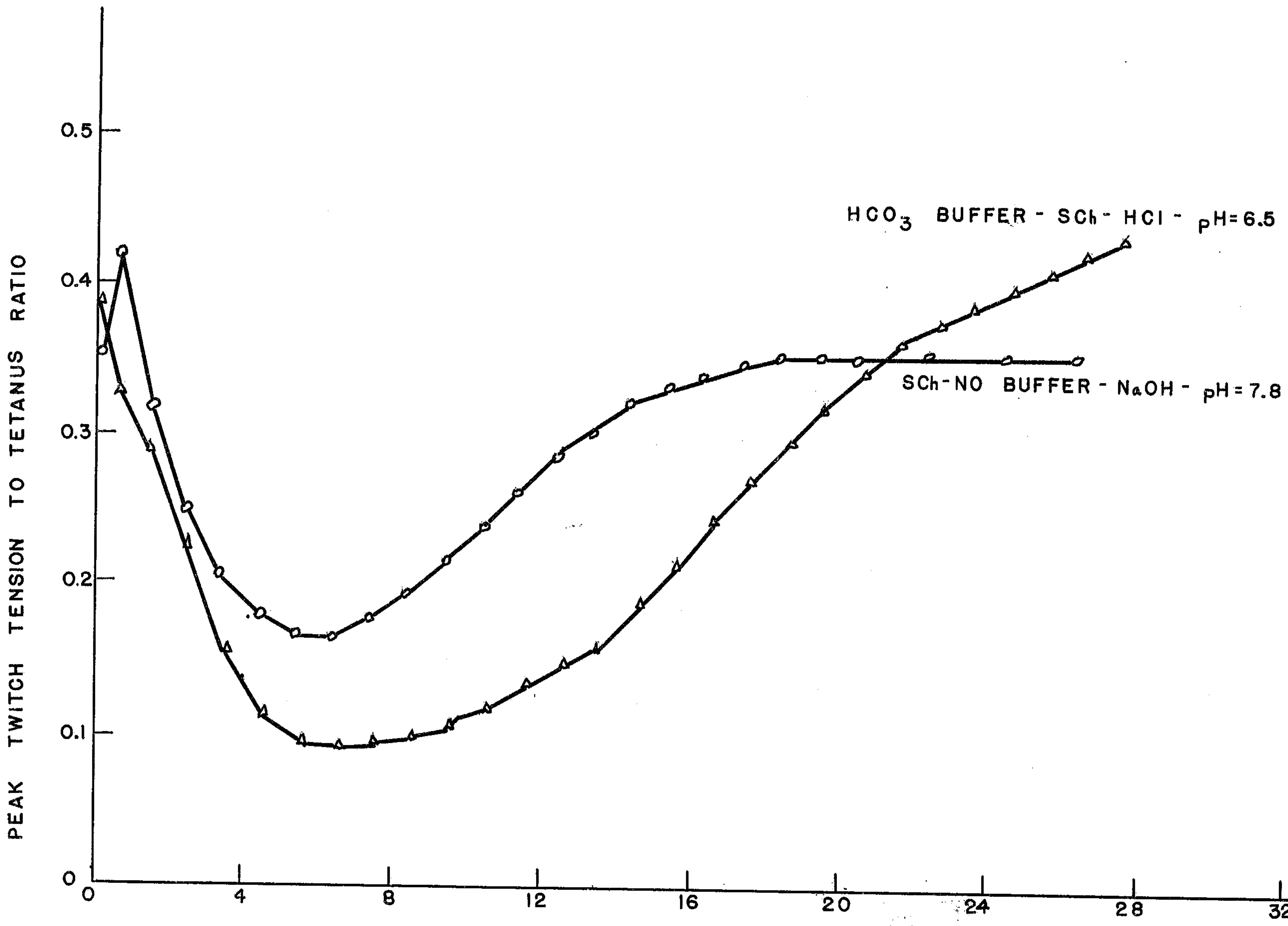

Fig. 15. The effect of adjusting the pH on the twitch tension.

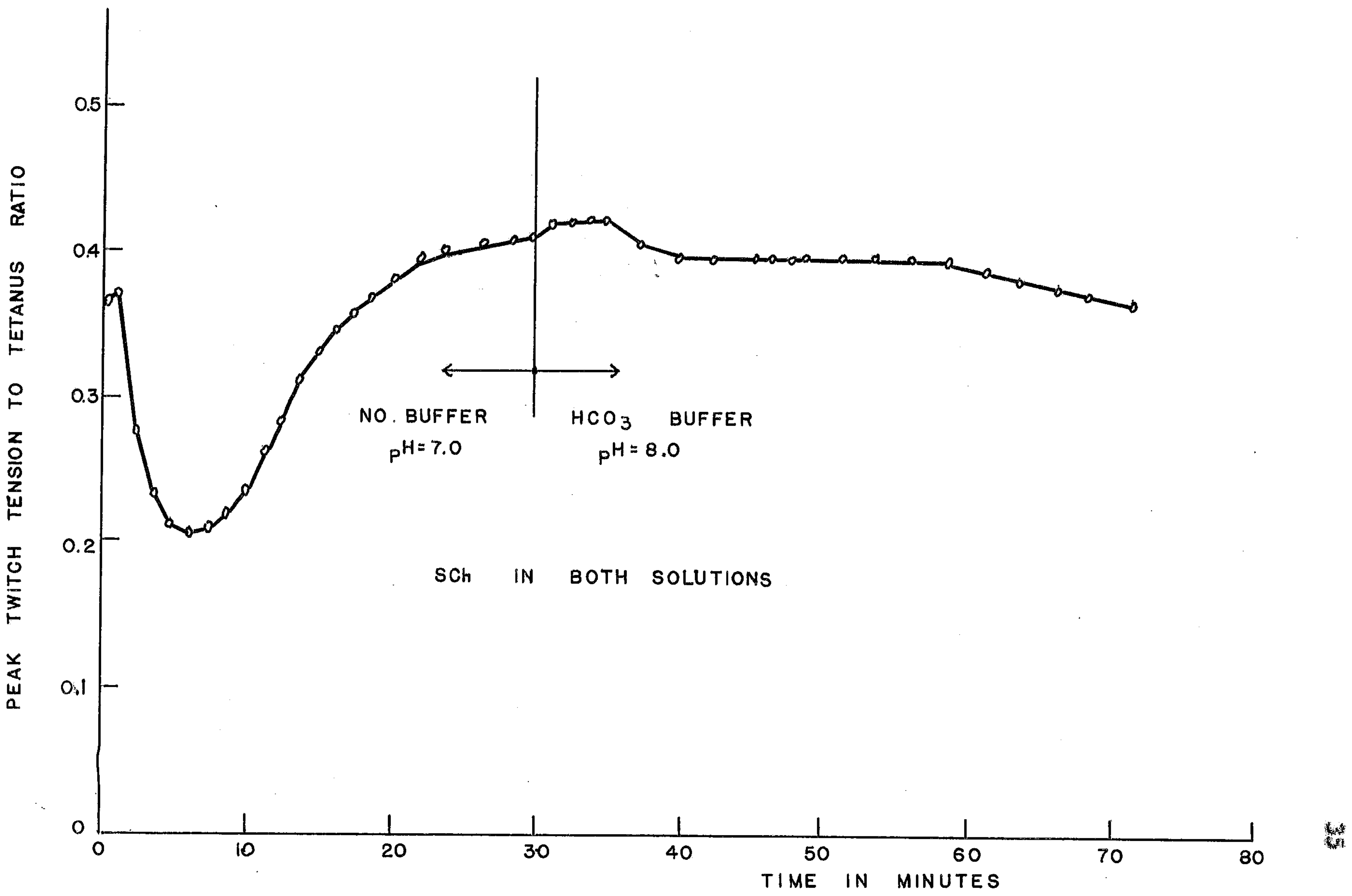

Fig. 16. The effect of switching solutions on a single muscle.

VIII. Discussion

From the results of the previous section, we can see that two of the factors listed in Table 1 must be altered in order to obtain a maintained reduction of peak twitch tension after the addition of SCh. Only the combination of high pH and bicarbonate buffer solution resulted in a reduction of tension. With this knowledge, the membrane events during depolarization can be examined more critically.

One of the most prominent objections to the acetylcholine theory of conduction has been the failure of externally applied acetylcholine to depolarize fiber membranes except at the synaptic regions. Hence, the acetylcholine system has been postulated to be a special mechanism present only at synapses. The use of detergents and snake venoms to pretreat nerve fibers will allow externally applied depolarizing substances to depolarize the fiber and hence block conduction. To explain these results, a barrier to depolarizing agents has been postulated and pretreatment of the fiber is supposed to remove this barrier. The use of a bicarbonate Ringer's solution with a high pH also allows depolarizing agents to cause depolarization. Apparently the barrier, if one actually exists, will allow depolarizing agents to pass if given the appropriate chemical environment.

It is desired to attempt an explanation of the experimental findings assuming the barrier to be the fiber membrane itself. A reasonable picture can be obtained by use of the acetylcholine theory of conduction. One postulated structure for the membrane

is a double layer of lipoid material with protein lined pores appearing periodically (Giese, p. 276). The action of snake venom, which contains proteolytic enzymes, may be the removal of some of the protein from the "pores" allowing the molecules of the depolarizing agent to pass more freely into the fiber. The corresponding leakage of small ions may not be great enough to depolarize the entire membrane during the course of an experiment. The action of a detergent would be to rip "holes" in the lipid layers. The use of high pH and bicarbonate ions in the bathing solution will result in a higher permeability to weakly basic molecules (Giese, p. 232) which would include depolarizing agents.

Other experimental findings could be reasonably explained on the basis of the above picture. Miledi (1960) found, using an unbuffered Ringer's solution, that, the nerve supply to the sartorius muscle of a live frog is cut, then the region sensitive to depolarization by external application of acetylcholine spreads outward from the end-plate region. The amount of spreading depends on the time interval between the cutting of the nerve and the removal of the muscle from the frog (of the order of weeks). It is well known that, when a nerve supplying a muscle is cut, the muscle atrophies. The region near the end-plate probably deteriorates first resulting in increased permeability in these regions--hence a spreading of the area in which external application of depolarizing agents is effective. The fact that end-plate regions are always sensitive even in normal muscle and in low pH and unbuffered solutions is discussed below.

Another experiment which can be interpreted by means of the acetylcholine theory has been performed recently by Katz and

Miledi (1964). Here, a sartorius muscle is cut transversely into two pieces in a live frog and the wound is allowed to heal for about a week. Sensitivity to externally applied acetylcholine developed on the cut end of both portions. Again, damage to the muscle resulted in deterioration of the membrane near the wound resulting in increased permeability to the acetylcholine molecules.

The above interpretations do not explain the results obtained when curare (d-Tubocurarine Chloride) is applied to muscles and nerves. Curare is a substance which has the property of being able to block neuromuscular transmission without any membrane depolarization. Del Castillo and Katz (1957) have investigated the action of curare and found that, when curare is applied externally to the end plate region of the frog sartorius muscle, an action potential reaching the end of the motor nerve fails to depolarize the end plate region. This results in a block of neuromuscular transmission. They also found that curare itself will not depolarize the end plate region. They injected curare into the muscle fiber just underneath the end plate region and found it had no effect on transmission. They concluded that curare competes with acetylcholine released by the nerve for sites in the end plate region by binding to the molecules in the membrane that are responsible for the permeability properties and preventing acetylcholine from affecting them. Since injected curare did not block transmission, it was concluded that these active sites occur only on the outside surface of the membrane.

Ochs, et.al. (1959 and 1960) also used curare and found that depolarization of the fiber membrane could be prevented by

acetylcholine and succinylcholine by pretreating the muscle with curare. They concluded that the curare has a generalized effect that is not restricted to the end-plate region (under the conditions of their experiments). However, curare did not block conduction of action potentials. If the acetylcholine theory of conduction is correct, curare would block the action of internal acetylcholine and hence conduction. One other possibility is that the membrane has active sites on both the inside and outside surfaces of the membrane. In this case, the presence of bicarbonate ions and a high pH does not result in the penetration of depolarizing agents into interior of the fiber. Rather, the agents act on receptors on the outside surface. Hence, the postulated barrier is probably not the membrane itself.

If the acetylcholine system is necessary for conduction of action potentials, curare must be able to block conduction. Since external application does not have any effect, curare injected into the fiber must have some effect. It was mentioned above that del Castillo and Katz (1957) did not observe a conduction block when curare was injected into the frog sartorius muscle. Also, Hodgkin and Keynes (1956) injected curare into the giant axon of the squid with no effect despite the high concentrations of curare used. Curiously enough, del Castillo and Katz (1957) and Hodgkin and Keynes (1956) both used unbuffered salt solutions and the latter group made a special effort to keep the pH of their solutions close to 7. These are the same conditions under which negative results were obtained for externally applied substances.

Can adjustment of the bathing solutions yield results similar to the case of externally applied substances for the case of injected substances? Grundfest, et.al. (1952) reported that injected curare was very effective in producing a conduction block. Unfortunately, the details on composition of their bathing solution were not published. These results mean a conditional yes in answer to the above question.

If one assumes the correctness of the acetylcholine theory of conduction, then the barrier to substances affecting conduction that is present under unbuffered conditions must be present on both sides of the fiber membrane. Since externally applied curare does not block conduction when it is able to penetrate the barrier, the site of the acetylcholine system of the cell must be inside the cell. Further, when injected curare is unable to penetrate the barrier, conduction remains. This implies that the acetylcholine storage area in the cell must be between the inside barrier and the fiber membrane.

The action of depolarizing substances at the end plate region can now be discussed. The ability of the muscle end plate region to respond to externally applied substances even when the chemical environment is such that the barrier is impermeable to these substances indicates the lack of any barrier there. What explanation can be given for the presence of a semi-permeable barrier over a large portion of the inside and outside of the muscle fiber membrane but not at the end plate region? The region is adapted to accept external substances. According to the acetylcholine theory of transmission, acetylcholine released from the

nerve ending diffuses 500 to 700 angstroms to the post synaptic region resulting in a conducted action potential. If the acetylcholine system was used only for conduction and electrical currents used for transmission, one would not expect a hole in the barrier where transmission was to take place. The region is adapted for reception of external acetylcholine and hence a chemical transmitter (as opposed to an electric current) would be a more probable means of transmitting an action potential from a nerve ending to the muscle fiber.

The nature of the barrier has not been discussed since very little information is known about it. Also, the application of these results to smooth muscle and cardiac muscle have not been discussed. Cardiac muscle presents a problem to any theory of conduction because of the long time course of the cardiac action potential and its inherent rhythmicity. The high value of twitch to tetanus ratio found when a muscle is bathed in bicarbonate buffered solutions is not easily explained. If the cause is due to a change in the shape of the muscle action potential, a modification of the acetylcholine theory may be needed.

IX. Conclusions

The composition of the solution bathing a muscle has been shown to affect the results obtained when depolarizing substances are applied externally to skeletal muscle fibers. The use of high pH and bicarbonate buffered salt solutions results in a maintained reduction in twitch tension when succinylcholine is added to the solution. On the other hand, only a transient dip in tension is observed when low pH and unbuffered salt solutions are used and succinylcholine is added.

Various experiments involving the acetylcholine theory of conduction and transmission were discussed. It was concluded that there is a barrier to depolarizing substances present on both sides of the membrane. Also, if the acetylcholine system is actually present along the fiber, it must be located between the barrier and the inside surface of the fiber membrane. Further, a more complete theory of conduction and transmission of action potentials must include the effects of ionic concentration, including effect of the hydrogen ion on the nerve or muscle fiber.

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