May 24, 1957

To the Graduate Council:

I am submitting herewith a thesis written by Edgar B. Darden, Jr. entitled "Electrophysiological and Electrolyte Changes in Frog Striated Muscle After Irradiation". I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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ELECTROPHYSIOLOGICAL AND ELECTROLYTE CHANGES IN FROG STRIATED MUSCLE AFTER IRRADIATION

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy

---

by

Edgar B. Darden, Jr.

June 1957

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	11
	Muscle preparation	11
	Intracellular electrodes	13
	Silver-silver chloride electrodes and recording equipment	16
	Micromanipulation equipment	21
	Radiation sources	23
	Morphology and histology	24
	Determination of potassium	24
III.	RESULTS	27
	Membrane potentials in normal fibers	27
	Effect of radiation on membrane potentials	31
	Effect of radiation on muscle potassium	41
	Visible pathological and histological changes	47
	Membrane potentials in clotted fibers	54
IV.	DISCUSSION AND CONCLUSIONS	56
BIBLIOGRAPHY		70
APPENDICES		76
APPENDIX A		77
APPENDIX B		79
APPENDIX C		86

LIST OF TABLES

TABLE PAGE

I. Survey of Membrane Potentials in Surface Fibers of Three Muscles 28

II. Comparison of Membrane Potential Measured with Intracellular Electrodes Filled with Isotonic (118 mM) and 3 Molar KCl 30

III. Frequency Distribution of Potentials in Control and Irradiated Muscles at Intervals Before and After Exposure to a Dose of 122 kr 34

IV. Comparison of Median Potentials in Control and Irradiated Muscles at Doses from 33 kr to 220 kr 35

V. Effect of Radiation on Muscle Potassium 46

VI. Composition of Ringer Solution 78

LIST OF FIGURES

FIGURE		PAGE
1.	Lucite Muscle Rack	12
2.	Tips of Micropipettes	15
3.	Electrode-Muscle Bath Assembly	18
4.	Intracellular Electrode in Muscle Fiber (120X)	22
5.	Membrane Potentials of Individual Fibers at Intervals Following Irradiation	38
6.	Potential Along a Single Fiber Recorded at Intervals Over a Period of 70 Hours	40
7.	Membrane Potential Along a Fiber Immediately Prior to Formation of a Retraction Clot at Indicated Position	42
8.	Potassium Changes in Irradiated Muscle at Room Temperature	45
9.	Potassium Changes in Irradiated Muscle in Cold (2-3° C)	48
10.	Living Fibers Before and After Exposure to a Dose of 200 kr (100X)	52
11.	Stained Sections of Control Muscle and Irradiated Muscle Exposed to a Dose of 220 kr (215X)	53
12.	Parasite Engulfing Portion of Muscle Fiber (75X)	59
13.	Diagram of Pulling Device for Ultramicropipettes	81

FIGURE PAGE

14. Input Circuit for Measurement of Membrane Potentials Including Resistance and Voltage Calibration Test Circuits 84

15. Equivalent Circuit for Measurement of Input Resistance 85

16. Rate Along Axis Inside Cobalt-60 Source Cylinder (As of Early 1956) 88

CHAPTER I

INTRODUCTION

Muscle is regarded as one of the most radioresistant tissues in the body. As is the case in many other tissues, the effective dose varies greatly depending upon the nature of the radiation effect observed. Also, as in other kinds of cells, but strikingly so in muscle, the radiosensitivity of individual fibers exhibits an extraordinary degree of variation.

Warren (1943), in a review of the pathologic effects of radiation on normal tissue, has described some of the very early observations of injury to muscle from implanted radon seeds. The radiation source was surrounded by an immediate zone of severe necrosis, 4 to 15 mm in diameter, presumably attributable to the beta ray fraction of the radiation. This region was characterized by loss of striation and fragmentation of muscle fibers, homogenization of sarcoplasm and nuclear breakdown. Beyond this zone was a less severely damaged region of congestion and hemorrhage.

Gottschalk and Nonnenbruch (1923) observed that the rate of respiration of frog muscle brei was lowered by a few minutes' exposure to roentgen radiation. On the other hand, Wels (1924) reported no effect on the rate of oxygen consumption of frog muscle brei after exposure to intense X radiation for periods up to five hours. Kushner (1925), also in the days of inexact dosimetry, noted a slight increase of irritability of excised frog muscle after a three-minute exposure to

40 kv X rays. Fenn and Latchford (1931) found a moderate increase in oxygen uptake in excised frog muscle after exposure to a dose of 60 to 90 kiloroentgens of intense X radiation. This was interpreted as a secondary result of injury rather than a direct effect on oxidative processes.

In a comprehensive histological study which has not received the attention it should have, Fedder and Hellner (1928) investigated the effects of 170 kv X rays in doses up to several erythema units* applied locally to the gluteal muscles of rabbits, both through the skin and through the exposed muscles. They studied the irradiated tissues at intervals of from one hour to one hundred days after radiation. At two hours some nuclei and the surrounding sarcoplasm in scattered fibers exhibited signs of injury with progressive increase in severity until at twenty-four hours, karyolysis and Zenker degeneration was widespread with leucocyte and macrophage infiltration of the damaged and necrotic fibers. The authors were careful to point out that the irradiation did not appear to induce any changes unique in itself. Janzen and Warren (1942) noted that in a study of the effect of radium X rays upon rat peripheral nerve with doses up to 100 kiloroentgens, muscle appeared wasted in the vicinity of the radon implants. Henshaw (1944) described the almost immediate acute effects of massive single doses of 25 to 50 kr of whole-body radiation of rabbits and other animals and observed

---

*One erythema unit equals approximately 600 roentgens. B. Rajewsky, Strahlendosis und Strahlenwirkung (1956). Stuttgard, Georg Thieme, 2nd Edition, p. 78.

that the rabbits died in a state of muscular spasm. Histological specimens showed that not a tissue in the body escaped damage. Both striated and nonstriated muscle fiber exhibited noticeably cloudy swelling with destruction of sarcoplasm and collapse of sarcolemma at some points.

Bacq, Lecomte, and Herve (1949) studied the effects of 50 kv and 190 kv X rays and P^{32} beta rays on the contractility of the rectus abdominis muscle of the frog excited periodically by application of a 5 per cent KCl solution. With doses below 500 r, spontaneously reversible contracture developed after several excitations. After a dose of four kiloroentgens, excitation was rapidly followed by progressive loss of excitability and irreversible contracture (Lundsgaard effect).

Gerstner, Lewis, and Richey (1954) and Gerstner, Powell and Richey (1954) investigated the early effects of massive doses of X radiation on the contractility and work capacity of excised frog muscle and on the work capacity of leg muscle (triceps surae) in the intact rabbit. In frog muscle exercised during radiation, loss of function was observed beginning at about 50 kr and extending to 150 kr. Beyond this dose all function was abolished. In muscle exercised following doses greater than about 30 kr, progressive impairment of work capacity was observed along with gross morphological changes (alterations in shortening, coloration, and translucency) as compared with the control, until above 150 kr all function was abolished. Histologic changes were observed within eight hours in most of the muscles in the high-dose range. Immediately after exposure to 72 kr,

the mammalian muscle under workload also showed progressive functional impairment with 80 per cent loss of work capacity by twenty-four hours. By this time massive edema of the limb was present along with histological evidence of small hemorrhages and scattered necrosis of muscle fibers. Lewis (1954) of the same group made a detailed histological study of irradiated muscle from the legs of rabbits after localized exposures from 6 to 72 kr. Destructive lesions were present three hours after the highest dose and by twenty-four hours most of the muscle was necrotic including connective tissue and blood vessels. Lesser doses down to 12 kr produced coagulation necrosis of smaller regions and scattered fiber atrophy.

Wilde and Sheppard (1955) observed no significant loss of function up to twenty-four hours after localized irradiation of rat forelimbs with doses up to 72 kr. Severe edema was present throughout the limb but the muscle, except for its excessive water content, appeared to be unaltered. The muscle water as determined by inulin and Na^{24} measurements was confined to the extracellular region. No significant change in muscle potassium and only a borderline change in muscle sodium were found.

Fedder and Hellner (1928) deplored the fact that no systematic investigation had up to then been carried out on the effects of roentgen rays on muscle; now, thirty years later, the entire literature on the effects of ionizing radiation on muscle tissue is still quite modest in extent and partially contradictory.

There are at least two reasons for this relative scarcity of information on irradiation effects in muscle tissue. The most obvious

reason is that radiation injury of more immediate concern from the medical and experimental standpoint is more quickly and conveniently produced in most of the tissues at much lower doses. The other reason has to do with the nature of the studies. Until fairly recently most radiation studies on muscle tissue and perhaps other tissues were concerned primarily with descriptive pathological manifestations of injury, chiefly perhaps, because of the great technical difficulties involved in obtaining quantitative measurements relating to changes in the tissue. This type of obstacle is now being rapidly bypassed as a result of some of the more recent technical developments, particularly in electronic instrumentation. For example, the art of flame photometry by which analyses of Na and K are now routinely carried out in a matter of minutes instead of hours, has now developed to a sensitivity just slightly short of that which makes feasible microanalysis of single muscle fibers (Vallee and Margoshes, 1956). However, electronic progress has already enabled micro techniques to be developed which allow precise electrophysiological measurements to be made on many kinds of single cells. By means of this intracellular electrode technique it is possible for the first time to make direct measurements on individual muscle fibers with no detectable injury to the fiber instead of having to rely on information obtained mostly at the whole-muscle level by procedures often involving destruction of the tissue. With this tool much progress has already been made toward a fuller understanding of the electrical processes in normal fibers, particularly as related to their internal and external ionic environments. The integration of the new information with classical electrophysiological concepts has been ably and comprehensively reviewed by Hodgkin

(1951). On the other hand, little use of the new techniques has been made to date in studying injured fibers whether by irradiation, disease, or other means.

To record intracellular, or rather transmembrane potentials by the intracellular microelectrode technique, a glass capillary pulled out to a microtip and filled with a conducting solution is inserted by micromanipulation directly into the cell. The other electrode, not necessarily equipped with a microtip, is in electrical contact with the external conducting solution. If the diameter of the extreme terminus of the microtip is on the order of 0.5 microns or less, it penetrates without effort and without apparent injury to the fiber. With tips larger than 1 micron, the magnitude of the potential recorded is progressively reduced with increasing tip diameter (Woodbury, Hecht, and Christopherson, 1951). It is not known exactly to what depth inside the muscle fiber the microelectrode must be inserted to establish the potential. Under the proper conditions the slightest detectable penetration of the tip into the fiber gives rise to the full value of the potential.

The characteristics of this potential both in the resting and active states have been investigated extensively, particularly in relation to the ionic environment inside and outside the cell. Inasmuch as the present investigation is not directly concerned with the action potential component, it will not be discussed in any length. Unless otherwise indicated, the terms "membrane potential," "fiber potential," or "potential" will refer to the resting membrane potential.

Graham and Gerard (1946), employing micropipette electrodes with tips up to several microns in diameter were the first investigators to

measure membrane potentials in single impaled fibers of the excised frog sartorius. Potentials as high as 80 mv were measured but the values were not found to be very constant from one fiber to another. Ling and Gerard (1949A), using improved microtips having diameters of a few tenths of a micron, found the membrane potential actually to be a highly consistent parameter and constant within a few per cent in fibers of a given muscle, both in excised and in intact living muscle.

Nastuk and Hodgkin (1950) studied the resting potential in further detail and made the first intracellular electrode studies of the action potential in striated muscle fibers.

The resting membrane potential was shown to be critically dependent upon the external concentration of potassium in the external fluid, to be relatively independent of the state of stretch of the muscle and pH (Ling and Gerard, 1949A, 1949B, 1950) and to have a Q_{10} of about 1.1 from 4° to 30° C (Ling and Woodbury, 1949). The relation of the potential to metabolism was studied and evidence was presented that the potential is made up of two components; one called the "A" potential, from about 55 mv to the maximum value of about 80 mv; the other, the "B" potential, from zero to 55 mv. The value of the average "A" potential correlated rather well with the phosphocreatine (PC) content of the whole muscle and appeared to be supported by glycolytic metabolism; the "B" potential appeared to be more nearly related to the concentration of less labile intracellular constituents such as hexose monophosphate, proteins and other anions (Ling and Gerard, 1949C, Ling, 1952). The relation of potential to other electrical properties of the membrane was

studied with microelectrodes by Jenerick and Gerard (1953). The resting potential in mammalian mouse fibers in vivo has been studied by Bennett, Ware, Dunn and McIntyre,(1953).

Little information is available on the electrical and ionic changes brought about by injury to muscle cells. A leakage of potassium is a consequence of injury and if repair does not take place, K is apparently progressively lost until cell death. The minimum concentration of K compatible with cell survival or viability has not been established, but the normal content in comparison to the critical concentrations of some other ions appears to be large enough to represent a considerable safety factor (Steinbach, 1954). Injury also lowers the membrane potential and cell death abolishes it, but considerable membrane potential (about 39 per cent of normal) is still found after essentially all K has been removed by soaking and the muscle is waterlogged (Tobias, 1950).

The relation of localized mechanical or chemical injury to the membrane potential was studied by Graham and Gerard (1946). They demonstrated an inverse exponential relation between the amount of reduction of the membrane potential and distance from the injury until at about 10 mm the potential was back up to normal.

The importance of physical factors on injury potential, degeneration and death of injured fibers has been emphasized by Rothschuh (1950). Factors such as the electrical conductivity of the medium and the length of the fiber were shown to greatly affect the fall of potential (measured as the injury potential).

In the present work, evidence is presented that radiation injury in excised frog muscle may be very early reflected in a loss of membrane potential which occurs at loci more or less randomly distributed over the fibers available for impalement; i.e., the superficially located fibers. The number and severity of these electrical lesions increase with time and with dose and appear to be strongly influenced also by the temperature of the surroundings. Concurrently, potassium leakage is presumably occurring at these lesions, for in time a net loss of K can be demonstrated by gross analysis of the muscle bath and/or ash. At this time, or shortly thereafter, visible changes are initiated at sites which, according to the evidence presented, correspond largely with the loci of the original electrical lesions. This visible fiber alteration leads to a histological condition similar to that commonly seen in many kinds of muscle injuries and diseases and known as Zenker's waxy or hyalin degeneration, just as described by previous workers for mammalian muscle heavily irradiated in the intact animal. This pattern of events is not uniquely confined to radiation effects but appears to be of some general significance.

Before considering the details of the present work, a brief description of the pertinent characteristics of the biological material used will be helpful. The excised sartorius muscle of the frog has been the subject of a large amount of intensive electrophysiological investigation. It is a relatively long, somewhat ribbon-shaped muscle, more or less eliptical in cross-section, with its origin on the pubic bone. It extends obliquely along the inner thigh surface and narrows to a somewhat

fusiform shape at the tendon which is inserted below the knee. The muscle is composed of several hundred more-or-less parallel fibers, each approximately 80 microns in diameter, continuous from end to end and terminating in tendinous tissue at both ends with some of the fibers crossing over each other especially at the lower end. The bulk of the blood supply, if not all of it, comes from a single vessel which, along with the nerve, enters the sartorius at its medial border about a third of the way up from the insertion and immediately arborizes into several extensive branches with numerous offshoots and capillaries between the fibers. The pattern of vessels was usually sufficiently unique and conspicuous to provide convenient landmarks for locating specific fibers. The external surface of the muscle just beneath the skin is covered with a closely adhering skein-like sheet of connective tissue, showing considerable resistance to penetration by a microtip and obscuring visible details of the fibers underneath. The internal surface of the muscle is covered with a much thinner layer of connective tissue and more richly supplied with blood vessels; hence, the fibers constituting this surface are the ones in which impalements are usually made.

CHAPTER II

MATERIALS AND METHODS

Muscle Preparation

The frogs were a variety of Rana pipiens from Vermont and were kept in aquaria at about 3° C. Excised sartorius muscles (weighing 90-150 mgm each) of both sexes were used in pairs throughout. Immediately after pithing the frog to destroy both the brain and spinal cord, the muscle was excised, usually under low-power magnification, to insure minimal injury to the fibers especially along the boundaries and at the origin. To further reduce chances of injury at dissection, particularly in muscles with poorly defined boundaries, adjoining tissue, especially in the region of the origin, was dissected out attached to the muscle. (It was important to spot injured fibers since the potentials of such a fiber were likely to be already relatively low throughout much of its length). Each muscle, under sufficient tension to prevent sagging, was clamped by tendinous tissue at both ends in a small, adjustable plastic rack as shown in Fig. 1. Final trimming of superfluous tissue and inspection for injured fibers was then done by transillumination on the stage of a binocular miscroscope with the muscle stretched on its rack in a shallow dish of Ringer.

In the electrophysiological experiments the muscle was not disturbed from its position on the rack throughout the duration of the experiment. When potentials were to be recorded the rack was placed in a shallow transparent bath chamber clamping directly onto the adjustable

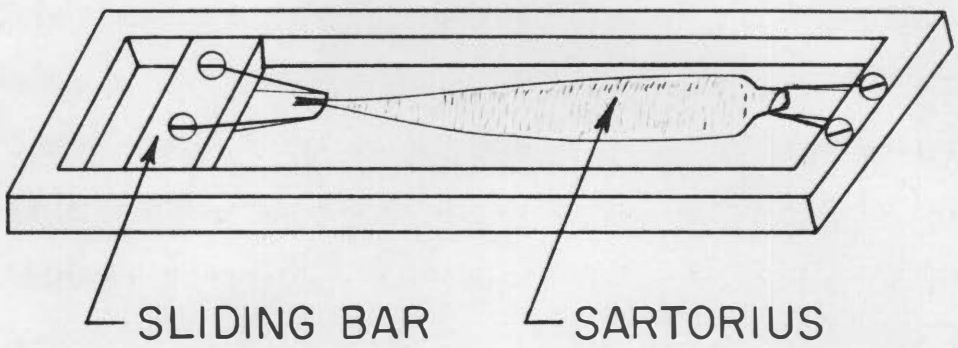


Fig. 1

Lucite Muscle Rack.

stage of the microscope. The volume of the bath was about 7 ml and it covered the muscle to a depth of approximately 2 mm. Impalements were generally made in the belly region of the inside surface, beginning near one border and working straight across to within a few fibers of the opposite border. Control and test muscles of a pair were customarily recorded one after the other. The bath solution was replaced before and after each series of impalements or at least every thirty to forty minutes if lengthy recordings were being made. The rack was kept in the same container for exposure to X-rays and for gamma-ray exposures the rack was hung inside a large test tube of Ringer. Muscle preparations when stored in the cold were at a temperature of approximately 3° C.

For the potassium experiments each muscle following inspection after dissection was removed from its rack and placed in a 25 ml Erlenmeyer flask containing at least 25 times the tissue volume of Ringer (at least 4 ml). The flasks served also as exposure vessels (gamma radiation only). The flasks were kept lightly covered (inverted 5 ml beakers) during irradiation and soaking procedures.

Phosphate Ringer solution containing 2.5 mm/liter of K with a pH of 7.35 $\pm$ .05 at room temperature was used for all procedure unless otherwise indicated. The composition is given in Appendix A.

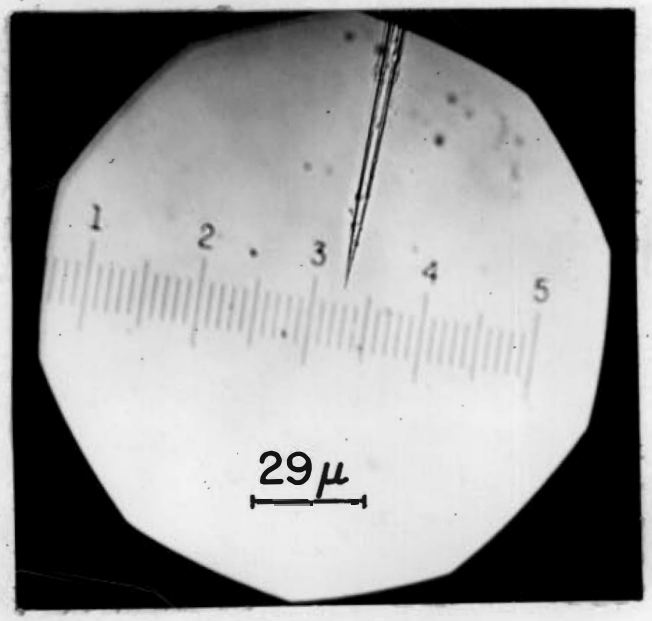
Intracellular Electrodes

The ultramicropipettes used and the intracellular electrodes were somewhat similar in size if not shape to those described by Ling and Gerard (1949A). After considerable experimentation with several kinds of capillary tubing a type made especially for microforge work

was selected. The details of fabrication and filling are given in Appendix B. It was found that smaller and better tapered micro tips could be produced if the stock tubing (1 mm O.D.) was first constricted to a diameter of approximately 0.3 mm before the final pulling. The latter was done by using a modification of an electric heating method described by Weale (1951), a variant of the drop weight method long employed by chemists for the pulling of extended lengths of capillary tubing.

Considerable trial and error was required to obtain the appropriate combination of heating time, tension, and temperature necessary to produce satisfactory tips. Micropipettes with satisfactory open tips less than 0.5 microns in diameter of the desired shape could then be repeatedly fabricated with a high proportion of successes. A photomicrograph of such a tip taken with a water immersion objective at 500X is shown in Fig. 2. The extreme terminus is at or below the limit of optical resolution. A tip of this shape is sufficiently flexible to resist breakage but still rigid enough to pierce through any thin fascia to the underlying fibers. An electronmicrograph of the terminus of a tip is shown in Fig. 2. The diameter of the tip is seen to be less than 0.2 microns and the diameter about a tenth of a micron.

The conducting fluid used was either Ringer or isotonic (118 mM) KCl. The d.c. impedance of micropipettes filled with these fluids may be several hundred megohms. A much lower value of impedance is often necessary in some types of investigations with intracellular electrodes, particularly studies involving action potentials. For this reason and for reason of limitations in equipment most workers using the



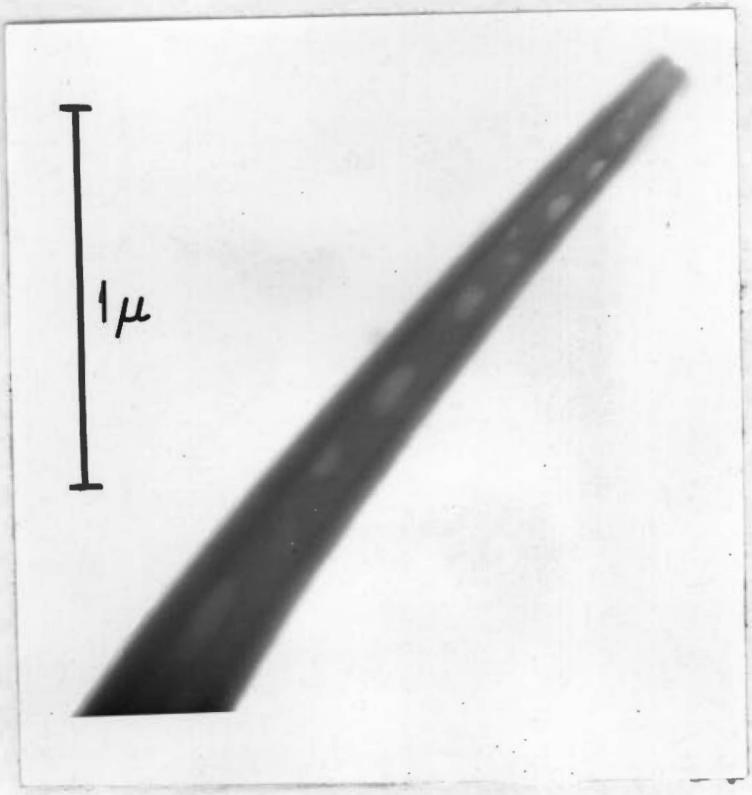


Fig. 2

Tips of Micropipettes

intracellular electrode technique have filled their micropipettes with concentrated KCl solution, usually 3 molar. In the present work the usable impedance was limited only by the sensitivity of the input impedance to external electrical influences, since the input impedance of the vibrating reed electrometer is on the order of 10^{17} ohms. In comparison to this the micropipette resistance was negligible. (The resistance of satisfactory pipettes generally fell in the range of 100-500 megohms).

The filling of intracellular electrodes is somewhat of a problem. At first the boiling method (Ling and Gerard, 1949A) used by a majority of the early investigators was tried. This method was found to produce intracellular electrodes which in a high percentage of cases behaved as though an excessive or unstable electric charge had been induced in the microtip, a condition also noted by others (see Adrian, 1956). After considerable experimentation with this and other reported filling methods (Bennet, Ware, McIntyre, and Dunn, 1953; Nastuk, 1953) a satisfactory injection technique was evolved as described in Appendix B.

The rise of fluid in the extreme ends of the microtips could be followed during the filling process and inspection of each tip for debris and air bubbles was routinely carried out with the aid of a 50X water immersion microscope objective, and a total magnification of 500X or 750X.

Silver-Silver Chloride Electrodes and Recording Equipment

In any system for measuring bioelectric potentials the junction of the aqueous phase with the metallic part of the circuit is a source of a junction potential which may interfere with the measurement of the desired

potential. An ordinary copper wire, for example, in contact with the electrolyte will slowly polarize as current flows. The concurrent changes in resistance and e.m.f. arising from the metal-liquid junction will be reflected in variations of the potential being recorded. If the variations are slow compared with the rate of change of the magnitude of the bioelectric potential such as, for example, an action potential, satisfactory measurements can in some cases still be made but, obviously, the system has to be electrically stable to properly measure steady bioelectric potentials. (To accomplish this objective some kind of nonpolarizable electrode is necessary. For bioelectric purposes silver-silver chloride electrodes are frequently employed. With very careful and involved preparation two such electrodes may be made to show a steady difference of only a few microvolts between each other when dipping into a chloride solution. For ordinary bioelectric measurements in which the potential is usually measured in tens of millivolts, such refinement is unnecessary and suitable stable electrodes which show a difference of a millivolt or less are much more easily prepared as described in Appendix B. The electrodes used in the present work are diagrammed in Fig. 3. Each consisted of a #20 B. & S. Gauge chlorided silver wire dipping in Ringer or KCl agar inside a glass tube about the size and shape of a medicine dropper. One electrode which served as the probe was fitted with a small rubber connector previously filled with KCl solution, to which was attached the micropipette. The other electrode served to make contact with the muscle bath and complete the circuit. The silver wires were led outside the electrode tubes through liquid-tight teflon

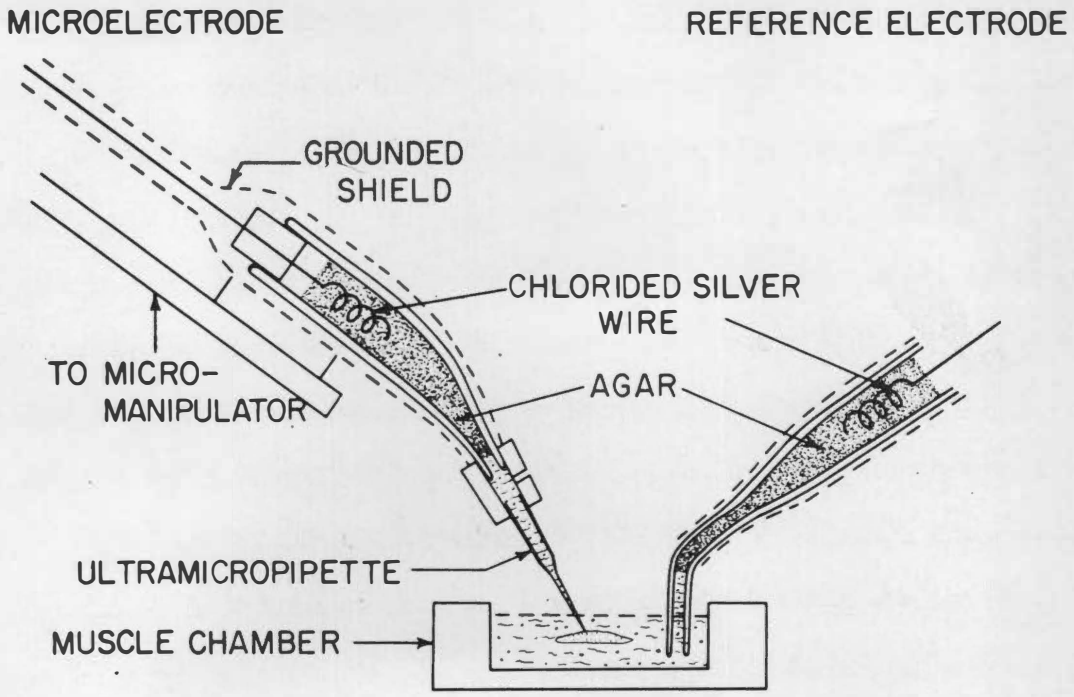


Fig. 3

Electrode - Muscle Bath Assembly.

bushings and were soldered to light-weight shielded leads connecting with the recording system. The electrode tubes were covered with a braided shield as shown. All shielding was connected together to a heavy copper lead attached to the building ground.

The input from the electrodes was connected to a vibrating reed electrometer along with auxiliary test and calibration circuits as shown in Fig. 14. (Appendix B). The input resistance of the electrodes, essentially that of the microelectrode, was measured by a balance method similar to that described by MacNichol and Wagner (1954) with appropriate modifications for use with the electrometer. The method which was based on a comparison of the unknown resistance against a high megohm precision resistor (Victoreen vacuum sealed) as a standard is described in Appendix B. A standard resistor value of 195 megohms was found to be convenient for measuring resistance from about 50 megohms to 1000 megohms. The input resistance was routinely measured before and after a series of impalements and after changes of bath Ringer.

A potential calibration circuit controlled through SW_5 (Fig. 14) enabled a known e.m.f. from the potentiometer to be applied in series with the input from the indifferent electrode to the feedback circuit of the electrometer. By this means the value of an unknown potential could be determined by balancing it against a known potentiometer voltage, or a potential of arbitrary magnitude and polarity could be applied across the electrodes for various purposes. As a matter of practice, calibration of the recorded potential was done only as an occasional check, since the output of the electrometer was recorded on a calibrated moving chart

recorder. The scale of the chart was 10 mv/inch and since it was a ten-inch chart, deflections were easily read to a fraction of a millivolt.

The baseline from which potential deflections were measured was always determined by placing both electrodes in the bath. The system was not considered to be functioning satisfactorily if the baseline exceeded about ± 5 mv as measured from zero mv (electrometer short-circuited), or was drifting or showed a noise band in excess of about ± 0.5 mv. An impalement was regarded as satisfactory if upon insertion of the electrode into the fiber, the potential rose immediately to its full value and remained constant within a millivolt. Generally, full deflection was obtained with the barest perceptible penetration of the microtip into the fiber. If the electrode had to be forced into a healthy fiber, a fouled or broken tip was usually indicated and the potential, if established at all, usually rose in one or more steps and crept slowly to a leveling-off value. A measurement of input resistance in this case usually showed a sharp change from the previous reading to an excessively high or low value depending on whether the tip was plugged or broken.

The tips of these pipettes, especially after being immersed in the bath for several hours, often picked up bits of fascia, et cetera, in the course of many impalements and, especially after several hours' immersion in the bath, acquired a perceptible coating of closely adhering organic material. In such cases it was usually possible to remove debris by "brushing" the immersed tip with a fine flexible glass whisker while under observation in the dissecting microscope. Contrary to expectation,

the extreme terminus was apparently undamaged by quite vigorous scraping with the whisker. This operation was also sometimes successful in restoring the proper magnitude of resistance, when it was excessively high.

In one case a single pipette with several such cleanings was used over a period of several days for almost four hundred impalements with the pipette resistance checks falling within the acceptable range.

Micromanipulation Equipment

A binocular microscope equipped with an adjustable stage was used in conjunction with a modified Emerson micromanipulator. Both were supported on a 3/4 inch steel plate mounted on rubber to reduce vibration. Bolted rigidly to the plate was a heavy brass vertical member supporting the manipulator in a horizontal position. This enabled the manipulator arm to operate in a vertical rather than the customary horizontal plane. The arm of the manipulator could be rotated down by coarse movement to bring the micropipette tip just over the surface of the muscle. To help prevent overshoot in swinging the arm downward, its axle was fitted with a reduction gear. By simultaneous adjustment of the manipulator and the stage, a target fiber could be quickly brought into position for impalement. Insertion of the micropipette into the fiber was then accomplished with a downwardly directed oblique movement so as to keep the microtip in view up to the instant of penetration (Fig. 4).

Illumination was provided by microscope lamp with a reflector and frosted glass disc to provide a large diffused source of light.

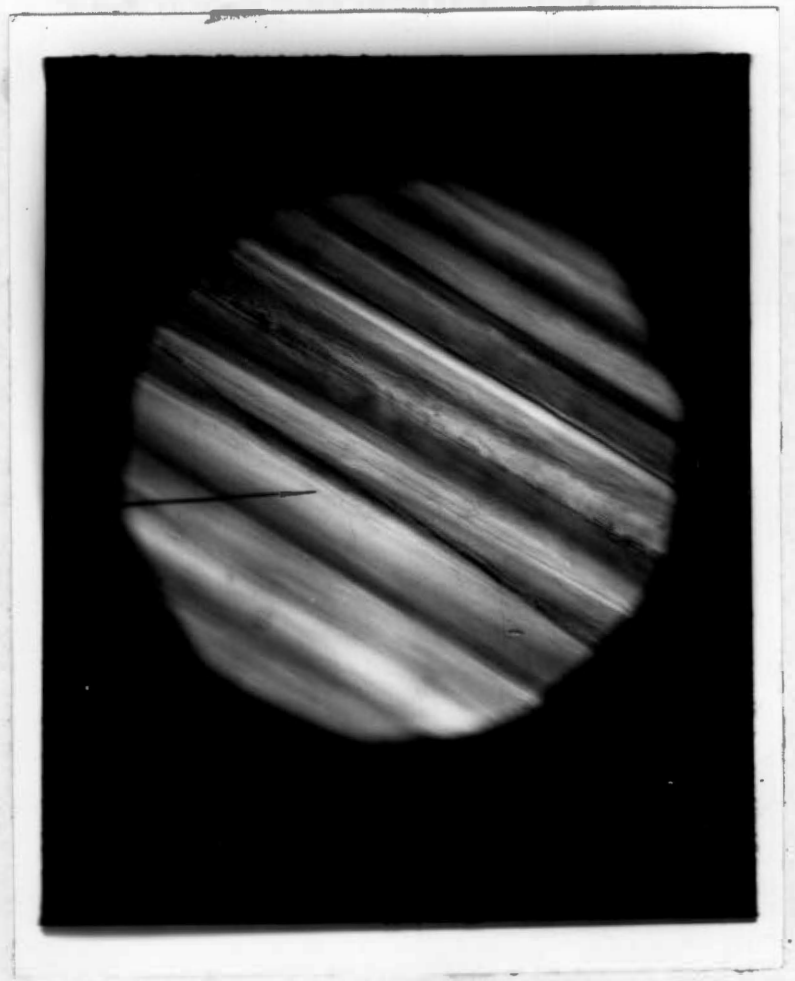


Fig. 4

Intracellular Electrode in Muscle Fiber
(120X)

With the binocular microscope proper balance of illumination was found to be critical in order to obtain maximum visibility of the microtip.

Radiation Sources

X-ray. Muscles were irradiated with 250 KVP X-rays, filtered by an added 2 mm of aluminum, from a General-Electric Maxitron deep therapy unit equipped with a beryllium window. Dose rates ranged from 1300-1900 roentgens per minute as measured by a calibrated Victoreen r-meter in air. The tissue dose was assumed to be essentially equivalent to the measured dose since the solution covering the muscle and the surrounding plastic dish presented only a few millimeters of material of low atomic number to be absorbed or scattered by the beam.

Co^{60} gamma ray source. A high intensity distributed-type Co^{60} source was used for most of the irradiation experiments. In addition to being available much more of the time than was the X-ray machine, this source possessed the advantage of an almost completely uniform field of radiation intensity, which needed to be calibrated only once. The facility was similar in design to a more intense source built for radiation chemistry studies described by Ghormley and Hochanadel (1951). The heart of the device was a hollow brass cylinder, 2 inches I.D. x 8 inches high inside, containing cobalt slugs distributed inside the walls in such a way as to give an almost flat field over the region of the air core. The cylinder, contained inside a massive cylindrical lead turret, was manipulated by a pushrod extending through the top of the turret. The latter rode on top of a massive lead block containing two vertical exposure wells. The procedure for making an exposure was simple. A

sample container of suitable size and shape was centered in one of the wells, the turret was sheeled directly over it and the source cylinder was lowered over the sample. Moving-part tolerances were close so that precisely reproductible geometry could be obtained with each exposure. The dose rate was approximately 1500 r/minute as determined by the value for the original calibration, corrected for decay. Details of the calibration are given in Appendix C.

Morphology and Histology

Observations on normal and irradiated muscle fibers in vitro were made with magnifications up to 750X with the aid of a water immersion objective.

A few muscles were fixed and stained with hematoxylin and eosin and with PAS (periodic acid Schiff) reagent. Fixation was in 10 per cent formalin followed by Zenker-formol. It was found unsatisfactory to drop the living muscle directly into either fixative because of the severe distortion in the fibers produced by the sudden and excessive shortening. To avoid this, a drop or two of formaldehyde was first added to the Ringer bath containing the extended muscle. This killed the muscle in a few minutes with a minimum of distortion after which it was fixed in the usual manner.

Determination of Potassium

All the potassium analyses were made on an internal standard flame photometer. In the first half of the work a Perkin-Elmer machine, Model 52C, was employed. Later, because of instability problems, this

instrument was replaced with a Baird-Associates flame photometer. The Perkin-Elmer instrument utilized a monochromator, together with photocells, light chopper, and an electronic amplifier to select and detect element lines. On the other hand, the Baird photometer used only narrow band-pass filters and photocells with no electronic amplification to accomplish the same purpose. In addition to its simplicity, this instrument operated at a much lower gas pressure than the other one. Regulation of gas pressure was found to be critical for acceptable operation. After considerable trial and error, excellent pressure control was finally obtained by utilizing an inexpensive low-pressure regulator with a large diaphragm, such as is used on city gas lines. In retrospect, it is probable that much of the instability experienced with the Perkin-Elmer instrument was traceable to this same factor in spite of the fact that both photometers were supposed to be capable of satisfactory operation without requiring precise control of gas pressure. In a few cases the same sample was determined first on the Perkin-Elmer and several months later on the new Baird. The agreement between readings was excellent.

For the radiation experiments sartorius muscles were isolated in pairs as before, blotted gently on filter paper, and weighed in tared 25 ml Erlenmeyer flasks. These flasks, covered with inverted 5 ml beakers, contained a measured volume, usually 4 ml or 5 ml, of Ringer solution. The muscle was irradiated in the flask, then at intervals of about two hours the muscles were removed following gentle agitation of the flasks to break up any ionic concentration gradients next to the

muscles, blotted, and transferred to a new pair of tared flasks. Samples for flame analysis were made by diluting the muscle bath or aliquots thereof following removal of the muscle, adding lithium chloride or lithium nitrate as the internal standard, and making up to the mark in 50 or 100 ml volumetric flasks. Repeated checks showed that the difference in volume of the bath before and after removal of the muscle, resulting from solution adhering to the muscle, was not large enough to be detected. For analysis of the whole muscle, a wet-ash procedure described by Sheppard and Martin (1950) was used. A small amount of concentrated nitric acid was added to each muscle with gentle warming to liquify it and the contents partially evaporated to reduce the volume. To oxidize the nitrates, formic acid was carefully added. After brown fumes of NO_2 had ceased to evolve, the resulting pale solution was diluted and made up in the usual manner for flame analysis. Blank checks in the flame photometer showed that the nitric acid in quite large concentrations exhibited no appreciable sensitivity in the potassium flame. Addition of the formic acid proved to be a tricky step because of the tendency of the contents to erupt rather violently. Even when the wet ashing was carried out in a flask equipped with a trap to prevent spattering, some slight loss of contents sometimes occurred. Therefore, in the later analyses hydrogen peroxide (30 per cent) was used as the oxydizing agent and found to be satisfactory with no spattering. Also, the resulting solution was completely colorless.

CHAPTER III

RESULTS

Membrane Potentials in Normal Fibers

The membrane potentials of fibers in healthy excised muscles bathed in Ringer solution containing 2.5 mM K per liter generally ranged between 75 and 85 mv as recorded at room temperature with intracellular electrodes filled with isotonic (118 mM) KCl. In isolated cases apparently healthy muscles were studied with potentials falling between 70 and 75 mv, but, on the basis of experience gained from the impalement of some two thousand fibers in nearly two hundred muscles, values below 70 mv, with technical errors ruled out, were generally indicative of unhealthy tissue.

To gain an idea of the consistency of membrane potentials in a given muscle surveys were made on three muscles on different occasions in which the potential was recorded in practically every available fiber straight across the middle region of each muscle surface with the exception of the first three or four fibers on each border. This survey was also prompted by an idea put forth by Conway (1954) suggesting the existence of a small component of sodium-rich fibers as part of the normal make-up of the fibers in a muscle. In support of this idea data were used based on the measurements of Nastuk and Hodgkin (1950). The present results, involving a total of 161 fibers, are presented in Table I. The potentials are seen to fall in fairly narrow, overlapping ranges in the three muscles. The mean values are

TABLE I

SURVEY OF MEMBRANE POTENTIALS IN SURFACE FIBERS OF THREE MUSCLES*

Date	Membrane Potentials										
Aug. 21:											
	76	70	78	78	79	79	78	79	76	75	76
	81	77	77	78	77	78	79	78	77	77	
	79	79	78	76	78	78	76	72	78	78	
	75	78	77	79	74	78	79	74	77	78	
	78	72	77	76	79	80	77	75	77	78	
Aug. 27:											
	75	78	77B	76	78	74C	78	77	77	77	78A
	75	79	79	78	77	77	77	77	77	78	75B
	75	79	78	78	77	76	77	75	78	79D	77C
	71	76	76	78	77	77	78	75	78		79D
	78	78A	79	78	78	77	76	77	77		
	(Lettered fibers were identified and afterwards recorded again to obtain values shown in far right column.)										
Sept. 11:											
	82	80	80	80	80	80	81	79	80	79	79	79	79
	83	79	79	80	80	79	80	78	79	78	79	79	79
	79	79	79	79	81	81	80	80	80	79	79	79	
	80	80	79	80	80	81	80	79	80	79	79	79	
	84	80	79	77	80	80	79	79	79	79	79	78	

Mean and Standard Error:			
	Aug. 21	51 fibers	77.2 ± 2.1 mv
	Aug. 27	48 fibers	77.0 ± 1.5 mv
	Sept. 11	62 fibers	79.4 ± 1.7 mv
	Total	161 fibers	78.0 ± 4.0 mv

*Recordings in each muscle were made in the order shown by reading the columns down and from left to right.

in general agreement with published values obtained under similar conditions. Thus, Ling and Gerard (1949A) give 78.4 ± 5.3 mv for the mean and standard error of a much larger sample of fibers from many muscles done under a broader range of temperatures in Ringer of slightly varying compositions. The significance of the present measurements in regard to sodium-rich fibers will be considered in the discussion.

In measuring membrane potentials, particularly in active states, a number of investigators have used intracellular electrodes filled with concentrated KCl, usually three molar, as a practical way of reducing the input impedance. For purposes of comparison with values so obtained, a few identified fibers in a single muscle were impaled and the potentials recorded, first with microelectrodes filled with three molar KCl, then with microelectrodes filled with isotonic KCl. As shown in Table II, the average value of the potential for ten fibers was 87 mv and 78 mv, respectively, in agreement with the value of 88 ± 1 mv for 161 fibers measured at a slightly lower temperature by Nastuk and Hodgkin (1950). If the single discrepancy in the values shown in Table II were excluded, the average membrane potential is 88 mv in agreement with them.

Environmental and physiological factors will be considered more fully in the discussion, but a few observations may be mentioned here pertaining to the maintenance of normal potentials in the fibers of excised preparations. At room temperature the potentials usually remained stable for not more than about ten hours as compared to several days when the muscle was kept in the cold. (In one instance a few

TABLE II

COMPARISON OF MEMBRANE POTENTIAL MEASURED WITH INTRACELLULAR ELECTRODES FILLED WITH ISOTONIC (118 mM) AND 3 MOLAR KCl

Fiber	Membrane Potential (mv)		Difference (mv)
	118 mM KCl	3 M KCl	
A	79	89	10
B	79	88	9
C	80	89	9
D	78	87	9
E	79	90	11
F	78	75	-3
G	77	88	11
H	77	87	10
I	76	90	14
J	77	90	13
Average	78.0	87.3	

fairly high values were recorded in a preparation twelve days old.) In experiments done in hot weather deterioration appeared to be accelerated at temperatures much above 30° C. No evidence of a need for special oxygenation or aeration of the preparation or of the need for continuously flowing Ringer was ever found, as described by Ling and Gerard (1949A). Apparently the periodic rinsing and changing of the bath provided sufficient agitation and/or aeration for the satisfactory maintenance of the potentials.

Effect of Radiation on Membrane Potentials

Individual fibers in an irradiated muscle exhibited wide variations in radiosensitivity as measured by changes in membrane potentials. With sufficient radiation dose an irreversible decline in potential eventually followed, but the latent period before detection of potential failure and the rate of decline appeared to be completely random variables in a given fiber. Also unpredictable was the extent to which a given fiber would be involved. In some fibers regions of low potential were highly localized, in others the potential was reduced more or less throughout the length of the fiber. This wide variation in the distribution and extent of radiation injury made it somewhat of a problem to present a consistent quantitative comparison of results in control and irradiated muscles. As a start, in most of the radiation experiments the mean and the variance were computed for each group of potentials recorded in each member of the control and irradiated pair at intervals after irradiation. It was found that the mean potential was roughly

an inverse function of the variance with increasing time after irradiation, thus expressing in an approximate numerical fashion both the decline and increasing spread of individual potential values with time. However, the variance was an unsatisfactory parameter because of its extreme sensitivity to low potential values, usually far out of proportion to relative numbers of these values. To avoid this objection irradiated and control groups were regarded as two populations in which no assumption was made as to the distribution of values within each population. Statistical methods for treating populations in which no assumption is made as to distribution, distribution-free methods, as they are called, are a fairly recent development in statistical theory and are found only in the more recent texts (Mood, 1950). Briefly, the method as applied here was to obtain the medians for each pair of control and irradiated groups of values and test them for significant differences. After the values of each group were ranked according to magnitude to obtain the separate medians, the two ordered groups were combined into a single ordered group and its median determined. The numbers of control and irradiated values lying above and below this median were counted, the resulting four numbers set up in a 2 x 2 contingency table, and a chi-square test of significance applied with one degree of freedom. A difference was considered significant if the probability of its occurrence by chance was less than 5 per cent. The test was also applied to samples of potentials recorded in each muscle prior to irradiation to ascertain that a significant difference was not present before exposure. Where an initial significant difference was

found, the data were rejected except in isolated cases in which the difference was not of extreme significance and the higher of the two medians was in the population from the test muscle.

While an analysis of this sort may be required to make a consistent statistical comparison of the results as summarized in Table IV, a better understanding of what is represented in the table can perhaps be obtained by looking more closely at the distributions of potentials. This is done in terms of the frequency with which potentials of various magnitudes occurred in the measurements. Such a type of analysis was applied by counting the number of fiber potentials falling in each 5 mv increment of potential from zero up to the maximum in each series of control and irradiated measurements in a given experiment. A typical example of this type of frequency analysis is given in Table III for a 122 kr experiment in which a definite radiation effect was found (Table IV). It is evident that a downward trend in the potentials of the irradiated muscle began by the end of irradiation or shortly thereafter. While no particular difference in the distribution of the control potentials is present in any of the three groups and all values are in the 75 to 85 mv bracket, no potentials above 55 mv were recorded in the irradiated muscle by the end of the four-hour post-irradiation period.

The statistical comparison of potentials in control and irradiated muscles is presented in Table IV summarizing results of experiments made with doses ranging between 33 kr and 220 kr. To make the results easier to compare, the P values have been placed in separate columns

TABLE III

FREQUENCY DISTRIBUTION OF POTENTIALS IN CONTROL AND IRRADIATED MUSCLES AT INTERVALS BEFORE AND AFTER EXPOSURE TO A DOSE OF 122 KR

	Control Fibers			Irradiated Fibers		
	Pre-Radiation	Post-Radiation		Pre-Radiation	Post-Radiation	
Increment (mv)	Hours 1-1/2	Hours 1/3	Hours 4	Hours 2	Hours 1/3	Hours 4
80-85	3		5	8		
76-80	5	6	5	3		
71-75					6	
66-70					2	
61-65					2	
56-60						
51-55						1
46-50						2
41-45						2
36-40						1
31-35						
26-30						1
21-25						3
Total	8	6	10	11	10	10

TABLE IV

COMPARISON OF MEDIAN POTENTIALS IN CONTROL AND IRRADIATED MUSCLES AT DOSES FROM 33 KR TO 220 KR

Dose (kr)	Temperature	Time After Irradiation (Hours)	Median Potential		P	
			Control	Irradiated	Not Significant	Significant
33	cold	52	54.5 (38)	63 (22)	.11	
50	cold	53	66 (66)	68 (64)	.70	
52	cold	21	ID 80 (19) RA 81 (19)	ID 80 (30) RA 81.5 (10)	.30 .03*	
88	room	2	ID 71 (12) RA 73 (8)	ID 66 (15) RA 63.5 (17)		.02 .02
97	room	7	78 (28)	79.5 (38)	.01*	
102	room	6	80 (39)	78 (39)		.01
122	room	4	79 (16)	57 (20)		.01
130	room	1	79 (11)	71 (13)		.01
150	cold	14	ID 76 (18)	ID 69.5 (34)		.01
220	room	1/2	73 (22)	67.5 (22)		.01

*Control median lower.

according to whether or not they were significant at the .05 level. The column headed "Time after Irradiation" gives the number of hours from the end of irradiation to the first group of measurements recorded that revealed a significant effect, or if there was no significant effect, the time given is to the end of the post-irradiation observation period. In some of the early experiments two designations of fibers were made as shown: identified fibers (ID), a group selected for ease of identification and mapped out prior to irradiation and followed as individuals throughout the observation period; and random fibers (RA), those chosen at random at the time of the measurement. The median potential, together with the number of fibers in parenthesis on which the value is based, is given in the fourth column for the control and irradiated populations after irradiation, and in the fifth and sixth columns are given the probabilities for a radiation effect obtained by a comparison of the pair of medians.

The temperature is given in column three for the experiment chiefly as a reminder that in post-irradiation periods exceeding about ten hours the preparations were stored in the cold (2 to 3° C.) between recordings to reduce deterioration.

It is seen that no significant radiation effect was present below about 50 kr. In the 52 kr experiment the median for the irradiated random fibers is actually significantly higher than the control median, and in the 97 kr experiment the irradiated population also shows a median significantly higher than the control. Even in the 150 kr experiment a significant radiation effect does not seem to have been

produced in the case of the random fibers. Most of the experiments above 52 kr, however, exhibit a reduction in potential generally becoming significantly lower at the 1 per cent level or less within a few hours after the end of the exposure. Further consideration of the significance of these results will be deferred to the discussion section.

The wide range of radiosensitivity as exhibited in individual fibers in a given muscle is demonstrated graphically in Fig. 5, based on data in the 122 kr experiment, which show the course of the membrane potentials in a number of identified fibers recorded one hour and four hours after irradiation. A significant radiation effect ($P = .01$) was produced by the time of the first recording (Table IV) and three hours later the typical progressive spread of potentials characteristic of the affected fibers is seen, although all were subjected to the same uniform insult. The deviation in the potential was to some extent a consequence of the fact that changes in potential in an affected fiber were usually found to show considerable variation along its length.

In fibers that had been followed electrically until the appearance of visible deterioration it appeared that some relation existed at least part of the time between low potentials and the subsequent development of histological lesions in the same region. Accordingly, distribution of membrane potential was studied in detail along a number of individual fibers. The method was to make a series of impalements at known distances along the fiber from an arbitrary origin selected for convenience and to repeat the measurements after an appropriate interval.

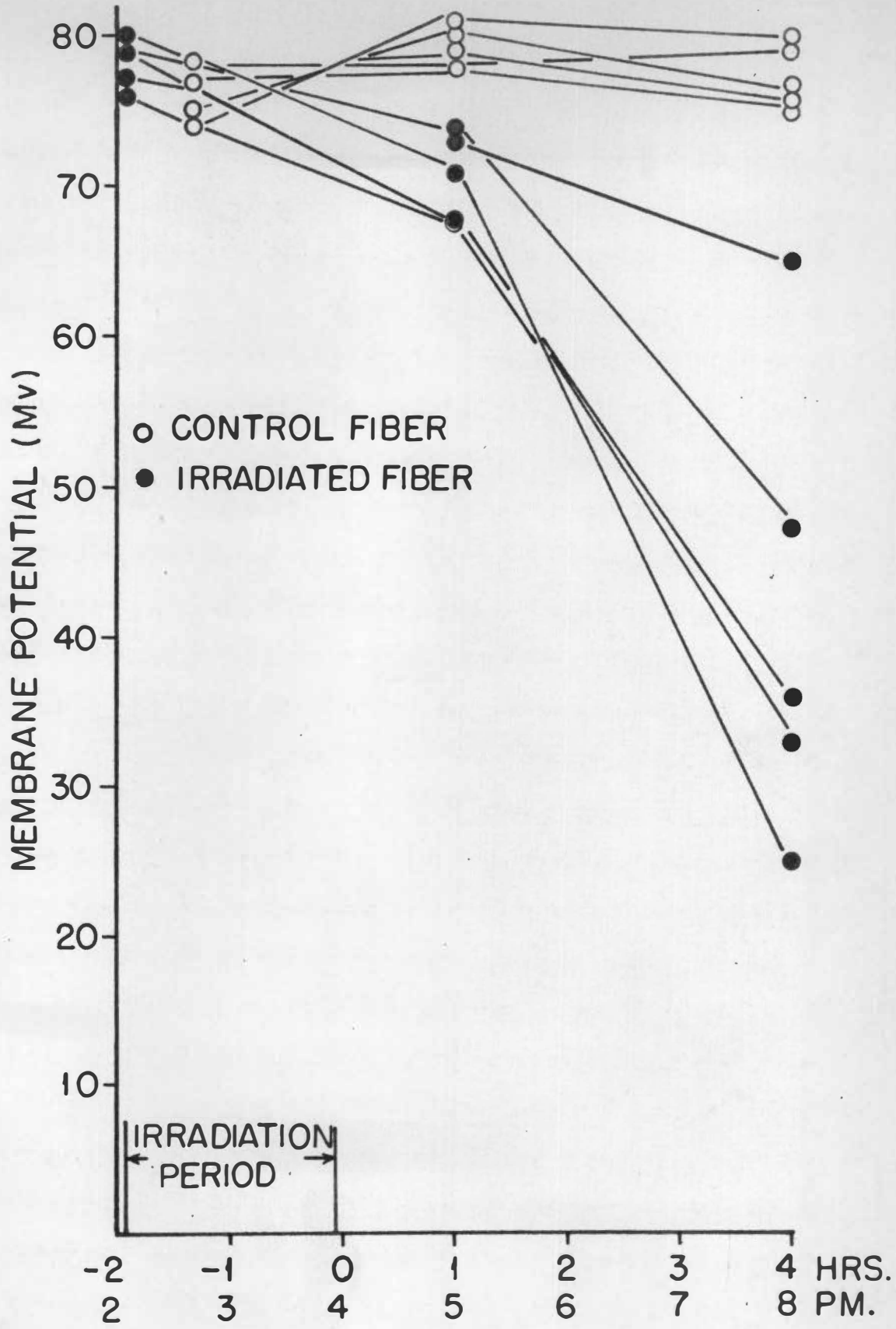


Fig. 5

Membrane Potentials of Individual Fibers at Intervals Following Irradiation.

Because of the tendency for adjacent fibers to overlap here and there, seldom could a fiber be followed for more than a few millimeters. Furthermore, repeated impalements over a short length of fiber occasionally appeared to hasten the deteriorative process. Since the fall of potential was quite rapid in some cases, it was not possible to show the potential along the fiber as it would have appeared at a particular time and what appeared to be a decline as a function of length was sometimes merely a decline in time from one impalement to another. Repeated series of measurements indicated whether or not a true minimum potential region was present. Where regions of minimum potential were established, they could often be correlated with the initial loci of visible fiber degeneration. The fall of potential along a particular fiber during a period of three days is shown in Fig. 6. In this case the initial recording at zero time (8:00 p.m.) showed a decline from 77 to 72 mv over a 4 mm length. Approximately fifteen hours later the level of potential was about 5 mv less and the distribution still fairly uniform. At forty-three hours most of the impalements still gave values around 70 mv, but two hours later the general level of potential along the fiber had fallen below 50 mv. On the morning of the third day, approximately sixty-three hours after the first recording, the potential range along the fiber was from 17 to 24 mv and six hours later, on removal of the preparation from the cold, a clot was found to have formed in part of the recorded region.

In another study in which the fiber was kept at room temperature, a clot was observed to originate on the spot at a point where the

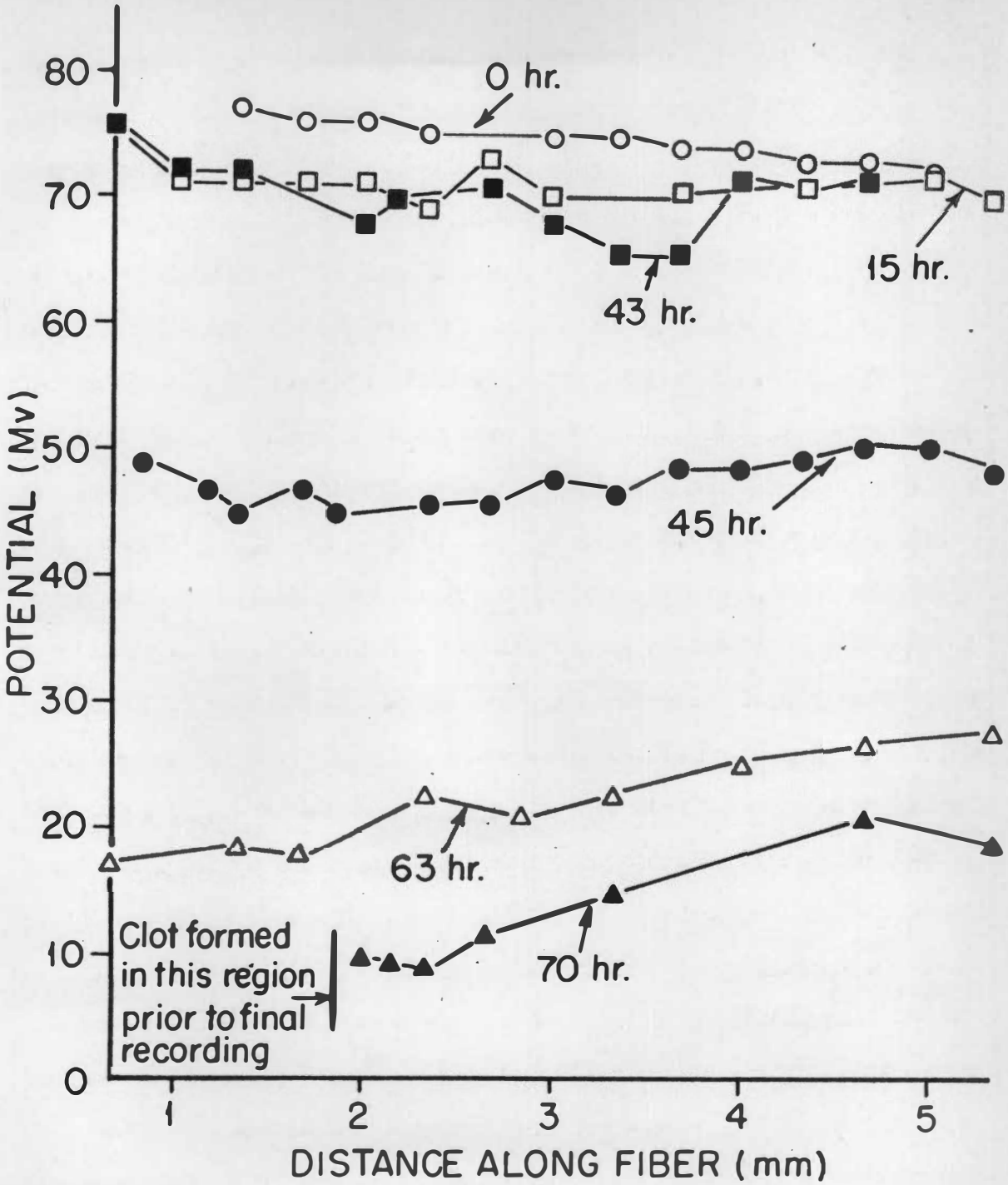


Fig. 6

Potential Along a Single Fiber Recorded at Intervals Over a Period of 70 Hours.

potential appeared to be at a minimum, as shown in Fig. 7. It is seen that the initial recorded values were already low and uneven. The recording was begun at the origin as shown and points were taken toward the right over a fiber length of 3 mm. A short time later, it was observed that the potential had fallen to 19 mv at the origin, some 22 mv lower than before. Potentials were then recorded and found to be declining toward the left. On the third impalement, made approximately 600 micra from the origin, the potential was down to 12 mv, and a tiny particle was seen, supposedly on the surface of the fiber, just below the point of impalement. At the same moment a twitch occurred (whether in the same fiber or not was not ascertained) with a momentary jump of a few millivolts in potential and, seconds later, the particle was observed to be the beginning of a single retraction clot. In the course of the next hour clotting had spread from this particular locus along a considerable length of the fiber.

Effect of Radiation on Muscle Potassium

Changes in concentration of ions such as K and Na have generally been determined in muscle by ash analysis of a sufficient number of muscles for each point in time to give a statistical average of the desired degree of accuracy.

In the case of potassium, thanks both to the large internal concentration as compared to that in the external medium and to the development of sensitive and rapid flame photometric equipment, internal changes can be inferred with reasonable accuracy by periodic analysis of

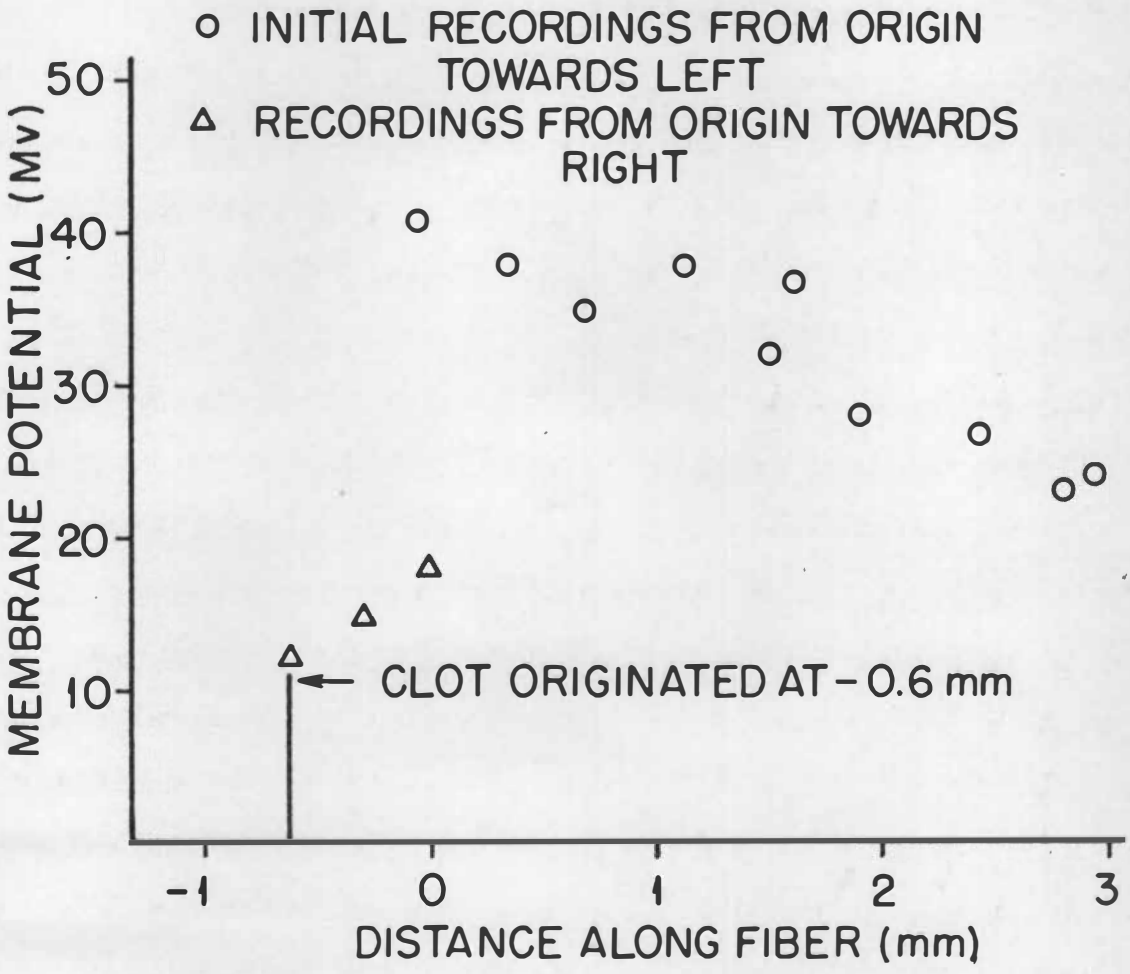


Fig. 7

Membrane Potential Along a Fiber Immediately Prior to Formation of a Retraction Clot at Indicated Position.

the immersion fluid. The absolute accuracy is not high since the small excess or deficiency of K in the bath is obtained as a difference between much larger numbers, but this drawback is more than outweighed for many applications by the advantage of following the changes, point by point, in the same muscle preparation. The method would seem to be most suitable for comparison experiments in which constant errors arising in the analysis of each member of the pair are largely balanced out in the end result. Furthermore, by analyzing the muscle ash for the residue of K at the end of the experiment, some estimate of the total K initially present in the muscle can be obtained.

In the present work the bath analysis technique was the method of choice as it was most sensitive for detecting the earliest K losses from the irradiated member of a pair relative to its control mate. In most cases final ash analyses were made also to provide confirmatory information on the total net K change.

As with the electrical data, results are divided into the two temperature categories, the experiments at room temperature lasting up to twelve hours and experiments in the cold lasting up to about sixty-five hours. The changes seen in K concentration were similar in both cases except for the time scale. Following excision and immersion in Ringer (2.5 mMK) an initial period of adjustment occurred characterized by a rapid temporary loss of K and weight. After this phase, equilibrium was apparently reached for the gain or loss of K from the muscle at room temperature usually became less than one mM per kilogram of wet weight of muscle per hour, and for muscle kept in the cold the net change of

K content became essentially zero. In the irradiated muscle K was increasingly lost with time after irradiation, the rate of loss depending upon the dose. No wet weight change of any consequence accompanied the K shift. In one or two instances a rapid final increase in weight occurred, attributed to terminal osmotic failure of dying fibers. In these cases visible fiber deterioration, in addition to swelling, was generally evident.

The results at room temperature are presented in Fig. 8, in which the mean rate of change of K, expressed in mM of K lost per kilogram of wet weight per hour, is shown for control and irradiated muscle pairs, three pairs at 150 kr and three pairs at 190 kr, along with the most probable slopes, assuming a linear rate change with time, as determined by the method of least squares. The mean rate was derived from the average of the sum of the number of mM K change for two consecutive soaking periods.

The change of the mean rates with time as given by the slopes is summarized in Table V. In both sets of experiments the mean rate of loss of K in the control muscles apparently declined with time as the rate of loss for the irradiated muscles progressively increased. The apparently large numerical discrepancy between the two control slopes is not statistically significant ($P = .24$). For the 150 kr muscles the slopes for the control and irradiated rates are not significantly different, but for the 190 kr muscles the difference in slopes is highly significant. The loss of potassium from irradiated muscles, particularly at the high dose level, is further indicated by results

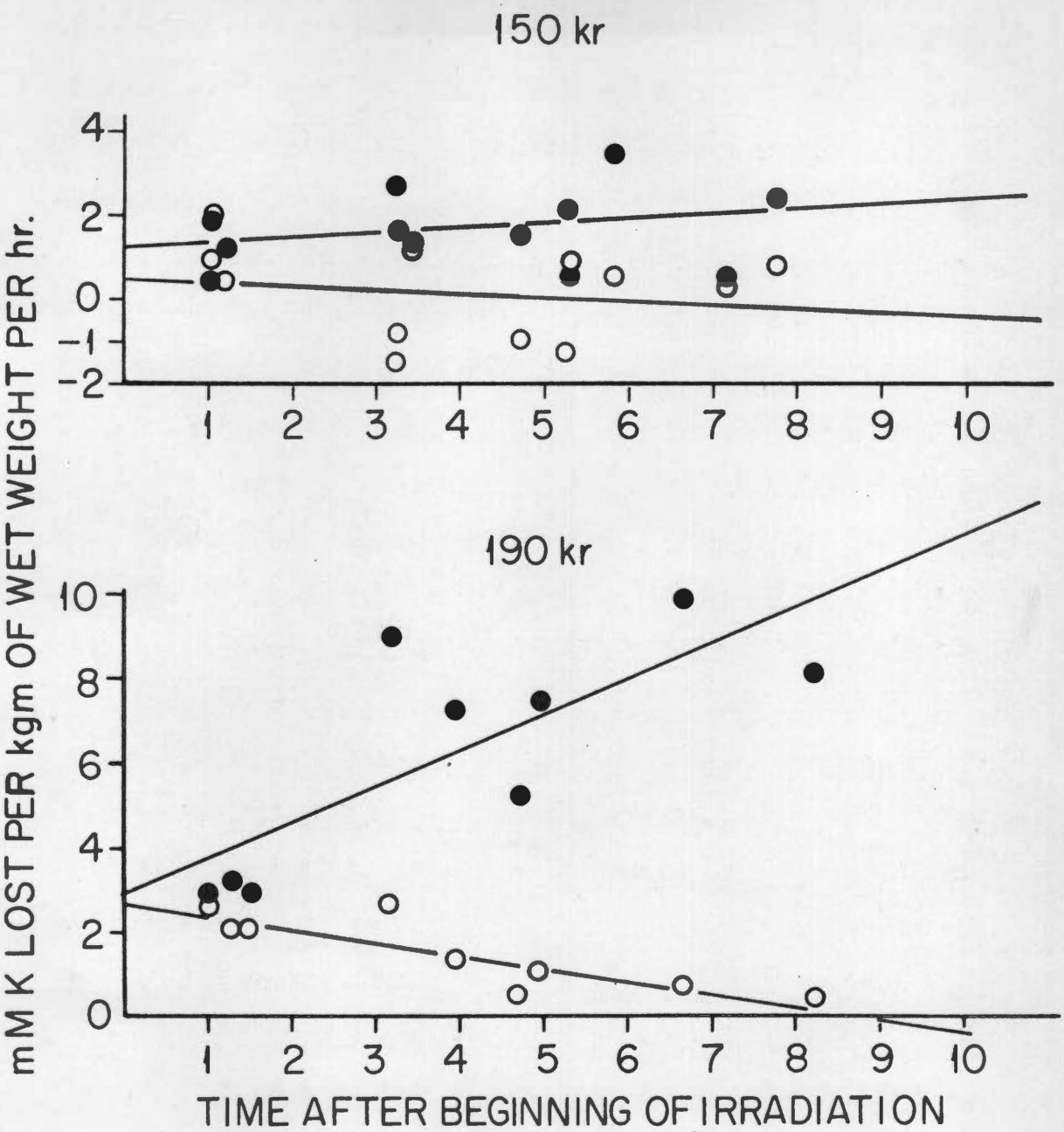


Fig.8

Potassium Changes in Irradiated Muscle at Room Temperature.

TABLE V

EFFECT OF RADIATION ON MUSCLE POTASSIUM

Dose (kr)	Room Temperature (Fig. 8) Mean Rate of Loss of Potassium (mM K per kgm wet weight per hour) Irradiated	Control	P
150	.0920	-.0843	.41
190	.856	-.299	.01

Ash Analysis
(mM K per kgm wet weight)

Room Temperature (Fig. 8)			In the Cold (2-3° C.) (Fig. 9)		
	Irradiated			Irradiated	
Control	150 kr	190 kr	Control	90 kr	190 kr
70	46		87	87	
79	66		77		23
83.5	77.5				
79		13.4			
85		9.2			

of the terminal ash analyses, as shown in Table V, for five of the six pairs of muscles used in these experiments. It is seen that in the case of the high dose muscles more than four-fifths of the potassium had leaked out by the end of the soaking period in comparison to the amount of the K residue in the controls.

In the experiments in which the muscles were soaked in the cold following irradiation the rate of loss of K was greatly reduced, even for the high dose, as demonstrated in the case of the experiments in Fig. 9 for doses of 90 kr and 190 kr. At the lower dose no significant difference in the two muscle mates is evident after more than two days of soaking, and, as near as can be determined, the two muscles seemed to have reached a state of equilibrium in which the net K change was essentially zero. Even in the high dose case the rise in the rate of K loss is seen to be relatively small, only about one-fortieth of the corresponding rise at room temperature for comparably irradiated muscles. These results are confirmed by the data on the terminal ash analyses shown in Table V. No terminal ash analysis was made of the particular muscle mates used in the 190 kr experiment shown in Fig. 9; the values given in Table V, 23 mM per kgm, and 77 mM per kgm, for the irradiated and control muscles respectively, are taken from data on a comparably irradiated and soaked pair of muscles, hence similar results would be expected.

Visible Pathological and Histological Changes

In a sufficiently irradiated or aged preparation the extent and rate of progression of histological alterations, as in the case of

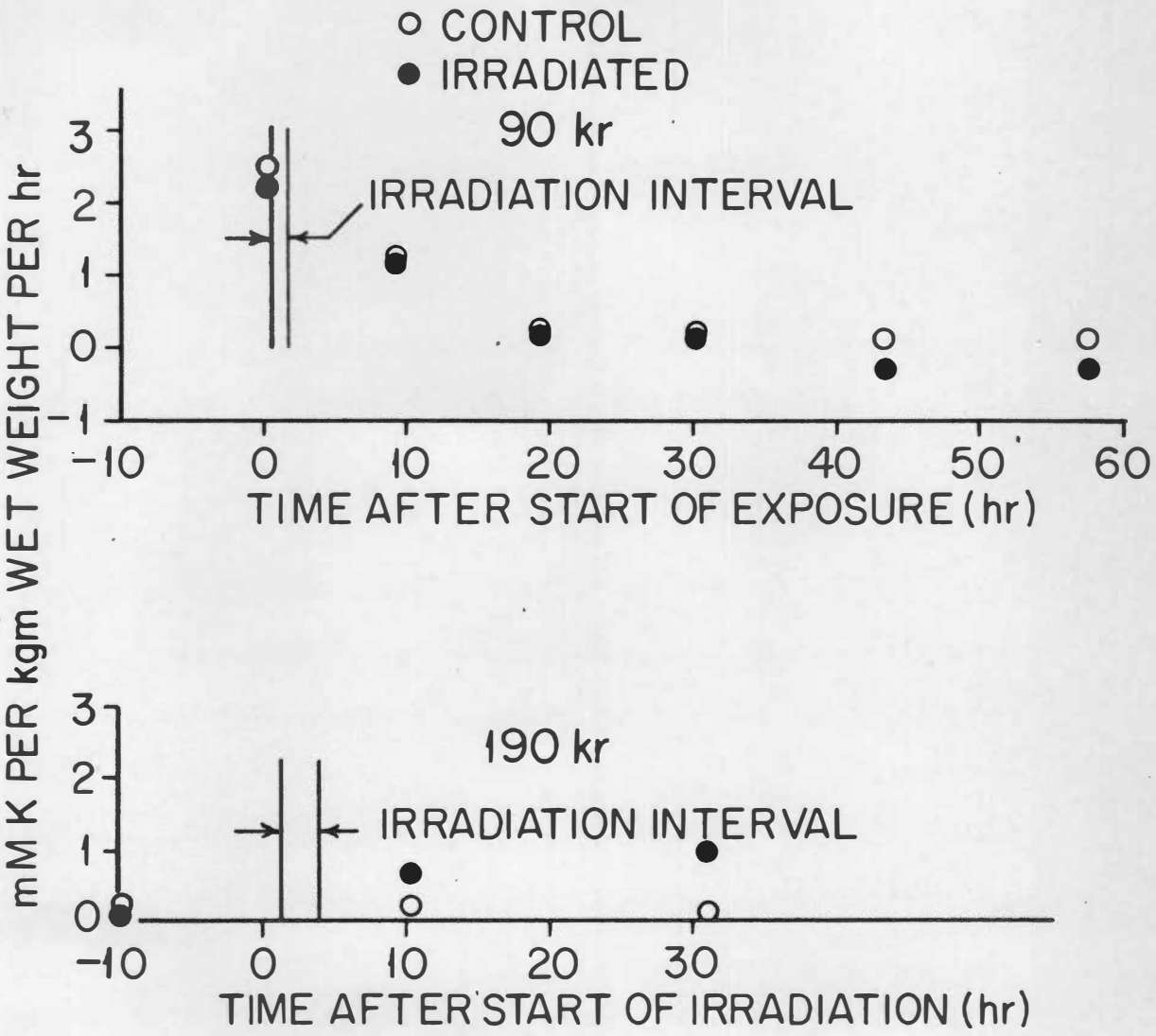


Fig. 9

Potassium Changes in Irradiated Muscle in Cold (2-3°C) (Mean rate of Change in K Concentration per Unit Wet Weight).

potential changes, exhibited wide variations in individual fibers. The randomness of the injury was sometimes modified, once degeneration had progressed to considerable swelling or clot formation in one or more fibers, in that adjacent, transparent, healthy fibers sometimes began to show signs of electrophysiological and visible injury apparently arising from the pinching and pressure thereby exerted. The initial visible sign of deterioration in a fiber was development of opacity in some regions, at first perceptible only in comparison to adjacent, healthy fibers. At the same time there was some tendency towards swelling, along with increasing resistance to impalement with a micro-needle or microelectrode. Where a potential was established it was often as though the electrode had first been forced through a cortical layer or wall of perceptible thickness underneath the sarcolemmal sheath. Magnification up to 500X or 750X with the aid of a water immersion objective revealed that the opacity and swelling was largely correlated with a decrease in the normal spacing of cross banding from about two microns to one micron or less, producing a region of localized extreme contracture in which the cross striations were so close together as to be barely resolvable. At the same time the outlines of individual myofibrils sometimes became more distinguishable than in the normal fiber and the fiber cylinder tended to appear roughened and full of streaks, probably as the result of separation of myofibrils. Nuclei were seen indistinctly in the living fibers.

Sooner or later localized coagulation of fiber contents became perceptible, resulting in the formation of the retraction clots or

retraction caps comprehensively described by Speidel (1938, 1939) in experimental fiber injury produced in the tail muscles of living tadpoles and in the muscles of many other species. This plug formation has long been referred to by pathologists as "waxy degeneration" (Heilbrunn, 1956). It appeared in the present studies that the clot formation was a stage following excessive and irreversible contracture in a highly localized region of the fiber. Occasional chance observations of the actual genesis of a clot indicated that it began as a tiny disturbance of the fiber contents as though a highly localized solation were occurring in two or three adjacent myofibrils resulting in partial or total loss of cross striated structure. This incipient retraction clot (Speidel 1939) extended rapidly across the diameter of the fiber as a sort of interface which served as a matrix for the progressive condensation of material in the form of a rapidly thickening, usually convex, opaque region constituting the retraction clot, approximately perpendicular to the fiber axis like a lens in a telescope. Individual myofibrils appeared to be flowing into the surface of the clot and causing a fusion of their cross striations as by a condensation process. Thus, myofibrillar structure disappeared and was replaced by a solid coagulum. Complete homogenization of fibrillar structure appeared to be temporary, since in the older regions of the clot occasional faint patches of highly condensed, almost unresolvable cross striations and myofibrillar boundaries could be discerned. Usually, lateral as well as longitudinal expansion of the clot occurred, causing greatly localized swelling and distortion with pressure against and displacement of adjacent fibers.

Also, compensatory stretching of the fiber regions on either side of the growing clot caused a progressive narrowing and stretching of the sarcolemmal sheath until rupture of the contents eventually occurred, followed by retraction of the broken ends inside the sarcolemmal tube and subsequent coagulation of each broken surface into a retraction cap, leaving a fluid-filled clear space between. Extension of clotting along a fiber was often quite rapid, sometimes as fast as 50 microns a minute as reported by others (Woodward, 1948). In a rapidly deteriorating fiber formation of an initial clot was usually followed by repeated fission and extension of the daughter clots like a string of dividing bacteria, often in such highly uniform sequence as to lend to the fiber the appearance of a jointed reed.

Histological changes such as these are shown in Fig. 10 for living fibers and Fig. 11 for stained sections. In Fig. 10 a field identified by the configuration of a blood vessel present was photographed, the muscle then irradiated with 200 kr, and the same field immediately rephotographed. A comparison of the two fields shows that noticeable fiber swelling has begun. One fiber has started to clot and the contents have pulled apart, leaving the clear sheath between.

In Fig. 11 stained longitudinal sections from control and irradiated muscles subjected to a similar dose of radiation are presented to demonstrate more clearly the striking alterations in fiber structure. In the section from the control muscle the myofibrillar and cross-striated structure of histologically normal fibers is clearly visible. The muscle nuclei appear as short dark streaks. The irradiated section

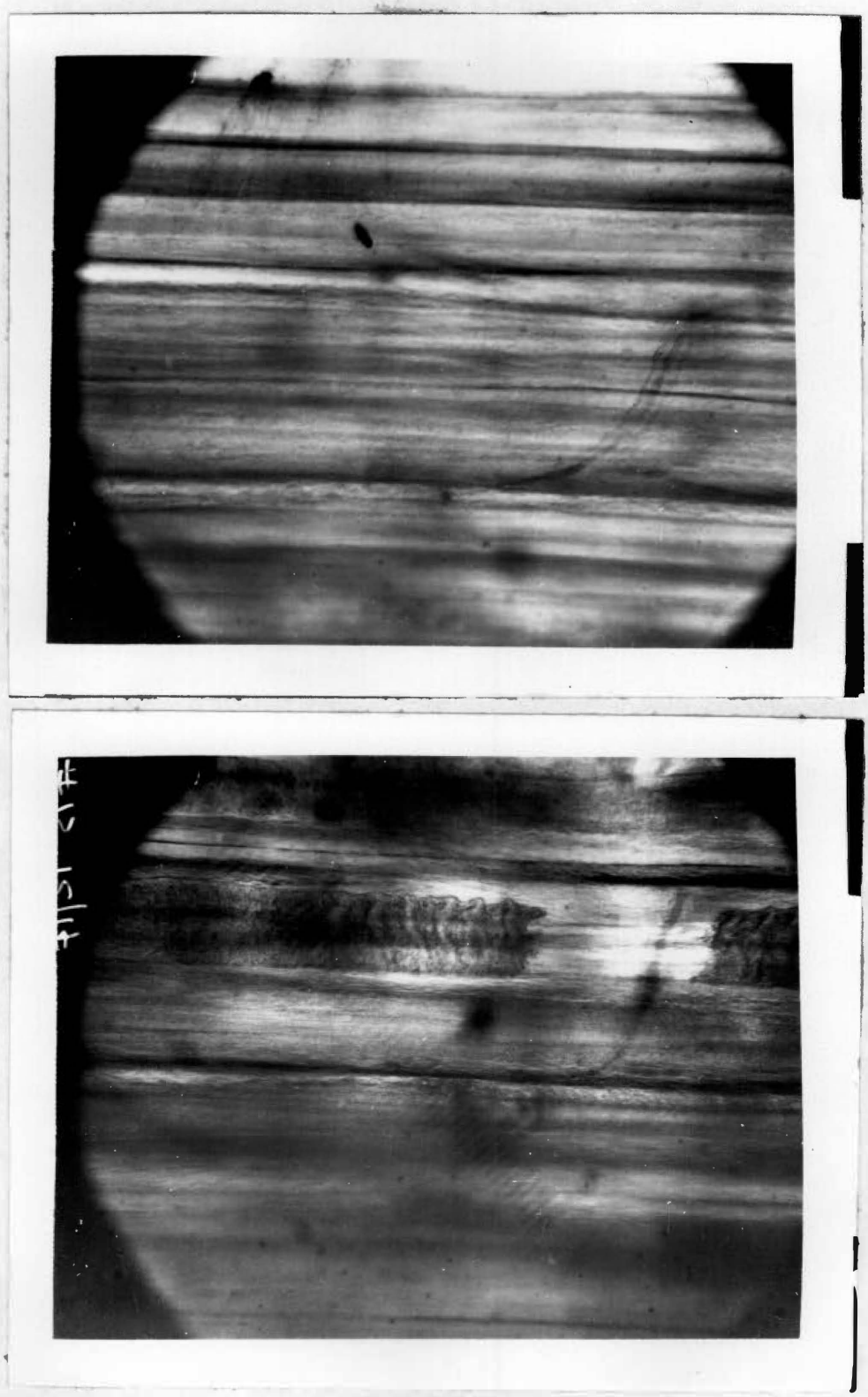


Fig. 10

Living Fibers Before and After Exposure
to a Dose of 200 kr (100X)

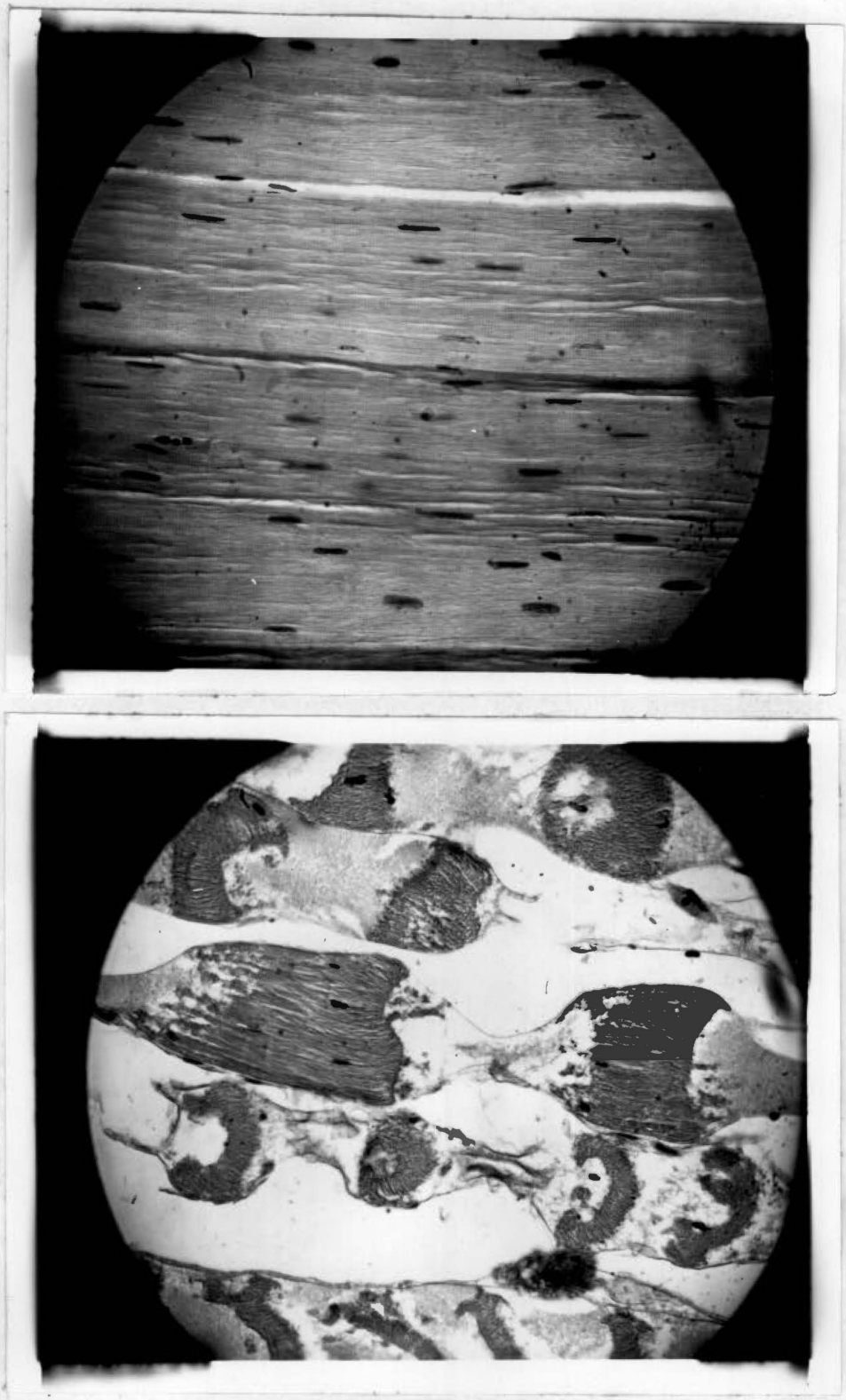


Fig. 11

Stained Sections of Control Muscle and Irradiated Muscle Exposed to a Dose of 220 kr (215X)

shows gross distortion and partial rupture of fibers as a result of multiple clot formation. The dark, well-developed retraction clots, some containing a suggestion of myofibrillar structure are seen interspersed with lighter colored, constricted regions where retraction of contents has left the empty sarcolemmal tube. Convex shaped masses of coagulated material referred to in the observations on clot formation are seen in the fibers just below the center one.

Membrane Potentials in Clotted Fibers

A number of points were brought out from an examination of the potentials in various regions of many degenerating fibers. First, the potential originates in the sarcous content of the fiber, not across the sarcolemma. Where the broken ends of a fiber have retracted leaving the empty sheath, impalements of the latter have never indicated evidence of a potential. Second, coagulated regions were not necessarily devoid of potential, especially if freshly formed. On one occasion impalements made at several loci on the freshly formed surface of a retraction cap revealed potentials up to 77 mv. In the empty clear sheath just in front of the cap the potential was missing as expected, while just behind the cap surface it was about 76 mv and of similar magnitude also at greater distances from the cap (500 microns or so). Continued probing in the deteriorated region as retraction progressed indicated that a very thin cortical layer was rapidly formed over the broken retracting ends as if a surface precipitation was taking place. Presence of a cortical layer in injured fibers has been

inferred from the behavior of oil droplets injected into the fibers (Rieser, 1949). The clotting phenomena in muscle fibers has been regarded as an example of the "surface precipitation reaction" studied in many kinds of cells by Heilbrunn and his associates (Woodward, 1948, Heilbrunn, 1956). This formed surface appeared to be the seat of the potential measured as above, but further observations were hampered by the rapid decline in potential accompanying the progressive thickening of the retraction cap. In general, however, potentials in clotted regions of fibers were very low or entirely absent. Third, there appeared to be a greater likelihood of significant potentials in fibers that were freshly clotted in a fairly fresh muscle than in the case of fibers both aged and clotted. In the example above the clot studied was the only one seen in the muscle.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

A number of investigators have presented evidence that the membrane potentials taken usually from a small number of fibers in a large number of muscles show a surprisingly high degree of uniformity for a biological parameter. The proposition of Conway (1954) suggesting the existence of sodium-rich fibers has raised a question about this uniformity since part of his evidence was based on potential data. Nastuk and Hodgkin (1950) reported that the potentials they observed in frog muscle usually fell in the range 80 to 95 mv, as measured with 3 molar KCl electrodes. Although a few potentials as low as 60 mv were encountered, these were rarely established abruptly on impalement and were regarded by the authors as evidence of faulty impalement. Conway, from a consideration of the statistics of the measurements, came to the conclusion that these low potentials actually could be normal potentials that would be characteristic of a small sodium-rich proportion he estimated to make up some 10 per cent of the total number of fibers.

The results of Table II do not support this interpretation of the data of Nastuk and Hodgkin. Of 161 fibers from three muscles only six values below 75 mv were recorded and none below 70 mv. If high-sodium fibers are present, it would seem that the potential is either not influenced or that only a very small graded differential in Na content is present, in which case the differences in potential would be probably masked by the normal small variations recorded. These tabulated

values cannot be said to represent a uniform sampling of muscle fibers since they are all superficial fibers, but, by the same token, neither can the data used by Conway since only inner surface fibers were included in either case. In the present work it was possible to pierce through the fascial sheath to a greater or less extent in some muscles and record a few fibers underneath. The data are certainly not conclusive but in these fibers of the external surface no evidence of a low-potential component was found.

Before turning to a consideration of the effect of radiation, some mention should be made of certain physiological and environmental factors affecting normal potentials and possible synergistic effects of these factors with radiation.

It is perhaps obvious that before meaningful experiments on biological material can be carried out in which the effect of a modifying agent is studied, the initial condition of the material must be satisfactorily assessed. In a tissue so thoroughly and frequently utilized as excised frog muscle, such a note of caution appears superfluous, but as methods of analysis have become progressively refined, differences formerly undetectable are now recognized. Thus, in the case of gross analytical methods, small irregularities arising from injury at dissection and perhaps other traumatic agents may, perhaps, be ignored, but at the level of the individual fibers, these formerly negligible factors assume new significance. This is true particularly in the study of membrane potentials, since, sooner or later, injury to a single fiber anywhere along its length may be reflected in a

loss of potential throughout the fiber. In this connection the importance of meticulously careful dissection cannot be overemphasized. It was especially easy to injure the extreme ends of the fibers when dissecting the pelvic end of the muscle away from its attachment. In such a case the ensuing decline in potential was sometimes so slow as to remain undetected for a long time.

An even more subtle factor influencing the potential was the physiological condition of the tissue at the time of excision. Muscles from frogs with obvious red-leg, for example, tended to have somewhat lower and more scattered potentials than muscles from healthy specimens, as reported by others (Graham and Gerard 1946). Parasitic nematodes, both in the encysted and active state, were a frequent finding in and around the sartorii of the frogs used in the present work, and in heavily infested muscles the potentials likewise tended to be lower and more scattered. Interestingly enough, the stability of the values did not seem to be greatly affected as judged in one case by recordings of identified fibers in a heavily parasitized muscle made over a period of three days. Since the occurrence of active parasites in frog muscle is said to be somewhat unusual, the picture shown in Fig. 12 of a longitudinal stained section through an extensively infested muscle is included as a matter of interest. An organism is seen to have been fixed in the act of engulfing a portion of a single fiber.

In connection with the initial physiological state of the tissue, no seasonal variations in membrane potential were discovered in frogs studied at different seasons of the year, although no active search was

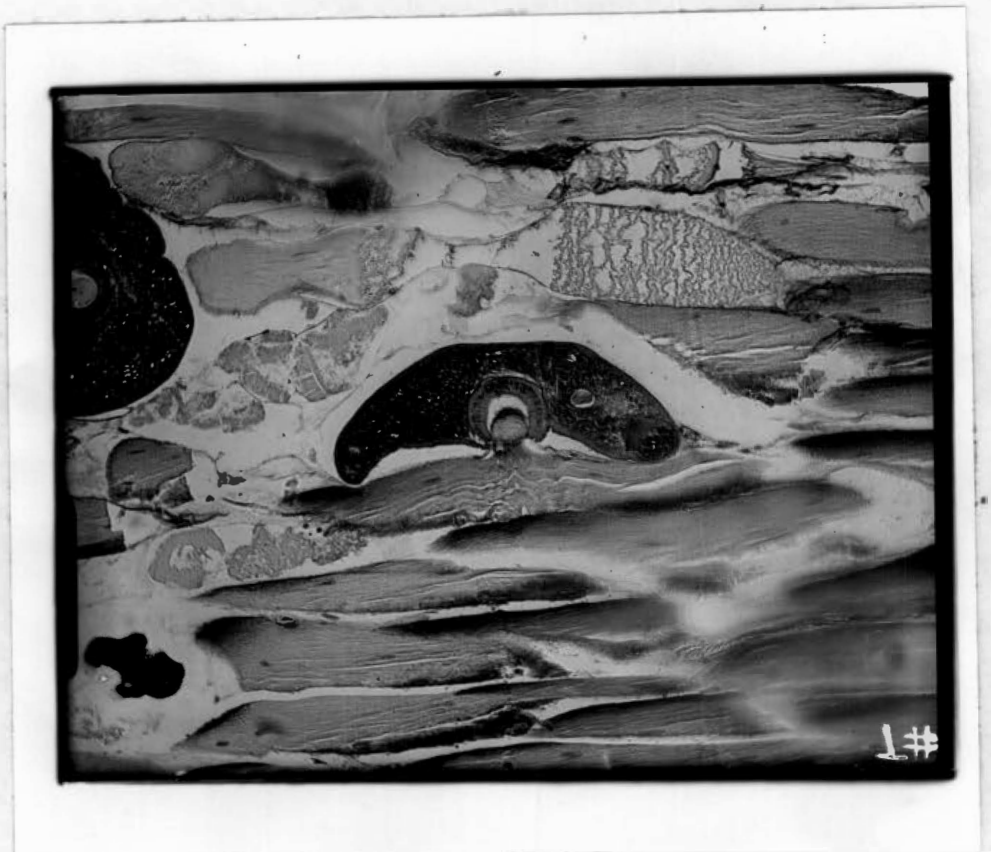


Fig. 12

Parasite Engulfing Portion of Muscle Fiber
(75X)

made for such fluctuations. The answer may lie in the fact that the frogs were kept in the cold following receipt of shipment until used. No significant difference was observed in the membrane potentials in muscles of frogs newly received and those from frogs kept in the cold for weeks. Negative results were reported in a long and extensive investigation of possible temporal variations in the K content of toad muscle (Shaw and Simon, 1955, and Shaw, Simon, and Johnstone, 1956).

Further consideration of physiological and environmental factors in relation to the potential will be made in connection with radiation effects.

Turning now to specific electrophysical and electrolyte changes found in irradiated muscle one may perhaps find some outline of a consistent physiological pattern of irradiation injury fitting in with the classical picture of histological alterations.

In this connection there is a unique property of ionizing radiation that makes it of value as an experimental tool in the study of pathological changes, namely, its capacity to deliver an insult uniformly and simultaneously to all parts of the tissue. The variation in response to injury in a given piece of tissue is then a property of the tissue itself and not a reflection of an inhomogeneous traumatic agent.

Another factor closely related to interpretation of the results is the use of the excised muscle preparation. It is hardly necessary to point out that in looking for an induced effect in a particular tissue, complicating systemic factors and the secondary effects

stemming from changes induced in surrounding tissue should be reduced to a minimum. An example of the striking influence of surrounding tissue in the study of irradiated muscle in vivo will be considered later. In the present work the excised muscle may be regarded as a collection of living fibers, which, in the resting state at least, enjoy a certain physiological independence as individuals. What happens in one fiber, so to speak, is of no concern to the others. Up until quite recently this distinction was of little importance, since physiological investigations in resting muscle were limited to effects measurable at the whole tissue level. The technical advances that have led to the successful use of intracellular electrodes have provided an instrument of sufficient power to overcome this handicap.

From the results of the present investigation utilizing this development, it is possible to formulate a concept of injury that could hardly have been demonstrable otherwise, namely, that one of the early physiological alterations following a sufficiently high dose of radiation is a failure of the integrity of the muscle cell surface at small loci, more or less distributed at random spatially and in time over the fibers of a muscle. There seems little doubt that the surface is involved when penetration of the microelectrode probably only a micron or so is sufficient to establish the maximum potential in the normal fiber. The localized early potential decline presumably reflects an early small leakage from inside the fiber before the ion has escaped in quantity detectable by gross analysis.

Briefly, then, what are the concepts relating potential to ionic state on which this interpretation is based, as seen from the standpoint of the classical Bernstein membrane theory of potential as modified by Boyle, Conway, Hodgkin, and others? (For comprehensive discussions, see Hodgkin, 1951, Conway, 1957). It is generally known that resting fibers maintain a high intracellular concentration of potassium and a low one of sodium in the face of an external ionic environment of roughly speaking, opposite concentration. The exact relation is complicated by the presence of appreciable extracellular space, and, of course, gross analysis does not distinguish between the extra and intracellular ionic components. For a concentration of K of about 85 mM per kilogram of wet muscle as has been found for the frog sartorius (Fenn, 1936, Boyle, and Conway, 1941) the actual intracellular concentration $(K)_i$ allowing for the extracellular space, has been calculated to be about 126 mM of K per liter of fiber water (Boyle and Conway, 1941). Corresponding values for Na were given as 24 mM per kilogram of wet muscle and 15.5 mM per kilogram of fiber water. Associated with this high ratio of K inside and outside the fiber is the resting membrane potential of a value that is in fairly good agreement with the number obtained by applying the Nernst equation in the form $E = \frac{RT}{nF} \ln \frac{(K)_i}{(K)_o}$ where R is the gas constant, n, the number of equivalents of ions, F, Faraday's constant, and T the absolute temperature. When the values for $(K)_i$ and the plasma concentration of K, $(K)_o$ outside the fiber are taken as 126 and 2.5 mM per liter (Fenn, 1936), respectively, with T as 298° for room temperature, the potential $E = 58 \log \frac{126}{2.5} = 99$ mv, a value

agreeing well with experiment when due allowance is made for junction potentials. The relation also holds quite well over a sufficiently wide range to cover ordinary biological conditions when the external concentration of K is experimentally varied. The potential has long been regarded as arising from a Donnan equilibrium of K ions on either side of the cell membrane. It seems, however, likely that bioelectric potentials in plant and nerve as well as muscle cells are not equilibrium potentials of the Donnan type but are maintained in the face of a tendency to run down (Davson 1951). This tendency to run down implies that to maintain the potential the cell battery must be kept charged by the continuous expenditure of energy of the part of the cell. Because of the demonstrated permeability of the cell to Na as well as K and Cl, it is widely held that the energy is used in some way to keep Na from passing into the fiber by diffusion from the high external concentration or to extrude it faster than it enters and so maintain the potassium equilibrium. Whether Na is actively extruded in the process or not, if the potential is maintained by an expenditure of cellular energy, interference with the energy sources or transfer mechanisms within the fiber would logically be expected to bring about a fall in potential.

What, then, initiates the failure of the cell battery when the cell is irradiated sufficiently or otherwise insulted? Little information is available on which to base an answer, since the maintenance of the electric field under normal conditions is not completely understood. The energy needed to maintain the potential is probably

derived from the splitting of high energy phosphate bonds. Ling and Gerard (1949C) in their investigation of the metabolic origin of the potential showed that it was strongly inhibited by poisons known to block some step in the glycolytic cycle. Furthermore, in muscles poisoned with iodoacetate, addition of pyruvate or lactate was found to delay the fall of potential and attendant development of IAA Iodoacetate or Lundsgaard rigor. The potential was not affected by cyanide in comparable concentration. In the present work a few preliminary experiments were carried out with inhibitors. Muscle was exposed to radiation (92 kr) following treatment with IAA, and the fall of potential following IAA poisoning was accelerated compared to the poisoned control. In CN treated muscles no synergistic effect of radiation (highest dose 115 kr) could be demonstrated. The experiments were too few to draw solid conclusions, but, taken with the findings of Ling and Gerard suggested that the glycolytic cycle rather than the oxidative pathway via the cytochromes may likewise be involved in radiation induced lesions leading to a reduction in potential. The work of Fenn, Koenemann, and Sheridan (1940) indicating that potassium retention in muscle is probably dependent on glycolysis rather than oxidation appears to be in harmony with this idea.

The observations of Bacq, et al (1949) and of Gerstner and associates (1954) were interpreted as probably indicating an interference of radiation in some way with biochemical energy sources, possibly high-energy phosphate bonds, required for normal muscle function. It seems quite likely that the reversible contracture at

low doses of a few hundred roentgens as reported by Bacq is a separate phenomenon. It is certainly not in accord with the concept of muscle as a highly radioresistant tissue as again demonstrated in the present work. No numerical data and only a brief description of this observation was given by Bacq so that attempts at this time to integrate it with the results which have been obtained at the higher doses are rather futile.

In regard to the interpretation of potential changes in connection with pathological alterations in fibers so little is known about the physiological significance of the visible lesions that little can be done but to speculate. It would be helpful to know if some critical internal concentration of ions exists, below which the physical integrity of the fiber cannot normally be maintained. Under ordinary conditions potassium ions are apparently present in high enough concentration to constitute a large safety factor (Steinbach, 1954). The terminal leakage of potassium from a cell is regarded as the result rather than the cause of death (Fenn, Koenemann, and Sheridan, 1940). With the generally low potentials found in deteriorating fibers and in consideration of the part played by potassium as a possible activator ion in some of the enzyme systems involved in the energetics of contractility, it is interesting if not accurate to regard the condensation of fiber structure to remain unfolded, either because of a fall of potassium concentration below a critical level required activation, or because of the final drying up of the last trickle of phosphate bond energy, or because of both.

The results of the present investigation appear to have a bearing on injury to muscle irradiated in the living animal, especially in relation to the contradictions raised by some of the previous studies. In this connection it is necessary to try to distinguish rather sharply the differences in the pathological changes found in tissue irradiated in vitro as compared to that irradiated in the intact animal. The designation probably used most often to characterize the changes found in experimental muscle injury is "Zenker's waxy or hyaline degeneration," originally described by him to characterize the changes found in the abdominal muscles of typhoid patients. Since the term has been referred to a number of times already, it may be well to describe briefly the pathology of waxy degeneration as given in a recent authoritative work on diseases in muscle (Adams, Denny-Brown, and Pearson, 1953). In the early stages cloudy swelling may be present with obscuration and/or preservation of cross striations or both, all in the same fiber. Discoid fragmentation of the fiber into horizontal segments as described by Bowman (1840) may be seen in some fibers, also slight nuclear swelling may be present. In later stages of the condition, the key feature is the presence of highly refractile, strongly eosinophilic masses within the fiber in which may be seen occasional faint traces of cross striations. The sarcolemmal tube may still be identifiable surrounding these masses and bridging the gaps between them. (Such changes are quite evident in Fig. 11, lower half). A few fibers may show vacuolation with partial preservation of striations. Large areas of the muscle may be transformed into hyaline segments,

showing an appearance similar to that produced by ischemia and certain physical agents. In all this alteration the change seems to be confined to the fiber substance, with no significant modifications to connective tissue and little infiltration by inflammatory cells. Scattered fibers or parts of fibers may retain a healthy appearance.

It appears that some features of Zenker's degeneration were present in excised frog muscle injured by radiation. A similar condition has been recognized in muscles irradiated in the intact animal, but here the picture has been obscured by alterations induced in the surrounding tissue with, perhaps, important systemic complications. An attempt has been made to separate some of the components involved through an extension (Sheppard and Darden, 1957) of the work of Wilde and Sheppard (1955) on the irradiated rat forelimb, and it seems likely that some clarification has already been obtained from the preliminary results. The events seen may be very briefly summarized as follows. As late as a day after a dose of 75 kr, no histological alterations in the muscle were seen beyond slight increases in the interfascicular spaces, traceable to overflow from the heavily damaged and highly edematous connective tissue region of the forelimb. Plasma space determinations in the leg and other observations indicated that vascular leakage of plasma proteins had begun within several hours following irradiation. By about forty hours after irradiation some evidence of severe vascular stasis was present along with small hemorrhages in the muscle tissue and widespread fiber alteration embracing many, if not all, of the changes described in irradiated

rabbit muscle, including the principal features of Zenker degeneration. There is nothing to suggest that the muscle injury was not merely a secondary phenomenon arising as a consequence of the direct effects of radiation on the more radiosensitive connective and perhaps vascular tissue. Vascular permeability was probably altered by substances released by injury to the connective tissue. It is suggested that the difference in sensitivity to radiation apparently present in rabbit and rat muscle reflects a higher or rather more rapid rate of development of the changes in the rabbit, stemming, perhaps, from a lessened ability of rabbit tissue to tolerate ischemia.

In conclusion, the interpretation of the potential and electrolyte changes seen in irradiated muscle may be summarized as follows. Fibers of excised frog muscle are highly resistant to irradiation. Potassium leakage was not detected following doses less than 150 kr, although significant decreases in the membrane potentials of individual fibers were observed beginning in the 50 to 100 kr range. The results suggest that early irradiation damage to the fiber was reflected in a loss of the integrity of the cell surface beginning at small scattered loci. If the dose was high enough, K leaked out in progressively larger quantities until the loss became detectable by gross analysis. It seems likely that at the lower doses the loss of K can be controlled; otherwise, the leakage in this case would be expected to eventually become large enough to be measured. Visible degenerative changes in fiber structure were largely correlated with fiber regions showing minimum potential, suggesting that the maintenance of the ordinary

architecture of the muscle cell was closely related to, if not dependent upon, preservation of some minimum level of ionic concentration. Nothing was found to suggest that these potential, electrolyte, and histological changes were unique to injury induced by irradiation; in fact, they appeared to be so much a part of the aging process exhibited eventually in all excised muscles that it is perhaps not too incorrect to look upon irradiation damage in excised muscle as essentially an acceleration of aging. The reason for the failure of the potential is not understood, but interpreted in light of current concepts requiring a source of energy to maintain the potential, the present findings, along with those of other workers, are in accordance with the idea that an interference with the metabolic processes associated with either synthesis, storage, or utilization of phosphate-rich sources of energy is involved.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, Raymond D., D. Denny-Brown and Carl M. Pearson. 1953. Diseases of Muscle. Paul B. Hoeber, Inc., New York.

Adrian, R. H. 1956. "The Effect of Internal and External Potassium Concentration on the Membrane Potential of Frog Muscle," Journal of Physiology. 133:631-658.

Bacq, Z. M., J. Lecomte et A. Herve. 1949. "Action des Radiations Ionisantes sur le Muscle Strie de Grenouille," Archives Internationales de Physiologie. 57:142-153.

Bennett, A. L., F. Ware, Jr., A. L. Dunn, and A. R. McIntyre. 1953. "The Normal Membrane Potential of Mammalian Skeletal Muscle Measured in Vivo," Journal of Cellular and Comparative Physiology. 42:343-357.

Bowman, William. 1840. "On the Minute Structure and Movements of Voluntary Muscle," Philosophical Transactions of the Royal Society. 130:457-494.

Boyle, P. J. and E. J. Conway. 1941. "Potassium Accumulation in Muscle and Associated Changes," Journal of Physiology. 100:1-63.

Conway, E. J. 1954. "Some Aspects of Ion Transport Through Membranes," In Active Transport and Secretion, Symposia for the Society of Experimental Biology VIII. University Press, Cambridge. pp. 297-324.

_____, 1957. "Nature and Significance of Concentration Relations of Potassium and Sodium Ions in Skeletal Muscle," Physiological Reviews. 37:84-132.

Darden, Edgar B., Jr., and C. W. Sheppard. 1951. "A Thimble-type Gamma-ray Dosimeter and the Measurement of the Radiation from Lumped and Distributed Type Sources," U.S.A.E.C. Document, Oak Ridge National Laboratory Research Report, ORNL 1002.

Davson, Hugh. 1951. A Textbook of General Physiology. The Blakiston Co., Philadelphia, Pa.

Dean, R. B. 1940. "Anaerobic Loss of Potassium from Frog Muscle," Journal of Cellular and Comparative Physiology. 15:189-193.

Falk, G., and R. W. Gerard. 1954. "Effect of Micro-injected Salts and ATP on the Membrane Potential and Mechanical Response of Muscle," Journal of Cellular and Comparative Physiology. 43:393-403.

Fedder, Ludwig, und Hans Hellner. 1928. "Die Veranderungen der Quergestreiften Muskulatur nach Rontgenbestrahlungen in Tierexperiment," Strahlentherapie. 30:682-706.

Fenn, Wallace O., and W. B. Latchford. 1931. "The Increased Metabolism of the Sartorius Muscle of the Frog Following Exposure to Roentgen Radiation," American Journal of Physiology. 99:454-462.

_____, 1936. "Electrolytes in Muscle," Physiological Reviews. 16: 450-487.

_____, R. H. Koenemann, and Eugenia T. Sheridan. 1940. "The Potassium Exchange of Perfused Frog Muscle During Asphyxia," Journal of Cellular and Comparative Physiology. 16:255-264.

Gerstner, Herbert B., Robert B. Lewis, and Everett O. Richey. 1954. "Early Effects of High Intensity X-Radiation on Skeletal Muscle," Journal of General Physiology. 37:445-459.

_____, Charles P. Powell, and Everett O. Richey. 1954. "Immediate Effect of X-Radiation on the Isolated Striated Frog Muscle," American Journal of Physiology. 176:9-14.

Ghormley, J. A., and C. J. Hochanadel. 1951. "A Cobalt Gamma-ray Source Used for Studies in Radiation Chemistry," Review of Scientific Instruments. 22:473-475.

Gottschalk, A., and W. Nonnenbruch. 1923. "Die Wirkung von Strahlenenergie auf die Gewebsatmung Tierischer Zellen," Strahlentherapie. 15:98-102.

Graham, Judith, and R. W. Gerard. 1946. "Membrane Potentials and Excitation of Impaled Single Muscle Fibers," Journal of Cellular and Comparative Physiology. 28:99-117.

Heilbrunn, L. V. 1956. Dynamics of Living Protoplasm. Academic Press, New York.

_____, and Floyd J. Wiercinski. 1947. "The Action of Various Cations on Muscle Protoplasm," Journal of Cellular and Comparative Physiology. 29:15-32.

Henshaw, Paul S. 1944. "Experimental Roentgen Injury. III. Tissue and Cellular Changes Brought About with Single Massive Doses of Radiation," Journal of the National Cancer Institute. 4:503-512.

Hodgkin, A. L. 1951. "The Ionic Basis of Electrical Activity in Nerve and Muscle," Biological Reviews. 26:339-409.

Janzen, Arnold H., and Shields Warren. 1942. "Effect of Roentgen Rays on the Peripheral Nerve of the Rat," Radiology. 38:333-337.

Jenerick, Howard P. 1953. "Muscle Membrane Potential, Resistance, and External Potassium Chloride," Journal of Cellular and Comparative Physiology. 42:79-102.

_____, and R. W. Gerard. 1953. "Membrane Potential and Threshold of Single Muscle Fibers," Journal of Cellular and Comparative Physiology. 42:79-102.

Kushner, Irving. 1925. "The Effect of X-Rays on the Irritability of Muscles in the Frog," Journal of General Physiology. 9:55-61.

Lewis, R. B. 1954. "Changes in Striated Muscle Following Single Intense Doses of X-Rays," Laboratory Investigation. 3:48-55.

Ling, G., and R. W. Gerard. 1949A. "The Normal Membrane Potential of Frog Sartorius Muscle Fibers," Journal of Cellular and Comparative Physiology. 34:383-396.

_____, and R. W. Gerard. 1949B. "The Influence of Stretch on the Membrane Potential of the Striated Muscle Fiber," Journal of Cellular and Comparative Physiology. 34:397-405.

_____, and R. W. Gerard. 1949C. "The Membrane Potential and Metabolism of Muscle Fibers," Journal of Cellular and Comparative Physiology. 34:413-438.

_____, and R. W. Gerard. 1950. "External Potassium and the Membrane Potential of Single Muscle Fibers," Nature. 165:113-114.

_____, 1952. "The Role of Phosphate in the Maintenance of the Resting Potential and Selective Ionic Accumulation in Frog Muscle Cells," Symposium on Phosphorus Metabolism. Vol. II. 748-797. The Johns Hopkins Press. Edited by William D. McElroy and Bentley Glass.

_____, and J. W. Woodbury. 1949. "Effect of Temperature on the Membrane Potential of Frog Muscle Fibers," Journal of Cellular and Comparative Physiology. 34:407-412.

MacNichol, E. F., Jr., and Henry G. Wagner. 1954. "A High-Impedance Circuit Suitable for Electrophysiological Recording From Micropipette Electrodes," Naval Medical Research Institute Research Report. Project NM 000 019.03.01.

Mood, Alexander McFarlane. 1950. Introduction to the Theory of Statistics. First Edition. McGraw-Hill Book Company, New York.

Nastuk, W. L., and A. L. Hodgkin. 1950. "The Electrical Activity of Single Muscle Fibers," Journal of Cellular and Comparative Physiology. 35:39-73.

Nastuk, William L. 1953. "The Electrical Activity of the Muscle Cell Membrane at the Neuromuscular Junction," Journal of Cellular and Comparative Physiology. 42:249-272.

Rieser, Peter. 1949. "The Protoplasmic Viscosity of Muscle," Protoplasma. 39:95-98.

Rothschuh, K. E. 1950. "Uber elektrische Entladungsvorgange an der Verletzten Skeletmuskelfaser und ihre Beziehungen zum Vorgang der Degeneration, der Regeneration, und des Absterbens," Pfluger's Archiv Fur Der Gesamte Physiologie. BD 252:445-467.

Shaw, F. H., and Shirley E. Simon. 1955. "The Nature of the Sodium and Potassium Balance in Nerve and Muscle Cells," Australian Journal of Experimental Biology and Medicine. 33:153-178.

_____, Shirley E. Simon, and B. M. Johnstone. 1956. "The Non-Correlation of Bioelectric Potentials with Ionic Gradients," Journal of General Physiology. 40:1-17.

Sheppard, C. W., and Edgar B. Darden, Jr. 1957. Work in Progress.

_____, and W. R. Martin. 1950. "Cation Exchange Between Cells and Plasma of Mammalian Blood. I. Methods and Application to Potassium Exchange in Human Blood," Journal of General Physiology. 33:703-722.

Speidel, Carl Caskey. 1938. "Studies of Living Muscles: I. Growth, Injury and Repair of Striated Muscle as Revealed by Prolonged Observations of Individual Fibers in Living Frog Tadpoles," American Journal of Anatomy. 62:179-235.

_____, 1939. "Studies of Living Muscles: II. Histological Changes in Single Fibers of Striated Muscle During Contraction and Clotting," American Journal of Anatomy. 65:471-529.

Steinbach, H. Burr. 1954. "The Regulation of Sodium and Potassium in Muscle Fibers," Symposia of the Society of Experimental Biology VIII, Active Transport and Secretion. University Press, Cambridge. 438-452.

Tobias, Julian M. 1950. "Injury and Membrane Potentials in Frog Muscle After Depleting Potassium and Producing Other Changes by Soaking in Potassium Free Salt Solution or Distilled Water," Journal of Cellular and Comparative Physiology. 36:1-13.

Vallee, Bert L., and Marvin Margoshes. 1956. "Multichannel Flame Spectrometer," Analytical Chemistry. 28:175-179.

Warren, Shields. 1943. "Effects of Radiation on Normal Tissues. XIV: Effects on Striated Muscle," Archives of Pathology. 35:347-349.

Weale, R. A. 1951. "A Method for Drawing Micro-capillary Tubes," Journal of Physiology. 112:4p-5p.

Wels, Paul. 1924. "Der Einfluss der Rontgenstrahlen auf die Oxydationsgeschwindigkeit in Zellen," Pflugers Archiv fur de Gesamte Physiologie. 203:262-273.

Wilde, Walter S., and C. W. Sheppard. 1955. "Electrolytes and Water in Muscles of Rat Forelimb After Intense Local X Irradiation," Proceedings of the Society for Experimental Biology and Medicine. 88:249-253.

Woodbury, Lowell A., Hans H. Hecht, and A. Rodney Christopherson. 1951. "Membrane Resting and Action Potentials of Single Cardiac Muscle Fibers of the Frog Ventricle," American Journal of Physiology. 164: 307-318.

Woodward, Arthur A., Jr. 1948. "Protoplasmic Clotting in Isolated Muscle Fibers," Journal of Cellular and Comparative Physiology. 31:359-394.

APPENDICES

APPENDIX A

TABLE VI

COMPOSITION OF RINGER SOLUTION*

Cation	mM/liter	mEq/liter	Anion	mM/liter	mEq/liter
Na^+	116.5	116.55	Cl^-	117.1	117.1
K^+	2.5	2.5	$(H_2PO_4)^-$	0.45	0.45
Ca^{++}	1.8	3.6	$(HPO_4)^{--}$	2.55	5.10
Total		122.65			122.65

*Source: Nastuk and Hodgkin, 1950.

APPENDIX B

ELECTRODE COMPONENTS, INPUT CIRCUIT, AND RESISTANCE CALIBRATION PROCEDURE

Preparation of Micropipettes

The tubing used was a special type made for microforge work (Aloe Scientific 48302) 1 mm O.D., supplied in 100 mm lengths. The tubing was thoroughly cleaned, rinsed with alcohol, and dried. It was cut into twenty-five mm lengths, then pulled by hand in a one-eighth inch gas flame to form a central constricted portion about 0.3 mm in diameter and 15 mm long. The final pulling to form microtips was done with a simple heating coil device, the essential elements of which are shown in Fig. 13. The heating element consisted of a one-sixteenth inch diameter coil of three to four turns of #34 B. & S. gauge chromel wire with a resistance of about 3.5 ohms. The set screw and clamp were made of a plastic (teflon) flexible enough to hold the capillary firmly without breaking it. With a weight of about fifty grams and a current of approximately 1.3 amperes, the coil was heated to sufficient redness to pull the glass apart in several seconds. The heat was cut off as soon as definite softening and elongation of the glass was evident in order to avoid melting the newly formed tips as they separated.

The pipettes were filled by first immersing them in the solution to be used as the conducting fluid long enough to fill the extreme tips by capillary action. The remainder of the filling was done with the pipette held securely in a horizontal position in a shallow pool of

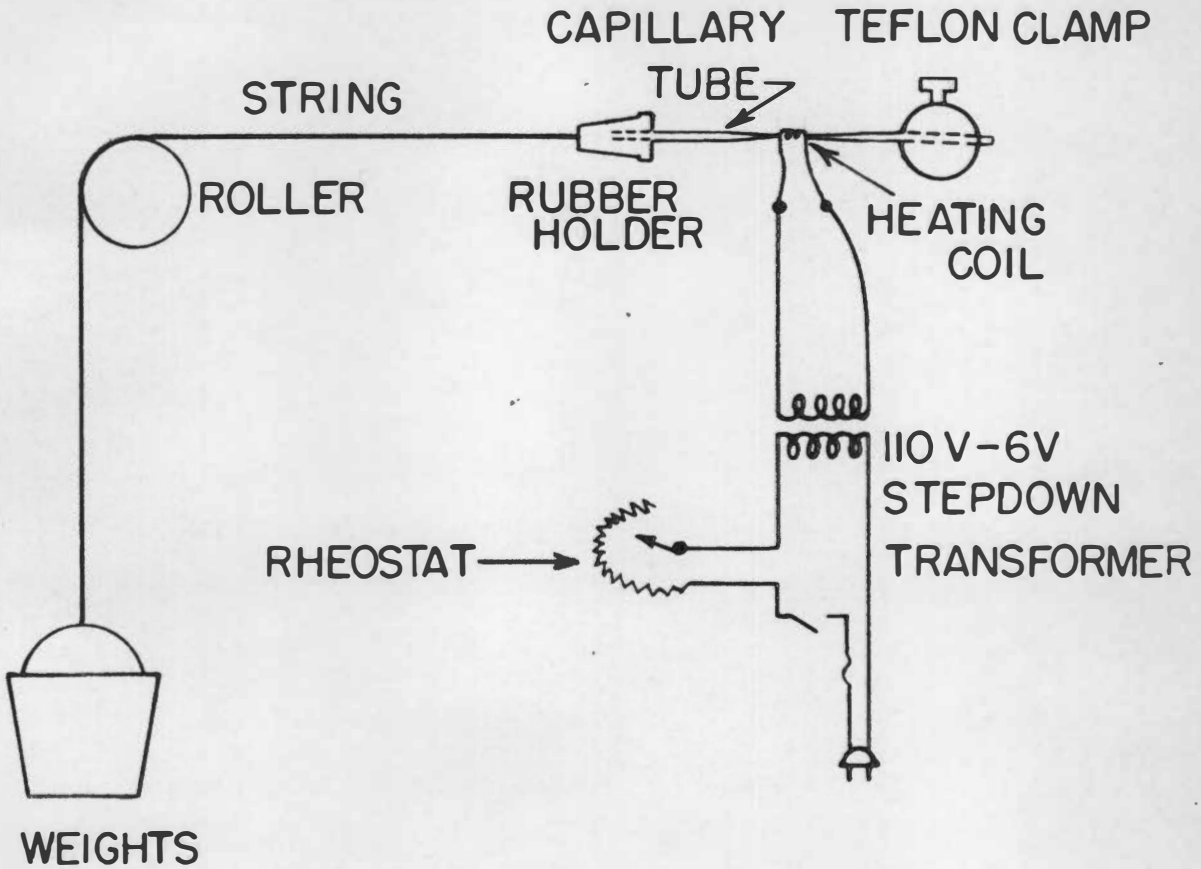


Fig. 13

Diagram of Pulling Device for Ultramicropipettes.

the liquid. A long slender glass pipette tapering to a diameter of ten to fifteen microns and attached to a small tuberculin syringe filled with the solution was inserted in the shank of the micropipette as far as it would go, to or almost to the level of the liquid in the tip and positive or negative pressure applied as needed to inject fluid or dislodge or suck out air bubbles, in order to fill the remainder of the micropipette.

The manipulations were readily carried out on the stage of a dissecting binocular microscope and after a little practice, some ten or twelve fillings could be accomplished in an hour.

Preparation of Ag-AgCl Electrodes

Short spirals of silver wire .032 inches in diameter (B. & S. Gauge #20) previously silver-soldered to light-weight shielded, teflon-insulated wires were thoroughly cleaned with nitric acid and soaked in concentrated HCl so that they were a dull white in color. Silver chloride was electrodeposited for one hour with the wire as the anode and a platinum electrode as cathode from a one N solution of HCl (Volumetric Standard grade) at a current density of approximately two milliamperes per cm^2. A small electroplating unit consisting of a 110 volt step-down transformer and selenium rectifier proved to be convenient for this purpose. The silver wires were now purple-colored from the presence of partly reduced (photoactivated) AgCl. They were then cleaned in ether and rinsed in alcohol to remove any traces of grease, a source of electrode instability, then washed for at least

twenty-four hours in running water.

The electrodes were tested for electrical stability in Ringer solution before mounting them in tubes. Over a twenty-four hour period the potential difference across the electrodes after an initial decrease from 0.5 mv, was 0.2 mv ± .05 mv. Each Ag-AgCl wire was inserted in its glass tube and warm agar made with Ringer solution (0.2 gm per 10 cc solution) was sucked up in the tube to cover the spiral and allowed to harden. The teflon insulation bushing in the top of the tube, along with the protruding lead, was thoroughly sealed in with ceresin wax (Fig. 3).

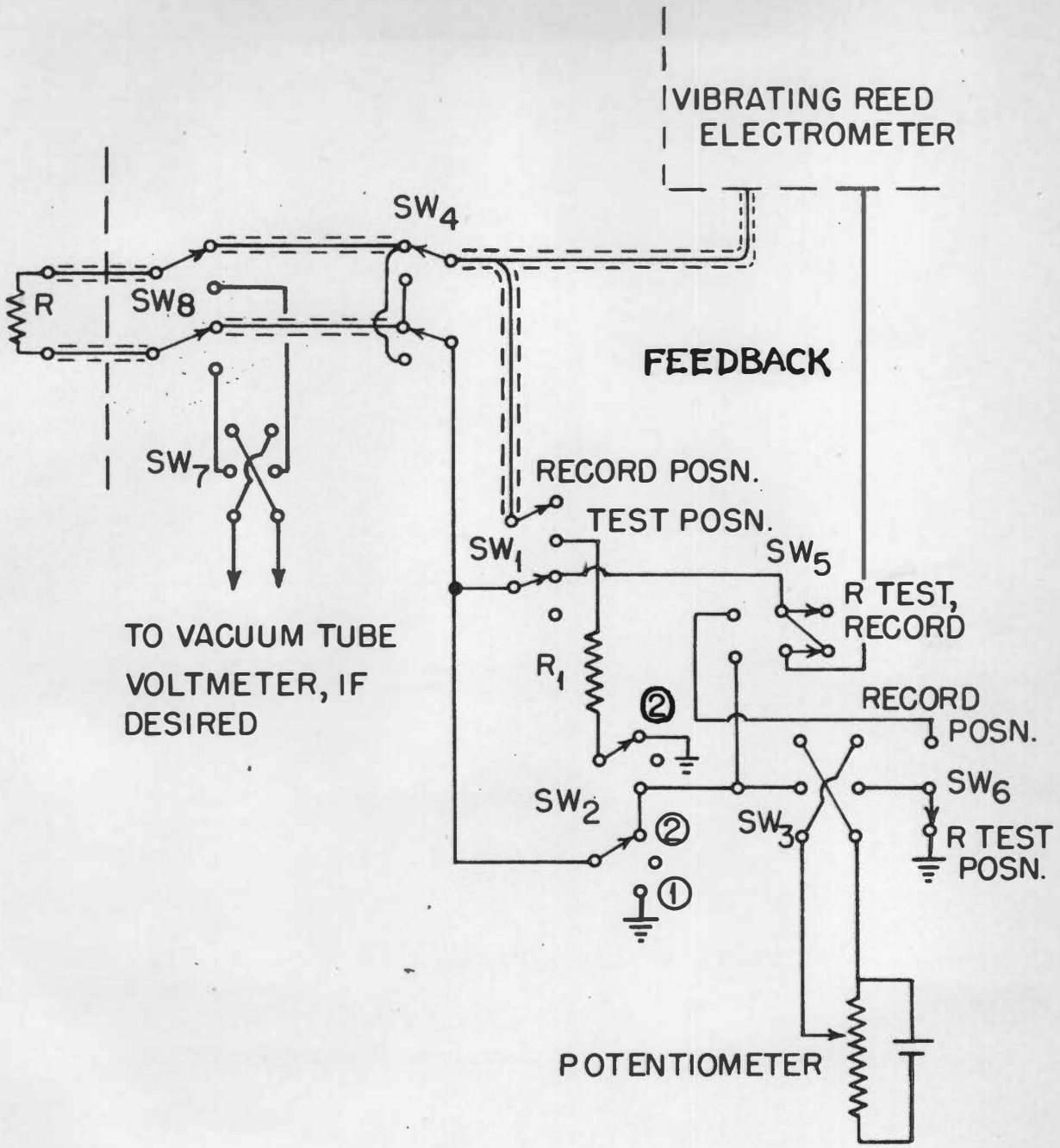
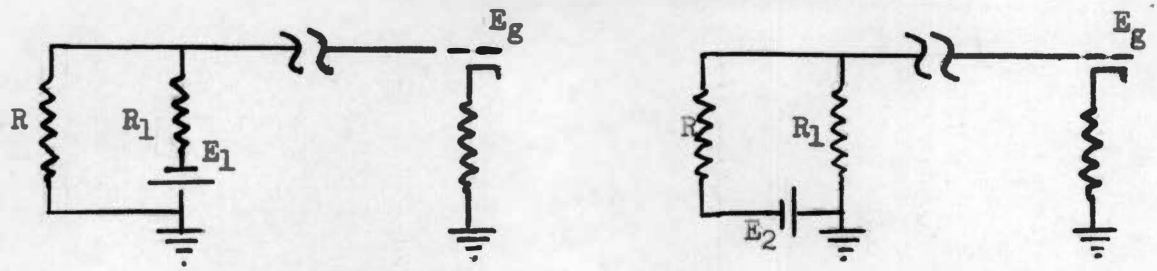


Fig. 14

Input Circuit for Measurement of Membrane Potentials Including Resistance and Voltage Calibration Test Circuits.



Position 1 Position 2

Fig. 15

EQUIVALENT CIRCUIT FOR MEASUREMENT OF INPUT RESISTANCE

With SW_1 (Fig. 14) in the test position measurement of the input resistance between electrodes was done in two operations represented by the two simplified circuits in Fig. 15. First, the input electrode circuit of unknown resistance R was grounded by throwing SW_2 (Fig. 14) to position 1. Then the potentiometer (Fig. 14) was adjusted to apply an arbitrary voltage E_1 across R and standard resistor R_1 resulting in a grid voltage E_g on the first input tube of the electrometer and a signal on the electrometer output meter. Second, the standard R_1 was grounded and the electrodes ungrounded by throwing SW_2 to position 2. The potentiometer voltage was adjusted to a value E_2 to give a signal E_g of the same magnitude as before. Then, in position 1, $E_g/E_1 = R/R + R_1$, and in position 2, $E_g/E_2 = R_1/R + R_1$. Eliminating the constant terms:

$$R = E_2/E_1R_1$$

Since R is fixed (195 megohms was the value used), the ratio of the two potentiometer settings E_2/E_1 enabled an immediate estimate of R to be made.

APPENDIX C

CALIBRATION OF DOSE RATE OF COBALT 60 EXPOSURE FACILITY

The calibration was made prior to the present investigation.* The dosimeter used was a thimble-type gamma ray ionization chamber having a volume of about 2 cc, attached to a polystyrene probe five-eighths inch in diameter. Its response in roentgens was determined both by the Bragg-Gray principle and by repeated calibrations against a radium needle of known strength certified by the Bureau of Standards, as described in a previous publication (Darden and Sheppard, 1951). In a field of such high intensity as present in this source, it was found that even with collecting voltages of almost 900 volts, complete ion current saturation was not achieved. However, by an investigation of saturation characteristics from 600 to 870 volts, it was estimated that at 600 volts the current measured was at least 99 per cent of the saturation value. Also included in the final calibrated value was a small correction of about 2 per cent for the distortion of the field due to the shank of the thimble chamber, experimentally determined by the use of a dummy shank.

The field inside the source cylinder was surveyed both along the axis and at points off the axis up to five-eighths inch, the limit of lateral displacement of the center of the chamber. The high degree of uniformity of intensity along the axis is shown in the graph in Fig. 16, in which dose rate is plotted as a function of distance from the lower end of the cylinder. The field along the inner third of the axis where

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*The writer wishes to acknowledge the advice of Dr. C. W. Sheppard in whose group the work was done.

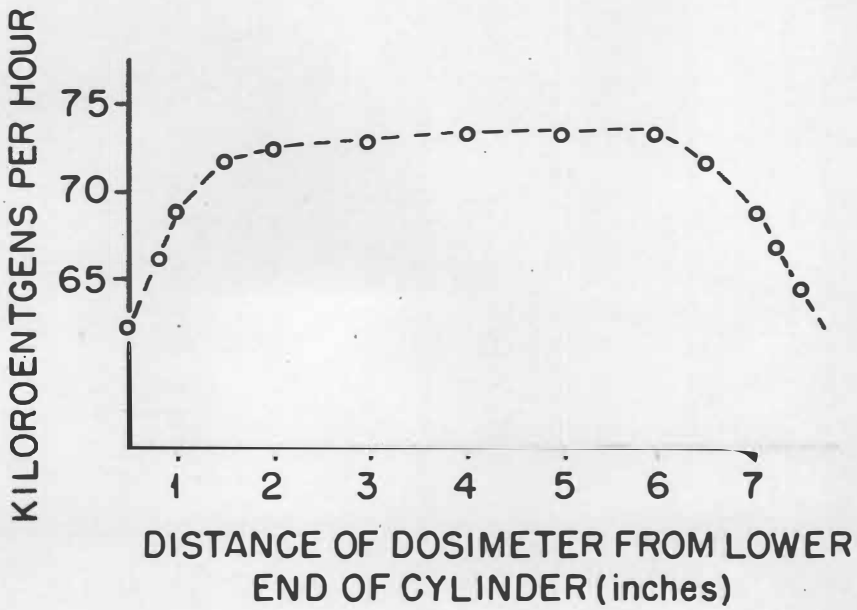


Fig. 16

Rate Along Axis Inside Cobalt-60 Source Cylinder (As of early 1956).

samples were generally placed was flat to within ± 2 per cent. The maximum increase in intensity measured off the axis was 8 per cent.