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GRADUATE COLLEGE

INVESTIGATIONS INTO THE MECHANISM OF ACTION OF A FISH PITUITARY HORMONE

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INVESTIGATIONS INTO THE MECHANISM OF ACTION

OF A FISH PITUITARY HORMONE

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INVESTIGATIONS INTO THE MECHANISM OF ACTION OF A FISH PITUITARY HORMONE

INTRODUCTION

Movement of water through an epithelium such as the toad urinary bladder, occurs by osmosis after active transport of solutes through the epithelium. The neurohypophyseal hormones antidiuretic hormone (ADH), vasopressin and lysine vasopressin regulate the permeability of the toad bladder epithelium in two ways. Either of these hormone increases the permeability of the apical plasma membranes of the mucosal cells to sodium ion,* water and urea. ADH probably also indirectly stimulates aerobic cellular metabolism and Na^+ transport from the bladder's lumen to the body cavity (Leaf, 1967, and Zadunaisky, et al, 1968).

A hormone with ADH like physiological properties has been isolated from the cypriniform fish mesadenohypophysis, a structure homologous to the adenohypophysis of other vertebrates (Pickford, 1959). The substance, which resembles luteinizing hormone (LH) in many of its physical-chemical properties, has a molecular weight of about 70,000, and has been named the "hydration factor" (Clemens, et al, 1964). This

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*Ions hereafter will be referred to by chemical symbols with their respective positive or negative charges.

factor is involved in two primary in vivo actions, in either the carp, Cyprinus carpio or the goldfish Carassius auratus, stimulation of a transient water accumulation in a variety of tissues (Clemens and Grant, 1964 and 1965) and stimulation of spermatogonial proliferation and spermatogenesis in the goldfish (Clemens and Reed, 1967b). When a crude distilled water extract of acetone dried pituitary was injected at low dosages (0.69 ug or less/gram of body weight based on Acetone extracted Dry Weight of Pituitary (ADWP) before water extraction), it stimulated water accumulation in muscle, kidney, operculum, ovary and testis tissue. As the dosages increased logarithmically (0.69 to 69 ug/g body weight), the testis and the operculum continued to accumulate water, reaching the highest water content at 22 to 24 hours after injection and declining to preinjection levels over the next three to four days. All the other tissues measured gained water for the first 8 hours, then lost it to preinjection levels during the next 14 to 16 hours at linearly proportional rates (Clemens and Grant, 1964). It was concluded that the end result of the testis hydration was primarily extracellular water accumulation since the water content of the seminal plasma increased at a dosage proportional rate (Clemens and Grant, 1965). These studies did not indicate whether the transient water changes in the other tested tissues were primarily extracellular or intracellular. Since there was a decrease in blood water percentage, an increase in hematocrit, and an increase in red cell count associated with the gonadal hydration effect, the water of the blood plasma may be the source of the water that is moved into the testis (Clemens and Grant, 1964).

Although the total volume of seminal fluid increased significantly and the sperm count per unit volume of semen decreased during testis hydration, there was no significant change in the total particle concentration or in the concentrations of Na^+, K^+, or Ca^{++} in the seminal plasma (Clemens and Grant, 1965).

While many events may be operating to cause the increase in water content of the testis (ibid.), the fact that there were no changes in total particle concentration or in the concentrations of the ions measured indicates that ion movement is a factor in the hydration process. If ion movements were not involved in the hydration effect, then the seminal plasma should have a significant lowering of Na^+, K^+, and Ca^+ concentrations with the increasing water percentages seen with increasing dosages of the hormone.

The "hydration factor" could act by increasing the passive permeability of the plasma membranes of cells lining the testis lobular lumen to water and Na^+ and the blood pressure would cause greater filtration of water and ions into the seminal spaces of the testis. Alternatively, the "hydration factor" could selectively increase the rate of ion, particularly Na^+, transport into the testis lobular lumen in an action similar to that of ADH on the toad urinary bladder. The increased rate of movement of Na^+ into the testis lobular lumen would induce an increased osmotic water flow. Since most cells are quite permeable to K^+ and Cl^- (Ussing, 1954) the result would be an increased volume of semen with essentially the same ion concentration as blood. The transient hydration seen in the other studied tissues (Clemens and Grant, 1964) might also be explained by these same mechanisms operating

in different degrees, i.e., passive permeability changes being more important than active transport. The action of the hydration factor may be a general effect upon most, if not all, of the fish's tissues, with its transient or continual hydration effects related to the arrangements and kinds of cells in the tissues studied.

A second effect of the "hydration factor" occurred when male goldfish with nutritionally regressed gonads were chronically injected with a pituitary extract containing the factor. Gonadal regression was induced by a daily diet limited to 2% of the fish body weight. After regression, a 30 day experiment was performed in which the daily diet was increased to 5% of body weight and the factor injected every other day, the ratio of testis weight/body weight x 100, the Gono-Somatic Index, increased 1,000% over control and saline injected fish (Clemens and Reed, 1967b). The final GSI in the experimental fish was the same as that of normal, prespawning, male goldfish. Histological studies of the testes of the above fish demonstrated that the extract injections had induced a proliferation of spermatogonia from the germinal epithelium and an increase in spermatogenetic activity.

The effects of the "hydration factor" in stimulating tissue hydration and cellular proliferation may be reflections of a single mechanism of action, or of multiple actions. Neither effect may be a direct one; i.e., the factor may be merely "turning on" a multiplicity of hormonal pathways.

Any attempt to study the isolated effects of the "hydration factor" on the testis tissue or other tissues requires the elimination of secondary responses from other body systems. Consequently in vitro

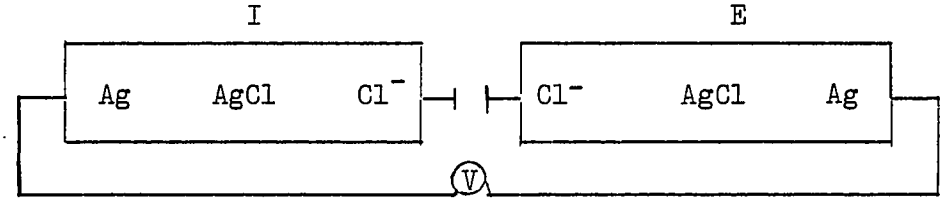
isolation techniques seem suited for studies of the various tissues. The method of tissue culture was chosen to isolate the testis tissue, for tissue cultured cells or cell masses are usually far enough apart to be used for testing ion exchange with the culture media. Tissue cultures can also provide a way to study the cell division stimulation effects of the "hydration factor" since cell counts and cell size measurements can be made repeatedly, after a time lapse, on the same isolated tissue. Initially, adult, prespawning male testis tissue was used as explant material. It grew poorly as cultured cells often do in the presence of excessive amounts of lipid. Extracellular lipid droplets could clearly be seen in the cultures. In addition, adult specialized cells have a lower proliferation rate (Moscona, et al, 1965). This tissue was also difficult to manipulate and study because of the large numbers of spermatozoa present. The testis of the nutritionally regressed male goldfish avoided some of these objections. There were few, if any, spermatozoa present and fatty substances in the connective tissue around the testis were greatly reduced. In vivo evidence was available that cell division could be stimulated in the regressed testis by hormonal preparations containing the hydration factor (Clemens and Reed, 1967b). This was a clue that cells of the regressed testes might more readily proliferate in tissue or explant culture if they were chronically treated with the pituitary preparations. Kidney tissue was taken from the nutritionally regressed goldfish to compare its growth responses with those of chronically treated and untreated testis tissue.

The changes in ionic concentration in the seminal plasma, the testes, the operculum, the muscle or the testes that would account for

the observed water percentage changes could not be detected by conventional methods such as flame photometry and freezing point determinations (Clemens and Grant, 1965). Small changes in ionic concentration are detectable as electropotentials (Ochs, 1965).

In all animal cells the intracellular and extracellular ions are in a state of dynamic balance. The primary extracellular ions are Na^+ and Cl^- while the primary intracellular ions are K^+ and undetermined anionic charges, probably associated with amino acids and polyelectrolyte proteins. Since the intracellular anions and the extracellular Na^+ are largely restrained from free diffusion by the structure of the plasma membrane, and since Na^+ transport out of the cell is linked with the inward movement of K^+ and Cl^-, a concentration gradient, inside to outside, is established for each ion. A definite ratio is usually established between the intracellular K^+ and the extracellular Na^+. This transmembrane concentration difference is measurable as a transmembrane electromotive force (EMF) or membrane potential and has values close to those which can be predicted by the Nernst equation using either the Cl^- or K^+ transmembrane concentration gradients (Hodgkin, 1951).

Transmembrane Cl^- potentials can be detected by penetrating the membrane with a small capillary containing a KCl salt bridge electrode which is connected to a non-polarizable Ag·AgCl half cell. The other half cell of the chloride concentration cell is a physicochemically identical Ag·AgCl electrode.



When the half cell bearing the KCl electrode salt bridge penetrates a plasma membrane, if there is a lower intracellular Cl^- concentration within the intracellular than in the extracellular compartments, an EMF between the two half cell electrodes occurs which can be measured. Extracellularly, Cl^- will diffuse to the electrode E and yield electrons to the measuring instrument V. Intracellularly Cl^- will diffuse from the half cell I as its Ag^+ is reduced by electrons coming through V from E (Frank and Becker, 1964). The Cl^- is prevented from reaching an equilibrium and thereby reducing the transmembrane EMF to zero by two mechanisms. Firstly the extremely high resistance (10^{10}) of the measuring instrument allows only an extremely small current flow ($\ll 10^{-12}$ Amp) in the penetrated cell, therefore the amount of Cl^- released is extremely small (Frank and Fuortes, 1955). Secondly the size of the capillary tip of the intracellular salt bridge is very small (less than 0.5 microns) and prevents rapid movement of Cl^- and K^+ into the cell (Grahm and Gerard, 1946). Only about 5×10^{-14} moles/sec of KCl will diffuse into a cat motoneuron (Coombs, et al., 1955a) or a frog muscle cell (Nastuk and Hodgkin, 1950) through a capillary of this diameter.

As has been previously stated Cl^- is distributed across the plasma membrane in a passive charge relationship with respect to the concentration ratio of Na^+ and K^+ (Na^+/K^+). When a permeability change

occurs in the plasma membrane resulting in an increase in the inward flux of Na^+, there is concurrent inward movement of Cl^- and a consequent outward flux of K^+.

When the transcellular EMF is detected as decreasing, this is because the intracellular Cl^- concentrations has increased. This indicates that a new equilibrium of extracellular to intracellular cations is present.

Cellular membrane permeability to K^+ can also be determined, by measuring the DC current flow through the half cell and the 3M KCl filled intracellular salt bridge (Coombs, et al, 1955b). The measured injection voltage necessary to maintain a constant flow of K^+ and Cl^- can be used with Ohms' law to determine plasma membrane resistance. If the resistance of a plasma membrane is decreased, those ions can be pushed through the capillary microelectrode (CME) and the cell membrane at a lower voltage. Generally speaking, an increase of permeability of a plasma membrane to K^+ and Cl^- also means there is a greater permeability for Na^+ (Eccles, 1957).

The primary objectives of this investigative dissertation are the following: To test the "hydration factor" on cultured fish testis and kidney cells in order to observe whether the factor can stimulate in vitro cell division; and to study the effect of the factor's ability to alter the permeability properties of the plasma membranes to K^+ and Na^+ of in vitro isolated fish tissue cells and to relate these findings to the in vivo effect of the hydration factor.

METHODS AND MATERIALS

Cultures were prepared in a special glass fronted cabinet equipped internally with germicidal ultraviolet lamps, dissecting microscope, hotplate, gassing lines and valves. Prior to each culture session, the cabinet was washed with a surface active, bactericidal and mycostatic agent (Cetylcide), and the ultraviolet lamps turned on for at least one hour. Air flow into the cabinet was regulated by a disphragm type pressure regulator. It was then cleaned by being passed through a water trap, then through a sterile, cotton-packed filter tube, and into the gas lines entering the cabinet. Carbon dioxide flow was processed in a similar manner. The pressure regulators of the two gases were adjusted to maintain the gas flow into the cabinet at about 95% air and 5% CO_2. The CO_2 was also used to adjust the pH of solutions in the cabinet. The eyepieces of a dissecting microscope (Carl Zeiss) projected through and were sealed in a cutout in the inclined glass front on the cabinet. The microscope focusing control knobs and the two variable intensity dissecting lamps (Bausch and Lomb) with their transformers were contained within the cabinet.

Glassware and instruments were washed in a 1% solution of a nontoxic, neutral, liquid, tissue culture safe detergent ("7X"); rinsed in running tap water and several changes of glass distilled water; and air dried. The glassware and heat stable solutions were steam autoclaved

at 15 to 20 lbs/in^2 for 30 minutes. Heat labile solutions were sterilized by ultrafiltration (Millipore filter, HA 0.45). Dissecting instruments were sterilized in 70% ethanol for 30 minutes before being passed into the culture cabinet. They were dried with sterile 4" x 4" gauze surgical pads.

Tissues were obtained from sexually mature and gonadially regressed male goldfish Carrasius auratus of 8 to 13 cm standard length. The animals were housed in termperature controlled (22 to 24° C) concrete tanks. The fish were supplied by the Fisheries Research Center facility of the Department of Zoology, Oklahoma University, located at Noble, Oklahoma. The fish were gonadially regressed according to the procedure of Clemens and Reed (1967a). Regression is accomplished by feeding 2% of body weight per day of commercial fish food, for a period of at least three months. During this time interval, the spermatozoa and spermatocytes disappear leaving connective tissue, seminiferous lobules, and primary germ cells (Clemens and Reed, 1967a).

The fish were removed from the concrete tanks and brain pithed. After two to five fish had been pithed, they were transferred to a work table, the pectoral fins, pelvic fins, and ventral scales were removed. The descaled region was flushed with Cetylcide, a nontoxic antiseptic containing benzalkonium chloride and cetylpryidium chloride. A ventral, horizontal, thoracic incision and a midventral, longitudinal incision were made through the body wall. The juncture of the two incisions was retracted to expose the organs of the visceral cavity.

The visceral mass was moved aside and the threadlike regressed gonads were removed with a pair of sterile forceps. Occasionally, other

types of tissue were removed from the fish through the same or other appropriate incisions for culture purposes. A similar procedure was followed with the sexually mature male goldfish. The tissues were rinsed briefly in Cetylcide and passed through three rinses of sterile, Simms' balanced salt solution (Simms, 1942), pH 7.4, containing 500 micrograms per ml of streptomycin sulfate (Squibb) and 200 units per ml of a potassium salt of crystalline penicillin G (Squibb). The tissue types were pooled and transferred into a sterile, screw cap, Ehrlenmeyer flask containing Simms' solution and antibiotics at room temperature (22 to 24° C). The flask was placed in a polyurethane foam container to maintain the temperature of the solution in the flask. The container was then transported the seven miles back to the culturing laboratory.

The tissues were poured, with the transport solution, into a container of sterile Simms' solution pH 7.4, 23 to 24° C inside the culture cabinet. Once inside the cabinet they were passed through four Simms' solution rinses (no antibiotics) and stored in Wolf and Quimby's (1962) modified Puck's, et al., (1957) culture medium, pH 7.35-7.4, as indicated by the phenol red in the medium. The culture medium contained 20% agamma horse serum (Hyland Laboratories), 10% Medium 199 (Baltimore Biological or Hyland Laboratories), and 70% Simms' solution. The culture medium also contained 100 units per ml penicillin G, 200 micrograms per ml streptomycin sulfate and 100 units per ml of a mycostatic agent (Squibb's Nystatin). During the dissection and mincing of the tissues, the cabinet temperature was 23 to 24° C. Under the dissecting microscope, the pooled tissues of each type were stripped of peritoneal connective

tissue and fat then minced into roughly cuboidal fragments about 0.1 mm^3 in size with irridectomy knives. These tissue fragments were the explants.

The explants were cultured in plasma clots on #2 40 x 40 mm coverglasses sealed over Maximow deepwell slides. A drop of culture medium containing 10 to 15 explants was stirred into a drop of chick embryo extract (Bast and Mills, 1963) on the coverglass. A drop of reconstituted chick plasma (Difco) was then added and the mixture stirred so that the explants were distributed throughout a clot, 10 to 15 mm diameter and about 0.5 mm thick. After the mixture had clotted, the coverglass was inverted over the deepwell of the slide which contained 2 ml of culture medium. The edges of the coverglass were sealed to the slide with melted paraffin applied with a camel's hair brush. The slides, held twelve at a time in slide holders, were incubated with the coverglass down for varying lengths of time at 20° C in a BOD incubator (Precision Scientific).

Inspection and Recording Growth of Explant and Peripheral Cells

Periodically the cultures were visually inspected and photographed. The sets of slides in holders were removed from the incubator. Each slide was examined with a phase contrast microscope equipped with a trinocular head, long working distance objective, ultraplan oculars (Bausch and Lomb) and a variable intensity, filtered source of illumination (American Optical). Cellular and explant details were recorded photographically through the trinocular barrel equipped with an Exacta II or Hasselblad 500 camera, using Kodak High Contrast Copy or Kodak Panchromatic films,

respectively. Exposures were timed with an electronic timer (modified Heathkit pt-15). The timer controlled the illuminator brightness switching and a solenoid (Tektronix) which operated the camera shutter (circuit designed by K. S. Mills).

A systematic photographic record was made of the histories of regressed, gonadal explants and of kidney explants from the same fish. These histories were compared with those obtained from gonadal and kidney tissues treated with crude putuitary extract. A Simms' saline extract of carp pituitaries was added to a series of slides in 200, 400, and 800 microgram amounts (based on original dry pituitary weight) per ml of culture medium. At zero hours in culture one slide from the untreated cultures and from each concentration series of each tissue was chosen at random. An explant of each slide was chosen and a circle drawn about it on its overlying coverglass. A photograph was taken of each encircled explant at the time intervals listed in Table 3.

The histories ended when the cells in the outgrowth region were present in such large numbers that the confluent cell sheet prevented microscopic resolution of individual cells within the sheet or until the peripheral cells of the chosen explant had large amounts of vacuolation indicating cellular degeneration. Occasionally the circled explant would dissolve the plasma clot. When this occurs, the cells and explant become microscopically unresolvable or sometimes fall out of the plasma clot. When this occurred, a new explant on the same slide was randomly chosen and photographed.

Preparation of the Simms' Saline Pituitary Extract

Commercial, acetone dried, carp, Cyprinus carpio pituitaries (ADWP) (Stoller Fisheries 7/14/60) were homogenized with a glass homogenizer (Kontes 88550) in Simms' saline solution, pH 7.4, at a temperature of 5° C. The initial concentration of the dried pituitaries in the homogenizer was 20 mg per ml. The homogenate was placed into a sterile, 50 ml, screw top Ehrlenmeyer flask, mechanically stirred at 20° C and extracted for one hour. The homogenate was centrifuged (Servall Model RC-2) at 10,000 x g at 0 to 2° C for 30 minutes. The supernate was decanted into sterile, screw cap vials in 1 ml portions and frozen. Shortly before use in the cultures the extract was defrosted at room temperature and passed through a millipore filter into a sterile container within the culture cabinet. The concentrations added to the experimental preparations are based on this original 20 mg ADWP per ml homogenate solution. After this reconstitution one microliter of solution contained 20 micrograms ADWP.

A second pituitary preparation was additionally used in the electrophysiological experimentation. This extract was prepared from a lyophilized sample supplied by technicians at the Oklahoma Fisheries Research Center, Noble, Oklahoma. The sample had been prepared from a homogenized, buffer-extracted sample of fresh frozen carp pituitaries. The principal process of purification was the disc electrophoresis method outlined by Ornstein (1964) and by Bogenshutz and Clemens (1967). A 50 mg sample of the lyophilized sample was dialyzed against several changes of distilled water and then relyophilized to determine the amount of active

material present. After lyophilization 1.7 mg of the original sample remained. Johnson (personal communication) claimed the difference in weight is due to the loss of the glycine buffer used in the gel electrophoresis. A second 50 mg sample of the buffer + active material, the original lyophilized sample supplied, was dissolved in 0.5 ml of Simms' saline to yield a concentration of 3.4 mg per ml of active fraction. This was added to the biopotential measuring chamber at a dosage of 0.01 ml/ml of medium to yield a final concentration of 0.034 mg per ml. The buffer in the sample changed the final pH of the medium in the measuring chamber to about 8.4

Preparation of the Electrophysiological Reference Tissue, Goldfish Back Muscle Strips

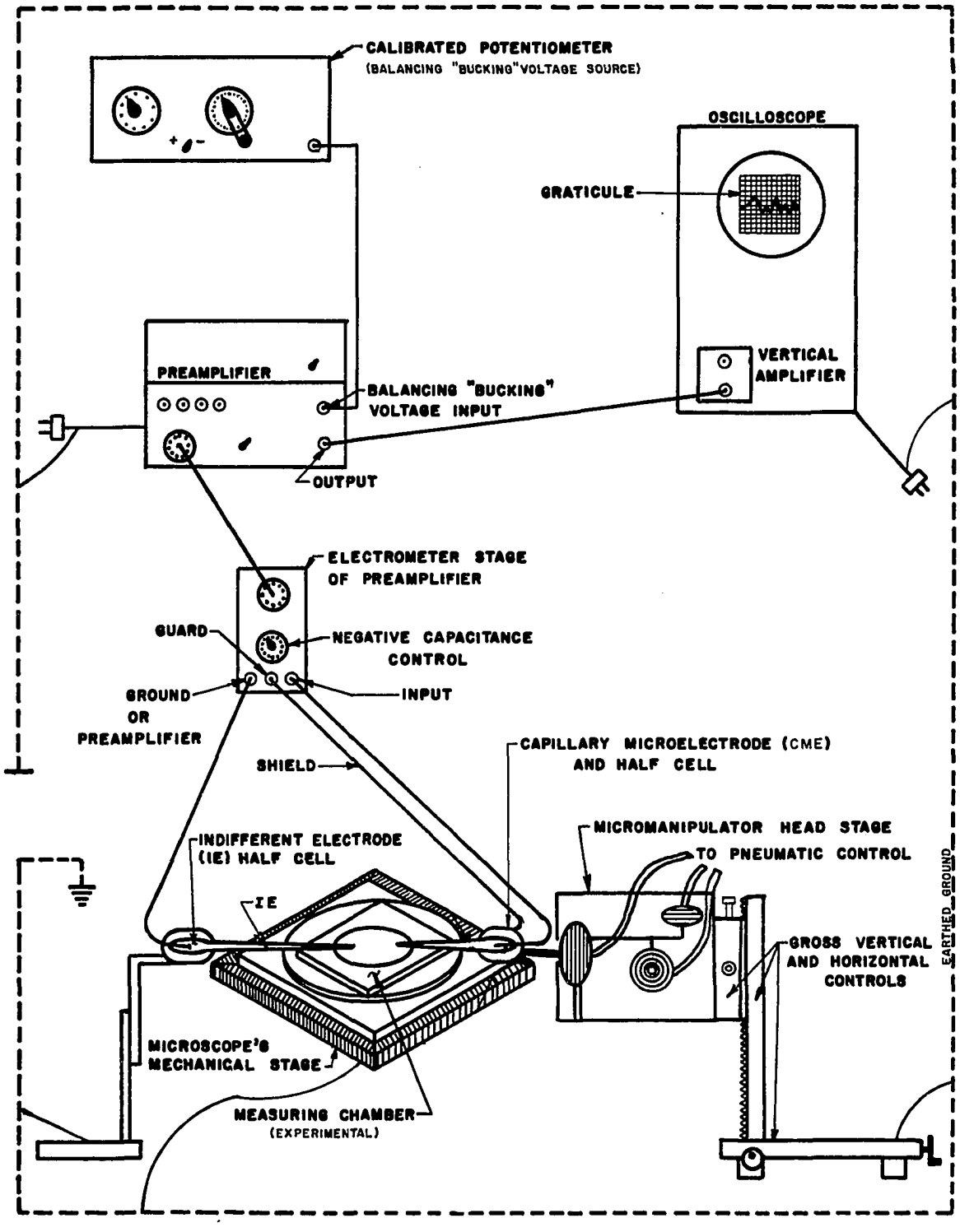
Strips of goldfish back muscle were used as a reference source of resting potentials and membrane resistance. The tissues were taken from both sexually mature and gonadially regressed male goldfish. After brain pithing a fish, its lateral scales were scraped away and the scraped area rinsed in tap water and in Simms' solution. A rectangular block of relatively homogenous muscle tissue about 1 cm x 6 $\pm$ 2 cm x 0.65 $\pm$ 0.05 cm was taken from the dorsal region just lateral to the dorsal fin of each fish. The skin was removed from the muscle mass. The mass was then placed into Simms' solution, pH 7.4, 5° C and cut into 2 mm x 2 mm x 20 to 30 mm longitudinal strips. Each end of a smaller strip was tied with cotton thread to a 1 mm x 40 mm pyrex glass rod. This procedure served two functions. It maintained the muscle at its resting length and served as a holding and transferring device for placing the muscle strip into the measuring chamber.

The Electronic Apparatus

Figure 1 illustrates the pieces of apparatus involved in the measurement of resting potentials and cell membrane resistances of the cultured gonadal cells and the in vitro isolated back muscle cells. The measuring chamber was made of acrylic plastic and had an optically flat, polished bottom. A cover slip, held in the chamber's upper movable section, could be rotated 360°. The lower section was held rigidly by the phase microscope's mechanical stage.

The capillary microelectrode (CME) (Nastuk and Hodgkin, 1950) and its Ag·AgCl half cell were mounted on the head stage of a pneumatically driven micromanipulator (de Fonbrune). The capillary tip was brought into approximate position by the auxillary, three directional coarse movement controls of the brass base plate. The electrical circuit was completed through the measuring chamber medium by the indifferent electrode (IE). Connections from the indifferent electrode and its half cell led to the switched ground terminal on the electrometer stage of the preamplifier which was connected with the circuits' common ground. Connections from the capillary microelectrode and its half cell led to the input terminal on the electrometer stage. An electrostatic shield surrounding the CME half cell was connected to the guard terminal on the electrometer head stage. This stage with its preamplifier, a negative capacitance electrometer (Argonaut LRA-043), amplified the bioelectric signals by 10 fold. The signal was further amplified 10 to 50 fold by the internal amplifier of the cathode ray oscilloscope (Tektronix 502a) and then displayed as a vertical displacement of the tracing on the face of the

Figure I



Schematic Diagram of the Experimental Apparatus Used in the Measurement of Membrane Potentials and Resistances

oscilloscope. These tracings were photographed for later analysis on Kodak Ortho Contrast film, gray base, with an oscilloscope camera (Beattie Coleman) equipped with interchangeable single pulse and continuous motion magazines. When the single pulse magazine was used, the variable horizontal trace sweep speed of the oscilloscope was used. When the continuous motion magazine was used, the horizontal trace speed was turned off and the oscilloscope electron beam spot placed at the center of the screen. The only deflection in this case was in the variable amplitude.

Potentials resulting from tip diffusion of KCl from the tip of the capillary microelectrode and/or resulting from half cell imbalance could be corrected by applying a positive or negative D.C. voltage from a calibrated potentiometer (Mills and Burkhart, 1956) to the preamplifier side of the capillary microelectrode half cell. Both the preamplifier and the oscilloscope circuitries were individually balanced to the ground level or zero voltage of the common ground. When the two instruments were added to the measuring circuit, the tracing on the oscilloscope represented ground or zero potential. This represented the D.C. baseline of the oscilloscope horizontal tracing.

The capillary microelectrodes were prepared from capillary, flint glass tubing approximately 0.8 mm inside diameter (Fisher # 5-962) by means of an electromagnetic pipette puller (Brinkman #F-30-40-00). This puller was modified to provide variable delay before pull time, heater current timing, and easier clamping of the capillary tubing. The optimal pull time and heater current time were determined empirically. Samples of a batch of electrodes pulled at any given setting were microscopically

inspected. When the inspected samples had broken tips, more than 10 to 1 change in length-diameter ratios for the last 1 mm of tip, or outside diameters greater than 0.5 microns, the batch was rejected, and new adjustments were made on the electrode puller. When an optimal setting was found, it was possible to make uniform electrodes until that supply of capillary tubing was exhausted. When a different supply of capillary tubing was used, the controls on the puller were again empirically adjusted. When samples of electrodes were optically within the limits set, the entire batch was filled and then tested electronically. Electrode filling followed the procedure of Tasaki, et al (1954); the electrodes were submerged with their tips always up in methanol and placed in a vacuum. The vacuum was released and the electrodes submerged in degassed KCl and again subjected to a vacuum. The electrodes were filled shortly before being used in most cases, although some were filled and stored in paraffin oil or distilled water up to a week before being tested and used.

Each electrode was electronically tested by being placed in the CME half cell holder and lowered, tip first, into the experimental medium or into a container of 3 M KCl. A salt bridge connected the input half cell with these solutions. A square wave current of constant magnitude was passed through the electrode. Those electrodes which showed a voltage amplitude change proportional to a resistance of less than 1 megohm or more than 20 megohms in 3 M KCl, or 5 to 100 megohms in the experimental media were rejected.

The silver-silver chloride half cells, used for the indifferent electrode half cell (IEHC) and the input half cell (IHC), were prepared according to the procedures outlined by Brown and MacInnes (1935) and

modified by Ives and Janz (1961). Platinum-iridium wire (0.8 mm D, 75% Pt, 25%Ir) was cut into 2 cm lengths. One end of each segment was fused in an oxygen-laboratory gas flame forming a spherical billet of 1.5 to 2 mm in diameter. The billeted end of each segment was sealed into a flint or pyrex glass tube 4 cm in length by 8 mm inside diameter such that the billeted end projected into the interior of this glass cup and the straight end projected through the seal. The cavity of the cup surrounding the Pt billet was filled with a solution of 10% $Ag_2(CN)_2$ and placed in the silver cyanide plating bath. The projecting wire served the unit as the cathode. A separate large platinum-iridium wire was immersed in the bath as the anode. Six half cells were prepared at a time, and connected in parallel. Each group was subjected to a total current of 2.0 to 4.0 milliamperes for six to 24 hours, until a uniform coating of silver had been deposited on each billet and shaft inside the glass tubing. After plating, each unit was washed for approximately 12 hours in concentrated ammonia solution, rinsed with 10 to 20 changes of glass distilled water and filled with 0.1 N HCl solution. The six units were then connected in parallel as the anode, with a platinum-iridium wire as the cathode, and placed in a 0.1 N HCl bath for chloridizing. The units were chloridized at a total current of 2.0 to 4.0 mA for about one-hour hour. A uniform "plum" color surface on each billet and shaft, indicated that proper chloridizing had occurred. An input lead of a shielded cable (Belden #8411) was twisted around and soldered to the projecting 10 to 15 mm of wire for each unit. This joint was wrapped with plastic electrical tape. A piece of faradic and magnetic shielding (Mu metal) was wrapped around the glass of the half cell and soldered to

the shield of the cable. An Amphenol #27-1 subminiature connector was attached to the other end of the 3 to 4" shielded cable. The half cells were paired and tested by connecting the cabled ends to the differential amplifier circuitry of the oscilloscope or to the DC leads of a vacuum tube volt meter (Heath Kit V7-A). The billeted input ends of the pair were placed in separate beakers of .3 MKCl connected through a 5 MKCl - 1.5% agar salt bridge placed in 3 MKCl, or placed in the same container of 3 MKCl. Paired electrodes exhibiting more than 1 mV potential difference were discarded.

The half cells were connected (Figure 1) to the head stage of a negative capacitance electrometer-preamplifier (Argonaut LRA-043) connected to its preamplifier and power supply (Argonaut LPS-043). Published specifications for this instrument are the following: Input impedance 10^{10} ohms, grid current less than 10^{-13} amperes, amplification of input signal 10 X. The input cable was further isolated from the external environment by means of a guard shielding surrounding the input cable. This shielding provided through its connection, as the electrometer tube cathode shield, a source for displacement of current that would otherwise have come from the experimental preparation. All instruments were grounded in common to a copper wire connected by a solder joint to a laboratory air line.

Electrophysiological Measurements

Cellular membrane potentials and resistances were measured for untreated, cultured, regressed goldfish testis tissue and for the goldfish back muscle strips. Either the saline extracted or the "purified"

extract of the carp pituitaries were added to the cultured gonadal tissue and the back muscle tissue.

After the paraffin sealing a coverglass to its deepwell slide was scraped away, the coverglass was lifted from the deepwell slide and placed into the measuring chamber. The plasma clot and tissue explants faced upwards. Simms' solution pH 7.4, 20 to 22° C. was added to the measuring chamber to cover the explants. The muscle strips, attached to the 40 mm glass rod, were held and studied in this same chamber. The rod fitted exactly crossways in the chamber.

All tissues were brought into the field at low power focus (about 70 X). The capillary microelectrode was brought into the low power field of focus by adjustment of the coarse controls of the brass support plate. The pneumatic controls of the micromanipulator moved the capillary microelectrode tip into or out of the desired part of the tissue under 200 X magnification. Measurements were taken at varying time intervals from 12 to 64 minutes before the experimental medium was removed. This removal was executed by withdrawing the entire medium volume from the chamber and replacing it with three changes of Simms' solution at the same temperature and pH. After the rinse, the membrane resistances and potentials were monitored for varying lengths of time. Occasionally other substances were added after this rinse to ascertain their effects on the membrane parameters (Tables 6 and 7).

Measurements of resting potential and membrane resistance were first recorded in the control solution (Simms' solution pH 7.4) in the experimental chamber. Either crude pituitary extract or the purified extract was added to the solution in the amount of 0.01 ml per ml of

chamber medium. The solutions in the chamber were mixed by drawing the bathing medium into and out of a 10 ml hypodermic syringe three times through a 3" 17 gauge needle. Membrane potentials and resistances were monitored as soon as possible after this mixing.

Photographs were made of the oscilloscope tracings representing the electrical results of the movement of the capillary microelectrode into and out of the tissues. Two criteria were used for the selection of the oscilloscope tracings as a membrane potential, the magnitude of the vertical deflection from the horizontal base line and slope steepness of the base line deflection as the microelectrode was withdrawn.

The cellular membrane impedence (resistance) was determined in two steps. A square wave current of constant magnitude was applied through the capillary microelectrode and its half cell while the electrode tip was in the measuring chamber medium. The resulting voltage deflection was compared to the voltage deflection obtained when the same magnitude current square wave was applied through the electrode while its tip was inside (Moore and Cole, 1963). The cellular membrane resistance value was calculated from the comparable oscilloscope tracing photographs. A sample calculation is shown in Plate V.

RESULTS

Plate I presents a series of photomicrographs of explants, taken at different time intervals, from a typical culture session. Two fundamentally different cellular forms were observed. Fibroblastic and epithelioid cells (Puck, et al., 1957) can be seen in the periphery of each explant at zero hours in culture. These cells continued to increase in number around each explant so that by 72 to 80 hours, the cells formed a noticeable layer. A few elongated, fusiform to clublike, fibroblastic cells extended into these peripheral layers from the explant. By 85 to 95 hours, the layers around each explant consisted of sheets of mixed epithelioid and fibroblastic cells. The cell sheets continued to extend from each explant within the clot probably along the fibers of the clot matrix (Weiss, 1959), and on the surface of the coverglass so that by 280 to 312 hours, the sheets had become confluent. Very often the growth of the cell layers was concomitant with dissolution of the plasma clot. Explants exhibiting such dissolution occasionally detached from the coverglass; or sometimes the confluent layers and explants moved toward each other resulting in a single tissue mass. When the clots dissolve, the explants usually detached from the coverglass. Since both photographic and electrophysiologic studies were dependent on the attachments of the explants, the slides with detached tissue masses were discarded. Sometimes the cell sheets ceased extension from their

respective explants. The cells nearest the explant began vacuolating and this process continued from 350 to 630 hours in culture, finally affecting the most peripheral cells of the sheet.

Measurements of the diameters of the epitheliod and maximum diameter and length of the fibroblastic cell were made on photomicrographs to ascertain whether changes in cell size were significant ($P \leq 0.05$) with respect to time. When the means of the epithelioid cell diameters and the fibroglastic cell lengths for each time interval are plotted as a function of time in culture, a series of time dependent variations in cell sizes becomes evident. When interconnecting lines are drawn from mean to mean, there appears to be a cyclic pattern to the cell size changes. However, the variation in these means is not statistically significant ($P \leq 0.05$). When testis tissue was grown in the presence of crude pituitary extract, the frequency of the cycles changed. Though these variations also are not statistically significant, there was an increase in the number of cycles per unit of time when the extract (200, 400, 800 micrograms original ADWP per ml of culture medium) was added (Plate II and III).

A comparison of the effects of the chronically present crude pituitary extract on the sizes of epithelioid and fibroblastic cells at 97 hours indicates there was a nearly two-fold increase in the mean length of the fibroblastic cells. When these cells were cultured in media containing 400 and 800 micrograms per ml of ADWP crude pituitary extract per ml (Table 1), at 123 to 126 hours, the mean diameters of the fibroblastic cells stay about the same. The mean diameter of untreated epithelioid cells in these culture sessions was 13.1 micra (Table 3).

When cultures were grown in 400 to 800 micrograms ADWP extract per ml, this value increased to 13.9 to 14.8 micra. These values were not significantly different from the control values. The mean value of 13.1 micra diameter was used to calculate the surface area of a "normal" cell.

When the total number of "in focus" cells at each time interval in the treated and the untreated cultures of 6/12/67 were plotted against time, there was no indication of a consistent pattern established between the dosage of ADWP crude extract administered and the form of the growth curve (Figure 6). There is also no consistent pattern to the total area under each growth curve. Inspections of both the early and late photographs, as well as the shapes of the growth curves indicate that the extract, especially in the higher dosages, stimulates an increase in the rate of cell proliferation during the 80-160 hour interval. In all series of photographs there is an increase in cell number with time.

Growth of Kidney Tissue from Gonadially Regressed Goldfish

As in the testis tissue, epithelioid cells were evident around the explants in the cultures of goldfish kidney tissue at time zero in culture. There is some evidence of time related cyclic variation in the mean diameters in these cells (Figure 7). This variation in size is not significantly different from one time interval to another, however ($P \leq 0.05$).

Fibroblastic cells first appear in these cultures at about 123 hours. The mean lengths of these cells for the various time intervals

are two to three times as long as the fibroblastic cells in untreated testis cultures of the same age.

In the kidney explants cultured in 800 ug ADWP extract per ml of medium, the mean diameter and cyclic distribution of size for the round cells followed a pattern similar to that of untreated explants (Figure 8). Fibroblastic cells are evident around the treated explants 12 hours after culture. These cells increased in number so that by 97 hours the cells were distributed in sheets peripheral to each explant. The limited depth of field of the phase optics and the poor resolution of the plasma membranes of cells in confluent sheets resulted in poorly resolved photographs, hence few "in focus" cells were obtained after 97 hours in the treated cultures. When the lengths of the resolved fibroblastic cells in the treated culture prior to 97 hours were compared with the lengths of cells in untreated cultures after 123 hours, the treated cells were one fifth the mean length of the untreated cells.

The population of epithelioid cells in the untreated kidney cultures has the form of a typical growth curve with a maximum at about 85 hours in culture and declining thereafter (Figure 9). The fibroblastic cells exhibit the early phases of a typical growth form. The increase in fibroblastic cells is correlated closely with the decline in epithelioid cells, although the fibroblastic cells could have masked the presence of a number of epithelioid cells.

The treated (800 micrograms per ml medium) kidney epithelioid cells do not have a typical growth curve. The data for the growth curve of the treated fibroblastic cells is derived from small cell numbers since

these grow into unresolvable sheets very early. The indications are that the extract treatment caused an increased number of fibroblastic cells. The variation in the epithelioid cell number may also be a result of a masking effect by fibroblastic cells.

Bioelectric Results

When the CME was pushed into the peripheral cell layer around an explant in the plasma clot, or into the explant (Plate IV), cell penetration was indicated on the cathode ray oscilloscope by a sharp upward deflection from the base line. Its rise time depended upon the speed of movement of the CME into the cells, (Plate IV, 3, B-2 and 3, C-1-3). These deflections represent a resting potential of a negative 5 mV. Frequently the rise and fall times of the resting potential were both very short (Plate IV, 3, C-1 and 3, C-2). This was an indication that the CME had penetrated and passed through a particular cell or had ruptured the plasma membrane. A maintained potential provided an indication that the cell membrane had sealed around the CME (Plate IV, 3, B-2).

The mean resting potential of the cultured, untreated gonadal tissue ranged from 2.0 mV to 59 mV, depending on the age of the cultures and the relation of the probe to the explants within the plasma clot. The few isolated peripheral cells that could be measured generally had lower membrane potentials than cells in explants of the same age. The values in Table 4 are pooled from peripheral cells and explant cells. The specific membrane resistance for cultured testis tissue cells varied from 44 to 615 ohms$\cdot$cm^2, and were generally dependent on the age of the culture. They do not appear directly related to the resting potential values.

The untreated goldfish back muscle cells had a grand mean membrane potential of 83.2 ± 12.8* mV with a range of means of 53.8 to 178 mV for the several replicates. Input membrane resistances for the muscle cells had a grand mean of 259 ± 46 M ohms with a range of the means from 60 to680 M ohms for the several replicates (Table 5).

Resting Potentials and Membrane Resistances of Variously Treated and Cultured Testis Tissue and Back Muscle Cells

There is a slight decrease in the resting potentials of the gonadal tissue, cultured 5/3/67 and incubated for 144 hours, in response to sudden administrations of 34 micrograms of purified pituitary extract per ml of measuring chamber medium (Table 6, Figure 10). There was no discernible response to a 10 x increase in potassium ion concentration, or to the addition of 1 milligram per ml of sodium cyanide. No trend in the membrane potential changes was observed with the addition of 200 micrograms per ml of original crude pituitary extract per ml (Figure 11).

The input cell membrane resistance was measured by injecting a square wave of current of constant frequency and amplitude through the intracellularly positioned CME (Plate IV, 3, D-3). A stepwise, treppe-like, increase in the current driving voltage was often seen as the ionic current was injected into a cell (Plate IV, 3, D-2). A series of deflections and artifacts associated with switching the current on within the cell often occurred also (Plate IV, 3, D-1-3). The current was turned off and the CME was removed from the cell. After the potential deflection,

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*1.96 SE.

associated with the membrane potential, had returned to the DC basal line, a second identical series of square waves were injected through the CME while its tip was in the measuring chamber medium (Plate IV, 3, E-3). The switching artifacts (Plate IV, 3, 1, and 4), were still present; but the steplike initial voltage increase at the beginning of the current injection was absent. The magnitude of the voltage driving the injected current decreased from an intracellular value of -28 mV (5.6 cm) to a value of -8 mV in the medium (1.6 cm). A calculation was made to relate the input resistance values to the resistance of a unit area of cell membrane, the specific membrane resistance.

In order to determine the specific membrane resistance of these cells, the input resistance values were multiplied by the average cell surface area determined from the mean epithelioid cell diameter of 13.1 micra (540 micra^2 = 5.4 x 10^{-6} cm^2) to obtain the specific membrane resistance values expressed in ohms·cm^2. Since the back muscle cell sizes were not measured, input resistance values alone were used for comparing the responses of the goldfish back muscle cells. The means of the specific membrane resistance in the treated cultures of 8/22/67 indicate no definite change with the addition of 200 micrograms per ml of crude pituitary extract per ml (Table 8, Figure 12). Both the potentials and resistances show slight decreases within the first five minutes after addition of the extract to the measuring chamber.

A significant ($P \leq 0.05$) 20% decrease in the resting potentials of the goldfish back muscle cells studied on 5/15/67 occurred within 15 minutes after the addition of 34 micrograms per ml of the purified hormonal preparation. The mean potential decline was from a -57 to a -47 mV

(Table 9, Figure 13). The addition of 1 milligram per ml of sodium cyanide caused a marked, significant 300% decrease from a negative 41 to a negative 10 mV mean potential. Two rinses of the muscle strip with Simms' solution produced a significant 200% increase in membrane potential.

The addition of 200 micrograms per ml of crude pituitary extract caused a significant 500% decrease in membrane potentials from 71 mV to 12 mV in 4 minutes in muscle cells studied on 7/6/67 (Table 10, Figure 14), and a significant 30% decrease from 178 to 124 mV in 10 minutes on those of 8/21/67 (Table 11, Figures 15 and 16). The decreases in membrane potential of Figures 30 and 31 (Table 12, Figure 17), while not significant at the 0.05 level, support trends of the date of 5/15/67, 7/6/67, and 8/21/67. The purified hormone and the crude pituitary extract do significantly lower the membrane potentials of cells in goldfish back muscle strips.

DISCUSSION

The cellular migration and growth pattern seen in the cultures of testis and kidney explants is typical for adult, differentiated tissues. After a 72 to 96 hour latent period some epithelioid and fibroblastic cells began to migrate from the edge of each explant. There is also a rough correlation between the age of the donor tissue and its premigration latent period in culture. These findings are similar to those reported by Doljanski, et al (1940) who cultured chicken cardiac tissues varying in age from 24 hours in incubation to two years posthatching. These same conclusions are expressed in a literature review by Abercrombie (1965). The onset of cellular migration may be due to a number of factors including the composition of the culture medium, the amount of cell free space available, and the amount of dissociation of the peripherally located cells from their normal cell to cell contact relationships (Abercrombie, 1965). The eventual filling in of the free space around each explant and the formation of a layer of epithelioid and fibroblastic cells can be explained by cell migration and/or cellular division.

Cellular division usually results in a halving of the cytoplasmic volume of the mother cell (Firket, 1965). In the time intervals studied, epithelioid cells could be found whose diameters were one half those of the largest cells in the culture, a range of from 9 to 20 micra. The

same relation was observed for the length ranges on the fibroblastic cells. The cyclic variation occurring in the mean sizes of both types of cells in these cultures can be explained, if during the minima of the cycles more "half size" cells were present and during the maxima more "full size" cells were present of each type. The counts of cells indicate that this is the case. This variation in numbers of the different sized cells can be explained, if the cells were approaching synchrony in their division patterns.

The total number of "in focus" cells as a function of time, in both the gonad and kidney cultures would be expected to generate a logarithmic curve (Engleberg, 1961), if the cell division times in the cultures were randomly distributed. The weakness of the correspondence between the experimental curves of numbers as a function of time and logarithmic numbers as a function of time provides an indication of deviation from randomness of cell division and a tendency towards a synchronous division in these cultures. This intermediate form of division has been called "para-synchronous" division (ibid.). The hypothesis, that the tendency towards synchrony in the divisions exists in these cultures, is supported by the synchrony of cell size changes observed.

Cultures chronically treated with a Simms' saline extract of acetone dried pituitaries exhibited shortened time intervals between the cycles mentioned above. There were more cycles per time interval and the number of cycles during a given time interval was roughly proportional to the dosage of extract administered. Within the above stated limitations on interpretation of the cycling event, this means that the hormone

probably increased the rate of cell division in the cultured cells. This tentative finding should be further tested with an experiment designed to provide a more rigorous count of all of the cells and measure of all cell sizes during various times in the cyclic pattern.

The presence of the factor increased the mean lengths of fibroblastic cells and the mean diameters of epithelioid cells of testis cultures. In chronically treated kidney cultures, fibrocytes appeared around the explants' periphery as early as 12 hours after culturing. In untreated cultures the fibroblastic cells did not appear until 97 hours after culturing. At 97 hours the chronically treated cultures of fibroblastic cells had formed confluent sheets on the surface of the cover glass and individual cell sizes could not be measured. In the kidney cultures, when the crude hormonal preparation was chronically present, the cells migrated, divided earlier and faster, and became somewhat larger than control cells.

This effect, the apparent cell division stimulation effect, may be the result of the addition to the culture medium of solutes from the extracted pituitaries. Clemens, et al. (1964) indicated that from 31% to 82% of the original acetone dried carp pituitaries would dissolve in water. If 82% is assumed to be the percent of Simms' saline extracted solutes in this study, then the amount of soluble pituitary material added to the cultures (0 to 656 microgram/ml) varied from 0% in the controls to a maximum of 3% of the total soluble protein in the culture medium--protein was present in the medium at a concentration of 10 mg per ml. The additional material added in the extract is also far smaller than the total variation in the medium constituents due to possible systematic

errors. That a pituitary solute has stimulated an increase in cell proliferation rate, is supported by the in vivo evidence of Clemens and Reed (1967b), who reported an increased rate of proliferation of spermatogonial cells in the gonadially regressed goldfish by daily intraperitoneal injections of a distilled water extract of carp pituitary. This effect was ascribed to a pituitary gonadotrophin. The carp pituitary has been shown to contain many different protein fractions, including a gonadotrophin (Bogenshutz and Clemens, 1967, and Pickford, 1959). The stimulation of cell division may then be due to a gonadotrophin, the "hydration factor," or some other hitherto unstudied pituitary component. The hypothesis that the stimulation of cell division is due to a gonadotrophin is supported by the evidence of Moszkowska (1956) who showed that the formation of seminiferous tubules was considerably promoted in embryonic chick testis grown in contact with homoplastic pituitary tissue in organ culture.

Another explanation of the effect of the extract may be that its active component increases the permeability of the plasma membrane to nutritional substances in the medium. Insulin seems to act in this way to increase the plasma membrane permeability to glucose and amino acids (Park, et al, 1959). Insulin also stimulates marked increases in cell division rate and tissue growth in tissue culture (Lasnitzki, 1965).

The mean of the membrane potentials for cells of cultured regressed goldfish testis tissue was different for each experiment, the replicates varying from -2 to -60 mV. Individual values range from -1 to -120 mV. At least two conditions may have played a role in the variability: the age of the culture and the physiological state of the donor animal.

There was a general decrease in the bioelectric potential of the cells with increasing age of the culture. Primary cell culture cells undergo a number of deleterious changes. The most pertinent to this study is the increasing cell membrane permeability.

A second feature that affects the viability of explant cultures is the physiological state of the animal at the time tissue was obtained for culture (Willmer, 1965). Since the fish holding tanks were in rooms with windows, lighting conditions were not constant. Seasonal changes in day length have been shown to play a considerable part in the determination of the physical state of the gonad (Harrington, 1957, Bogenshutz and Clemens, 1967, and Clemens and Reed, 1967a). As a consequence these gonadially regressed fish probably varied physiologically from season to season.

The potential values obtained for the gonadal cells are comparable to those reported in the literature for other non-excitable tissues in culture. They range from -15 to -50 mV (Wardell, 1966, Douglas, et al., 1967, Whittembury, et al., 1961, Hild, et al., 1965). Non-cultured, non-excitable tissues, measured in situ, have values from -70 to -90 mV (Giebisch, 1968, Kuffler, et al., 1966). Recently potential values for cultures of confluent layers of human fibroblasts have been reported to have values ranging from -70 to -75 mV (Swift and Tadaro, 1968). The in situ potential difference value for non-excitable tissues most closely resembles the larger mean values of the cultured gonadal cells of the goldfish (Table 4). The fish culture media might be altered and give rise to much higher, more stable potential values. Such alterations in media are empirical and not within the scope of this study. Very little

is known about the functional changes that occur in long term primary cultures or permenent cell lines that enable them to survive the tissue culture environment. The change from primary to permanent cell line has been known to result from chromosomal alterations (Willmer, 1965). Normal functioning cells could fail to multiply with a consequence selection of undifferentiated cells or there may actually be loss of specialized function (Eagle and Levintow, 1965). Lemkuhl and Sperelakis (1963) were able to mimic the in vivo potentials recorded in chick heart cells in primary cultures of these cells with several media modifications; lowering the pH, increasing the Ca^{++} concentration, and omitting phenol red. These media modifications imply that the cell membrane of the cultured heart cells could have been somewhat modified by the normal culture conditions.

Addition of the electrophoretically purified pituitary fraction containing the "hydration factor" to the bioelectric chamber medium lowered the mean membrane potentials of the cells of the testis tissue culture 43% from the control values in 6½ minutes. The crude extract had no effect on the size of the gonadal cells' membrane potentials. The displayed, lowered, potential difference of gonadal cells in the presence of the purified fraction may mean that increase in the internal Na^{+} concentration has occurred, resulting in a decrease in the ratio of intracellular to extracellular K^{+}. This was the original hypothesis of action of the hydration factor.

The grand mean potential of -83.2 mV of the fish muscle cells is very similar to those reported for other vertebrate skeletal muscle cells and electrically excitable cells. Nastuk and Hodgkin (1950) reported

mean values of -77 mV for frog sartorius muscle *in vitro*. Goodgold and Eberstein (1966) reported values of -70 to -75 mV for human muscle cells studied *in situ*. Coombs, *et al* (1955a) have reported *in situ* values of about -70 mV for the resting membrane potential in the cat spinal motoneurone.

Administration of either a purified pituitary fraction or a saline extract to the goldfish back muscle strips caused a significant lowering (average 47%) of the cellular membrane potentials within ten minutes.

The specific membrane resistance of 15 to 644 ohms$\cdot$cm^2 obtained from the cells of the testis tissue cultures are higher than those reported for other non-excitable tissues. Hild, *et al* (1965) report values of 10 to 30 ohms$\cdot$cm^2 for cultured ependymal cells. This may mean that the use of the average diameter of the "in focus" epithelioid cell in this study for calculation of specific membrane resistance provided too small a resistance estimate.

Such estimates were necessary because in phase microscopy there is a halo at the edge of an object. As a result the position of the CME tip was not discernable in the layers of cells around the explant or in the explant itself. Consequently, the exact cell penetrated could not be seen optically or photographed. Potential deflections on the oscilloscope screen, however, indicated when the CME had penetrated a cell.

A second likely contribution to the variability of the gonadal cell specific resistance measurements follows from differences in the degree of cellular contact in the cultures. Cultured cells in their early migration-growth stages from an explant are rarely arranged with their

plasma membranes in apposition (Abercrombie, 1965). Thus in the early culture stages there are large fluid spaces between the cells. These spaces would give these cells lower resistance values than when the cells are in confluent sheets with less intracellular space.

The specific membrane resistance values obtained for the treated gonadal cell cultures were not consistently different from controls. The degree of variability in the control values present probably mask indications of a response. In contrast, the resistance data for the in vitro isolated back muscle has less variation and in every case where administered, the crude pituitary extract lowered the mean input resistance. The depression occurred within 10 minutes after hormonal administration and the resistance was decreased 20 to 90%. The effect could not be completely reversed by washing the tissue.

The lowering of both the membrane potential in the cultured gonadal tissue and in the muscle tissue and the ion current lowering of the input resistance in the muscle tissue is due, at least in part, to some action of a common component of the purified and the crude pituitary extract. This component could have increased the plasma membrane permeability of cells of both tissues; thus new transmembrane Na^+ and K^+ ratios would be established resulting in the lowered membrane potentials and membrane resistances.

The hypothesis derived from the data of Clemens and Grant (1964 and 1965) was that the "hydration factor" altered the permeability properties of the testis lobular epithelium. This would permit an inward flux of ions into the epithelial cells. The cells would then transport the intracellularly increased Na^+ at an increased rate into the lobular

lumen. A passive, osmotic movement of water would follow the ion movement into the lumen thus hydrating the testis lobular lumina. The deceasing membrane potentials associated with hormonal treatments indicate an increased rate of Na^+ movement into the cells. The membrane resistance decrease of hormonally treated fresh in vitro isolated muscle tissue indicate that an increase in the ease with which K^+ passes through the plasma membrane has occurred. The implication is a general permeability increase of a variety of tissue to Na^+ and K^+.

The effect of the "hydration factor" on the water content of the testis of the adult male prespawning goldfish may be a reflection of a general cellular response, since at the lowest in vivo administered dosages, (probably close to the physiological ranges) the factor promoted hydration in all of the tissues measured (Clemens and Grant, 1964 and 1965). The permeability response could be of even broader basis than to Na^+ and K^+, if in fact it is the "hydration factor" promoting the in vivo spermatogenesis and the possible in vivo increased rate of cellular division. It could be that the factor promotes an increase in general cellular permeability to solutes of at least amino acid or glucose size.

SUMMARY

A technique is described for short term culturing of the testis and kidney tissues of gonadally regressed, sexually mature goldfish. It involves the placing of explants of these tissues into chick embryo extract--chick plasma clots on cover glasses and incubating them at 20° C in a medium composed of 10% tissue culture medium #199, 20% agamma horse serum and 70% Simms' saline solution. Antibiotics; penicillin, streptomycin, and mycostatin, were present in concentrations normally used in tissue cultures.

Normal growth patterns of the cultures were described from counts and size measurements of "in focus" cells from phase contrast photomicrographs. There is a general increase in cell numbers resembling a classical growth curve. There is a cyclic pattern of change in the sizes of epithelioid and fibroblastic cells. The cycles were interpreted as being related to the cell division intervals. A factor present in a Simms' saline extract of the carp Cyprinus carpio pituitary increases the number of cycles per time interval.

Two electrophysiological parameters of testis culture cells were measured and compared to those of freshly isolated muscle cells. Cellular membrane potential was measured by penetrating the cells with capillary microelectrodes and recording the electrical potential difference of the inside versus outside. Cellular membrane resistance was

obtained electrically by injecting a stream of ions into the cell from the tip of the CME and measuring the voltage necessary to drive the ionic current through the cell's total membrane surface. The mean membrane potentials for the cultured testis cells varied from -2 to -60 millivolts. The specific membrane resistance varied from 15 to 644 ohm$\cdot$cm^2. The saline pituitary extract had no describable effect on the cell membrane potential, whereas the electrophoretic fraction "hydration factor" noticeably lowered the membrane potential of the culture cells.

The comparison tissue, goldfish back muscle strips, had mean membrane potentials of $-83.2 \pm 12.2^*$ millivolts. Both the crude pituitary extract and the "hydration factor" significantly lowered the membrane potentials and membrane resistances of the muscle cells.

---

*1.96 SE.

PLATE I

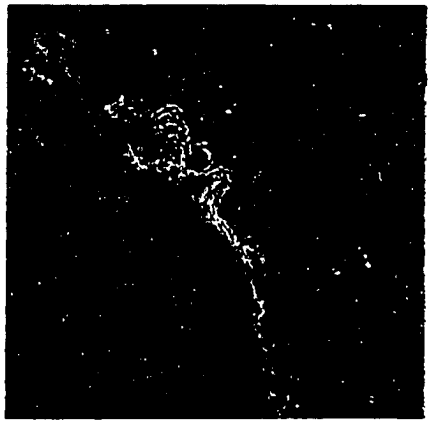
Phase contrast photomicrographs of the cellular outgrowth around goldfish regressed testis tissue explants cultured in normal medium.

Figure	Hours in Culture
1	12
2	24
3	48
4	96
5	159
6	280

PLATE I



1



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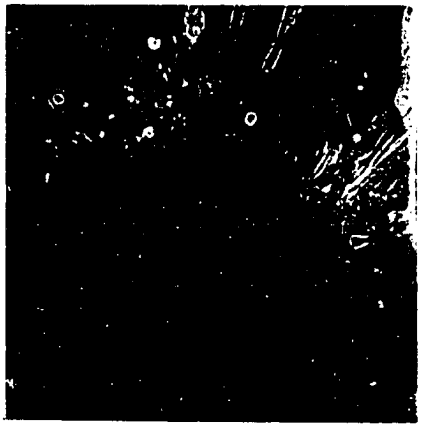

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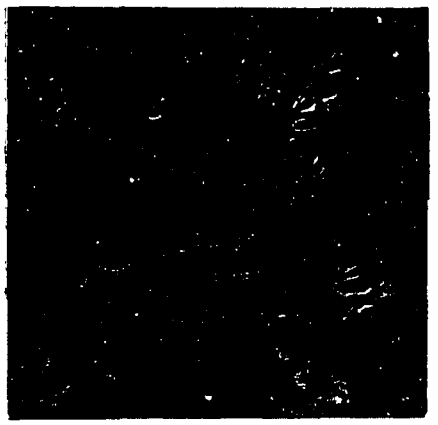
6

PLATE II

Phase contrast photomicrographs of the cellular outgrowth around testis and kidney tissue explants from gonadially regressed foldfish cultured for 97 hours in a medium containing various amounts of a saline carp, Cyprinus carpio, pituitary extract. The dosage is expressed in terms of original acetone dried weight of pituitary extracted (ADWP).

Figure	Tissue Type	Amount of Extract Added/ml of Culture Medium (micrograms)
1	Testis	0
2	Testis	200
3	Testis	400
4	Testis	800
5	Kidney	0
6	Kidney	800

PLATE II



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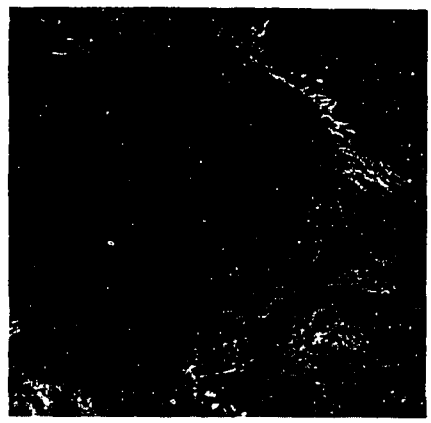

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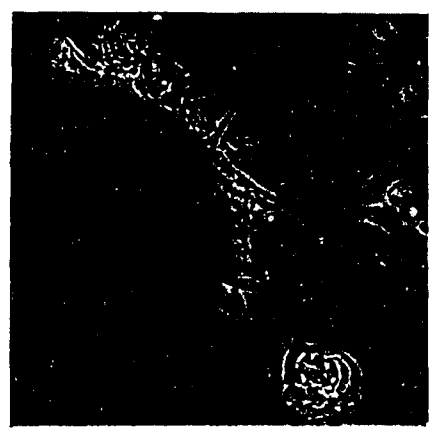

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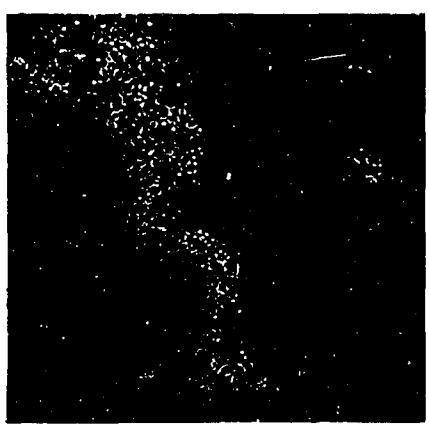

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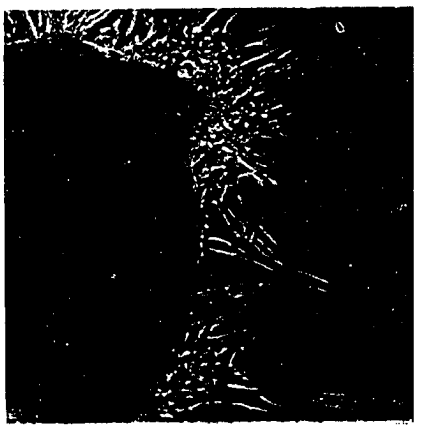
6

PLATE III

Phase contrast photomicrographs of the outgrowth around testis and kidney tissue explants from gonadially regressed goldfish cultured for 123 to 126 hours in a medium containing various amounts of a saline carp, Cyprinus carpio pituitary extract. The dosage is expressed in terms of original acetone dried weight of pituitary extracted (ADWP).

Figure	Tissue Type	Amount of Extract Added/ml of Culture Medium (micrograms)
1	Testis	0
2	Testis	200
3	Testis	400
4	Testis	800
5	Kidney	0
6	Kidney	800

PLATE III



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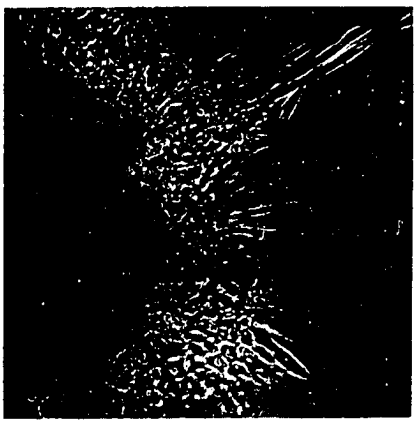

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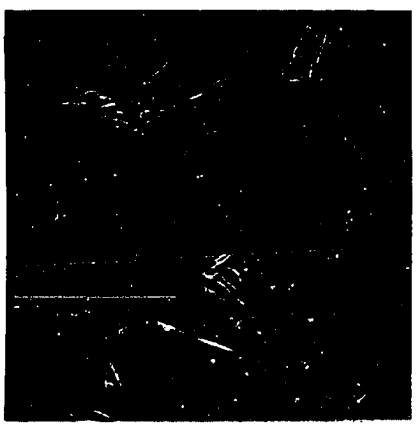

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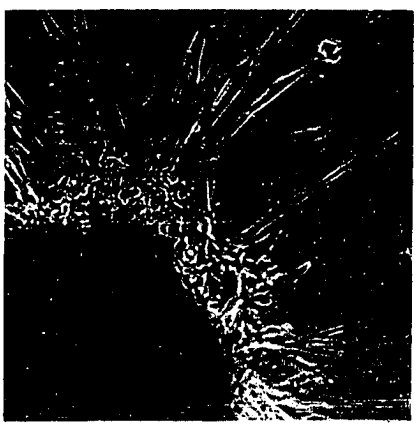

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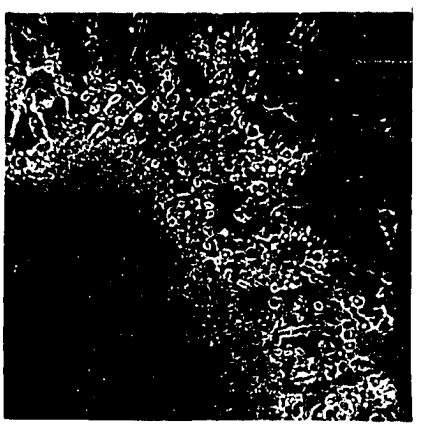

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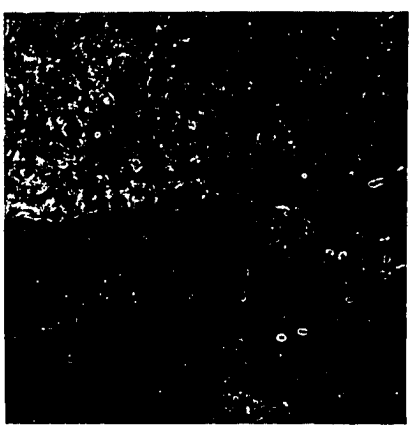

6

PLATE IV

Fig. 1. Regressed goldfish testis tissue, 150 hours in culture, no previous extract treatment, fibroblastic (FB) cells being approached by a capillary microelectrode (CME).

Fig. 2. Regressed goldfish testis tissue, 120 hours in culture, no previous extract treatment, explant (EXP) being approached by CME.

Fig. 3. Oscilloscope camera recording of 8/7/65. The tissue was regressed goldfish testis tissue 504 hours in culture. The cells were heavily vacuolated and the explant seemed to be loosing continuity. The recording was with a continuous motion magazine. The film moved horizontally from left to right at 8 inches/second. A vertical deflection of 1 cm represents 5 mV; the light horizontal lines spaced at regular vertical intervals are 1 cm apart (see B or E).

A-1,3. CME, indifferent electrode (IE), chamber medium in circuit.
A-2. Circuit switched to true ground.
B-1. CME, IE, and chamber medium in circuit.
B-2. CME in cells -5 mV deflection (1 cm) from B-1 voltage. Circuit includes CME, cell, chamber medium, and IE.
C-1-3. CME moved into and out of 3 different cells in an explant resulting in -7mV, -5mV, and -2mV respectively.
D-1. CME was pushed into an explant. A square wave of
D-2. current (100×10^{-12} A) was applied through the CME into a cell, resulting in a 25 mV deflection. The oscillations indicate the square waves of voltage driving the current.
D-3. Total circuit resistance was 250 Mohms.* Circuit includes cell, CME, IE, and chamber medium.
D-4. CME was removed from explant and cell. A current
E-3. of 100×10^{-12} A was applied resulting in a -7 mV deflection, a resistance of 70 Mohm for the circuit including the CME, IE, and the chamber medium.
E-4. Square wave current was turned off.

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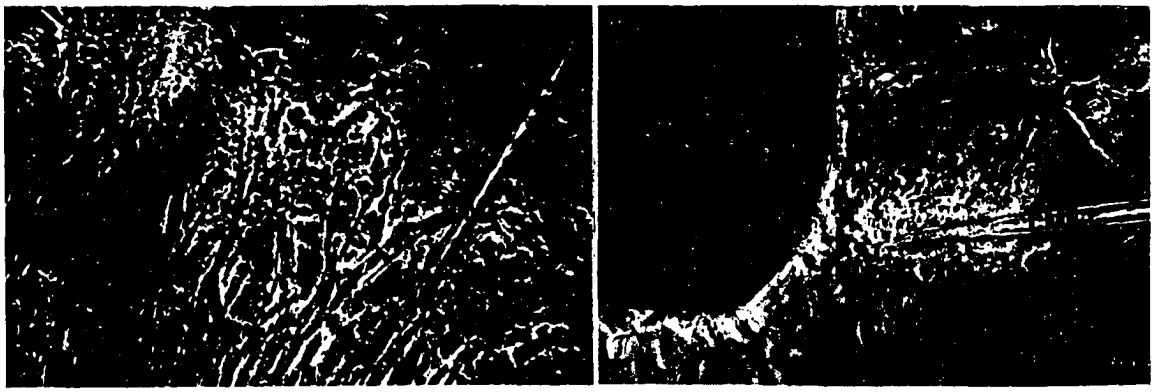
*See Sample Calculation (Plate V).

PLATE IV



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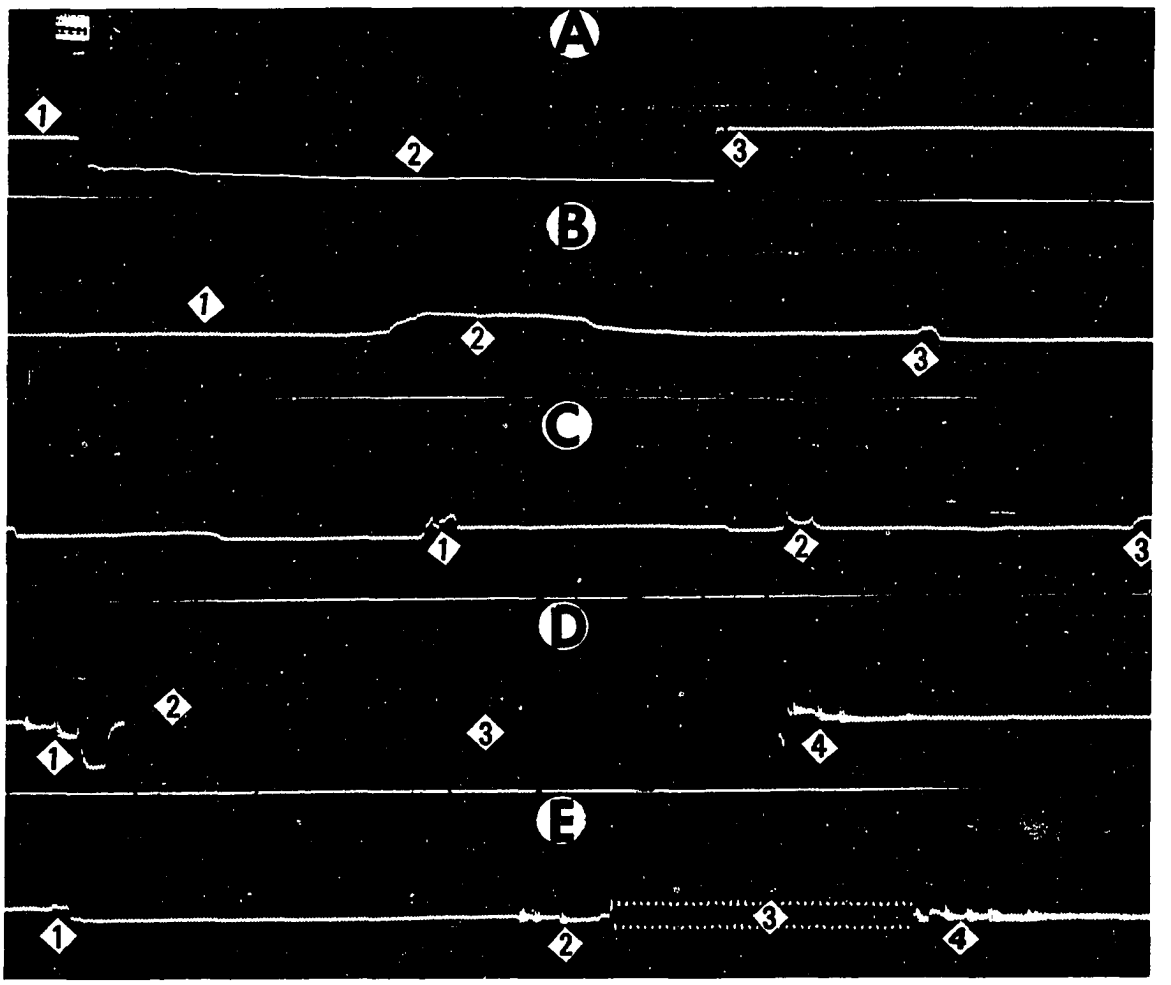


3

PLATE V

A portion of oscilloscope camera recording 0770 of 7/29/67.

The tissue was a strip of goldfish back muscle. Recorded with continuous motion magazine film moved P-S at 1/16" per second. A vertical deflection of 1 cm represents 50 mV. The light horizontal lines spaced at regular vertical intervals are 1 cm apart.

1. Zero potential, CME in bathing medium

2. CME penetrated cell DC membrane potential -85 mV

3. CME withdrawn from the cell

4. CME penetrates, and passes through or ruptures or is very quickly withdrawn from a cell causing a very short rise and fall time for the -75 mV membrane potential.

5. CME penetrated another cell

6. DC membrane potential -110 mV

7. 100 X 10^{-12} A of current was injected through CME into cell resulting in a driving voltage of -245* mV. Note the switching artifact at beginning of current pulse.

8. Current was turned off, the CME was withdrawn from the cell, and the voltage returned to near the base line level.

9. 100 X 10^{-12} A of current was injected through the CME measuring medium, IE circuit resulting in an -80* mV deflection.

---

*Sample Input Membrane Resistance Calculation - $(R_i) = R_1 - R_2$, where R_i = input membrane resistance; R_1 = the summed resistances of the CME, the cell membrane, the medium, and the circuit components; and R_2 = the summed resistances of the CME, the medium, and the circuit components.

$$R_1 = \frac{245 \times 10^{-3}\text{v}}{100 \times 10^{-12}\text{A}} = 2.45 \times 10^9 \text{ ohms}$$

$$R_2 = \frac{80 \times 10^{-3}\text{v}}{100 \times 10^{-12}\text{A}} = 0.8 \times 10^9 \text{ ohms}$$

$R_1 - R_2 = 1.65 \times 10^9$ ohms or

1650 Meg ohms = R_i

PLATE V

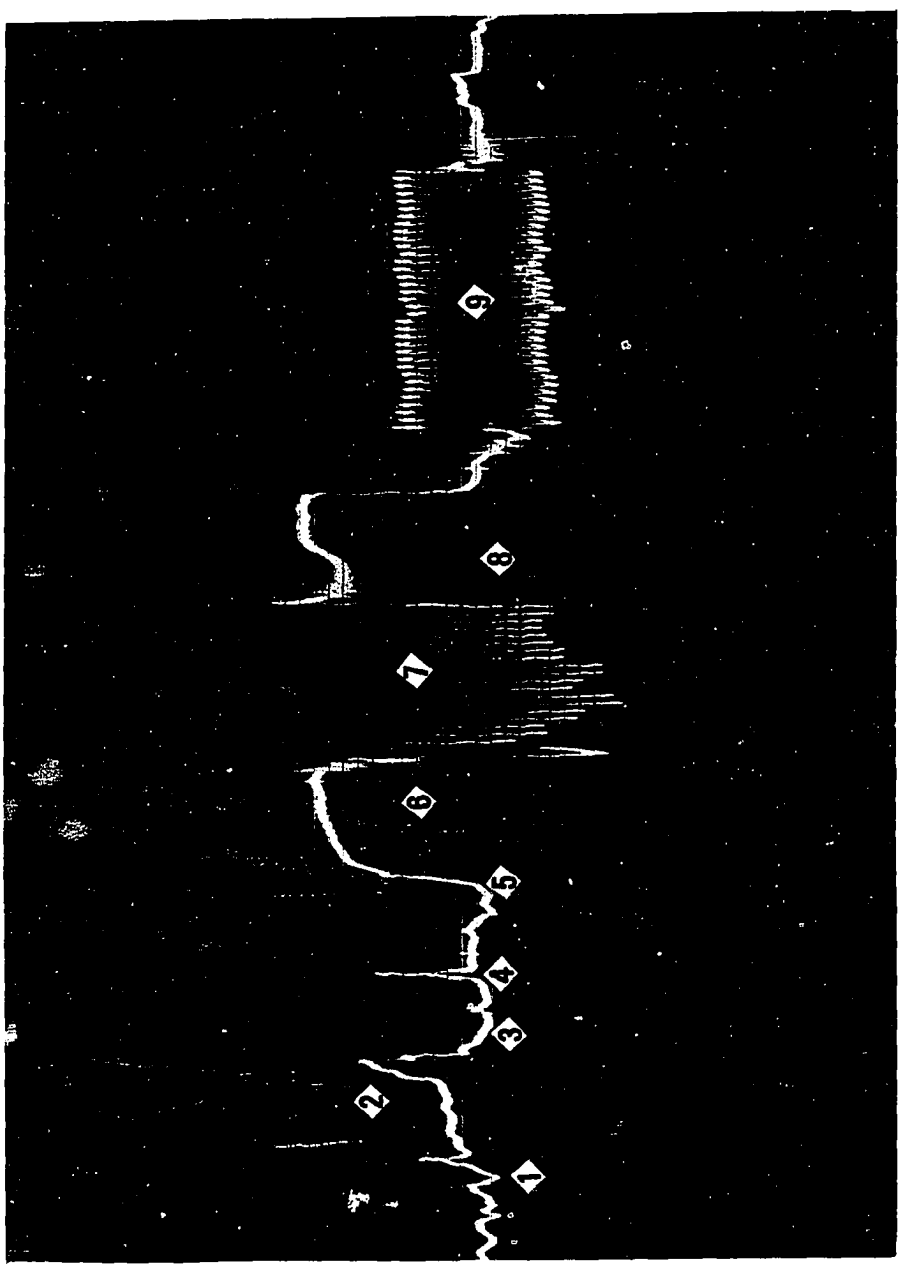


TABLE 1

Summary of the Sizes of the "In Focus" Cells around Explants of Goldfish Tissues Prepared and Maintained in Tissue Culture for 97 Hours

Appendix* Table No.	Amount of Extract (ug/ml)	Number of Epithelioid Cells	Mean Diameter (u)	Range Diameter (u)	Number of Fibroblastoid Cells	Mean Length (u)	Range Length (u)	Mean Width (u)	Range Width (u)
13a	0	12	15.8	10-19	11	52.6	20- 92	11.3	8-18
14a	200	4	11.5	9-16	17	48.6	20-109	9.5	7-18
15a	400	--	--	--	3	76.7	48- 88	11.7	8-16
16a	800	10	12.5	9-18	4	78.3	58-108	11.8	10-17
17b	0	27	10.0	7-18	Sheets of Cells--None in focus				
18b	800	3	18.3	10-25	Sheets of Cells--None in focus				

Note: Saline pituitary extract was added in amounts ranging from 0 to 800 micrograms per ml of culture medium.

*These data are from the Appendix, Tables 13 through 18.

[a]Regressed testis tissue.

[b]Kidney tissue.

TABLE 2

Summary of the Sizes of the "in Focus" Cells around Explants of Goldfish Tissues Prepared 6/12/67 and 6/16/67 and Maintained in Tissue Culture for 123-126 Hours

Appendix* Table No.	Amount of Extract (ug/ml)	Number of Epithelioid Cells	Mean Diameter (u)	Range Diameter (u)	Number of Fibroblastoid Cells	Mean Length (u)	Range Length (u)	Mean Width (u)	Range Width (u)
13	0	12	12.1	7-17	19	69.4	29-132	10.0	7-16
14	200	23	13.9	9-26	3	39.0	28- 49	10.3	10-11
15a	400	22	15.9	10-20	9	94.0	37-166	11.9	8-18
16a	800	6	17.7	11-21	18	123.9	40-216	11.2	7-16
17b	0	28	11.3	8-18	4	98.8	79-119	8.0	7-10
18b	800	26	10.2	6-18	Sheets of Cells--None in focus				

Note: Saline pituitary extract was added in amounts ranging from 0 to 800 micrograms per ml of culture medium.

*These data are from the Appendix, Tables 13 through 18.

[a]Regressed testis tissue.

[b]Kidney tissue.

TABLE 3

Diameter of the Epithelioid "in focus" Cells around the Explants of Goldfish Regressed Testis Tissue Prepared 6/12/67 and 6/16/67* and Maintained in Tissue Culture for Varying Lengths of Time.

Time in Culture** (hours)	Mean Diameter (microns) (D)	Number of Cells (F)	D X F (micra)
0	10	6	60
12	12	9	108
24,25*	13.3	12	159
36	11	3	33
47*,48	10.4	10	104
66	10.5	6	63
73	12.3	10	123
85	16	9	144
97	16	12	192
111	16	5	60
123, 126*	10.1	12	121.5
135	12.5	8	100
146*, 147	15.1	22	332
159	13.5	5	68
168*	17	4	68
		133	1735.5

Mean Diameter for 133 cells = 13.05 micra.

**Data was taken from the Appendix, Table 13.

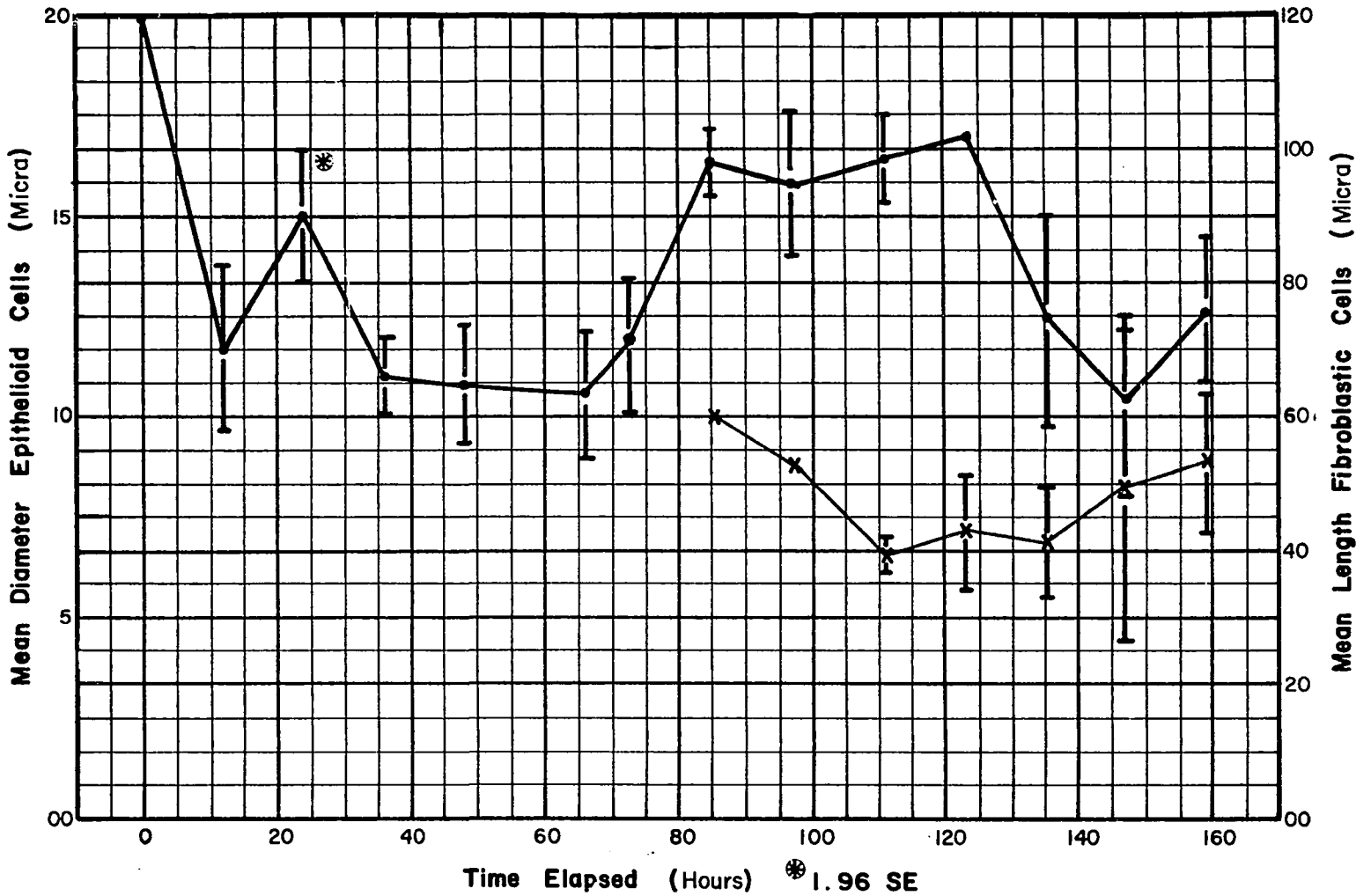


Figure 2

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish regressed testis tissue prepared 6/12/67 o = Epithelioid X = Fibroblastic

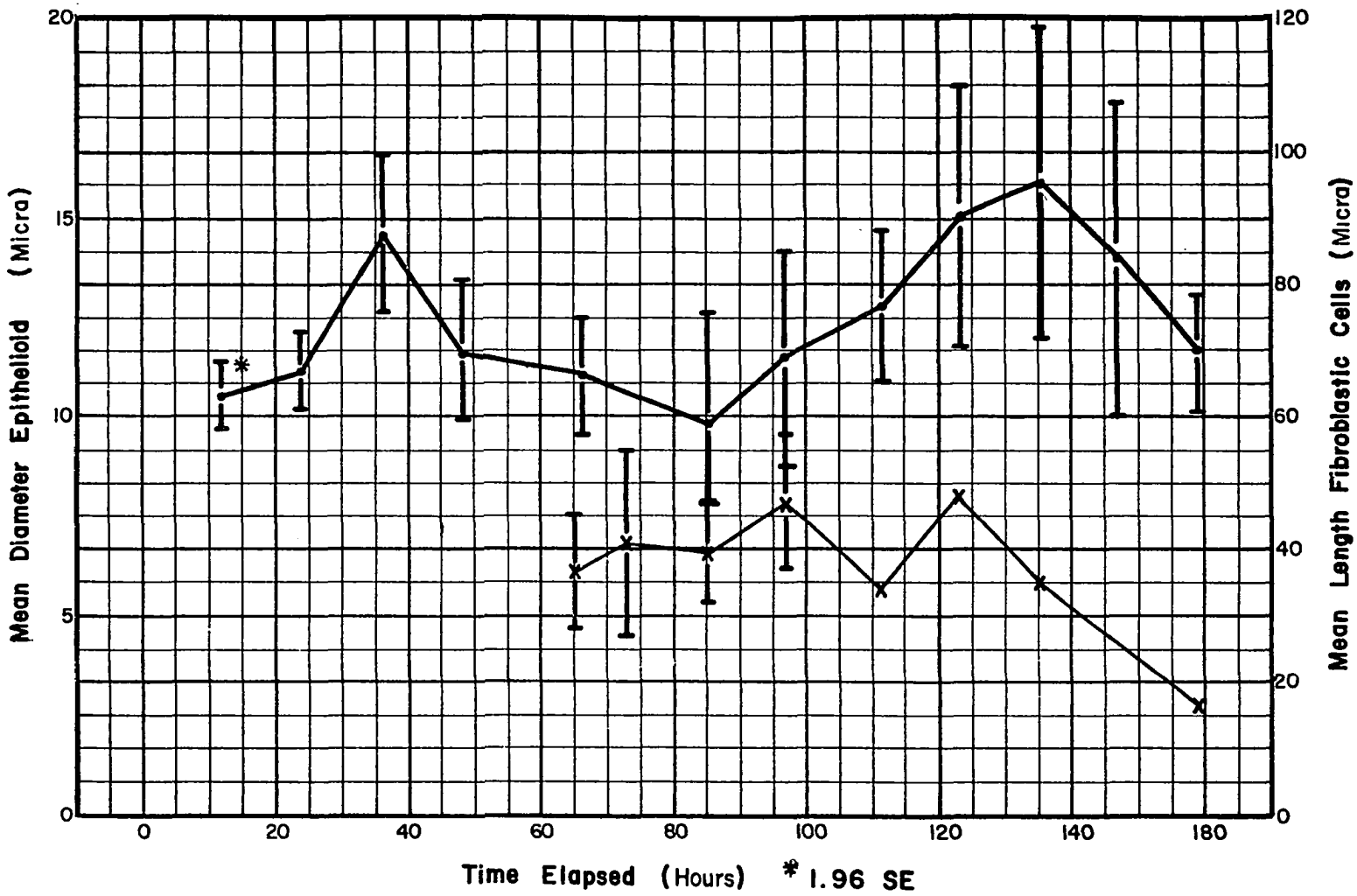


Figure 3

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish regressed testis tissue prepared 6/12/67 and chronically treated with 200 micrograms of saline pituitary extract (ADWP) per ml medium o = Epithelioid
X = Fibroblastic

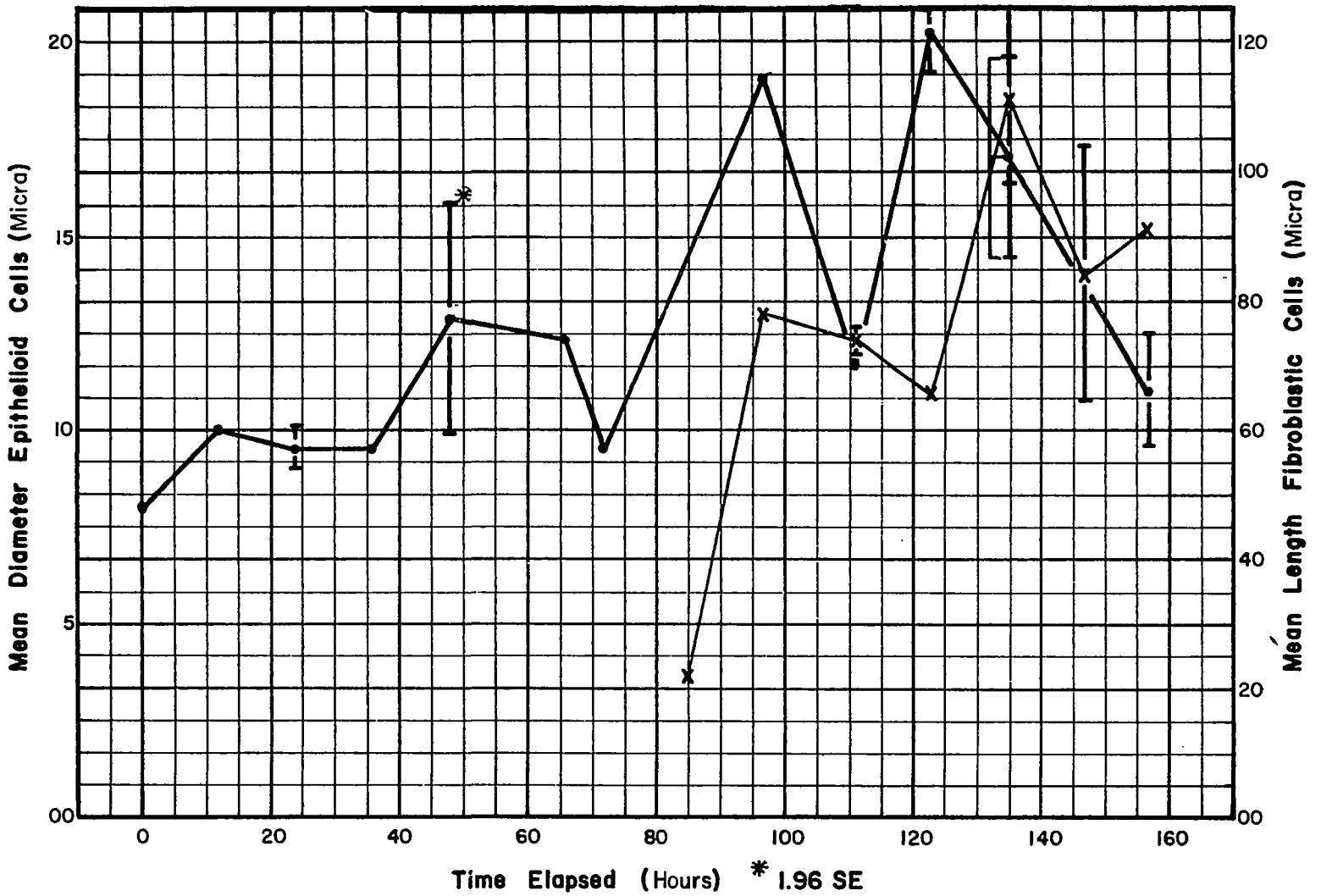


Figure 4

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish regressed testis tissue prepared 6/12/67 and chronically treated with 400 micrograms of saline pituitary extract (ADWP) per ml medium o = Epithelioid
X = Fibroblastic

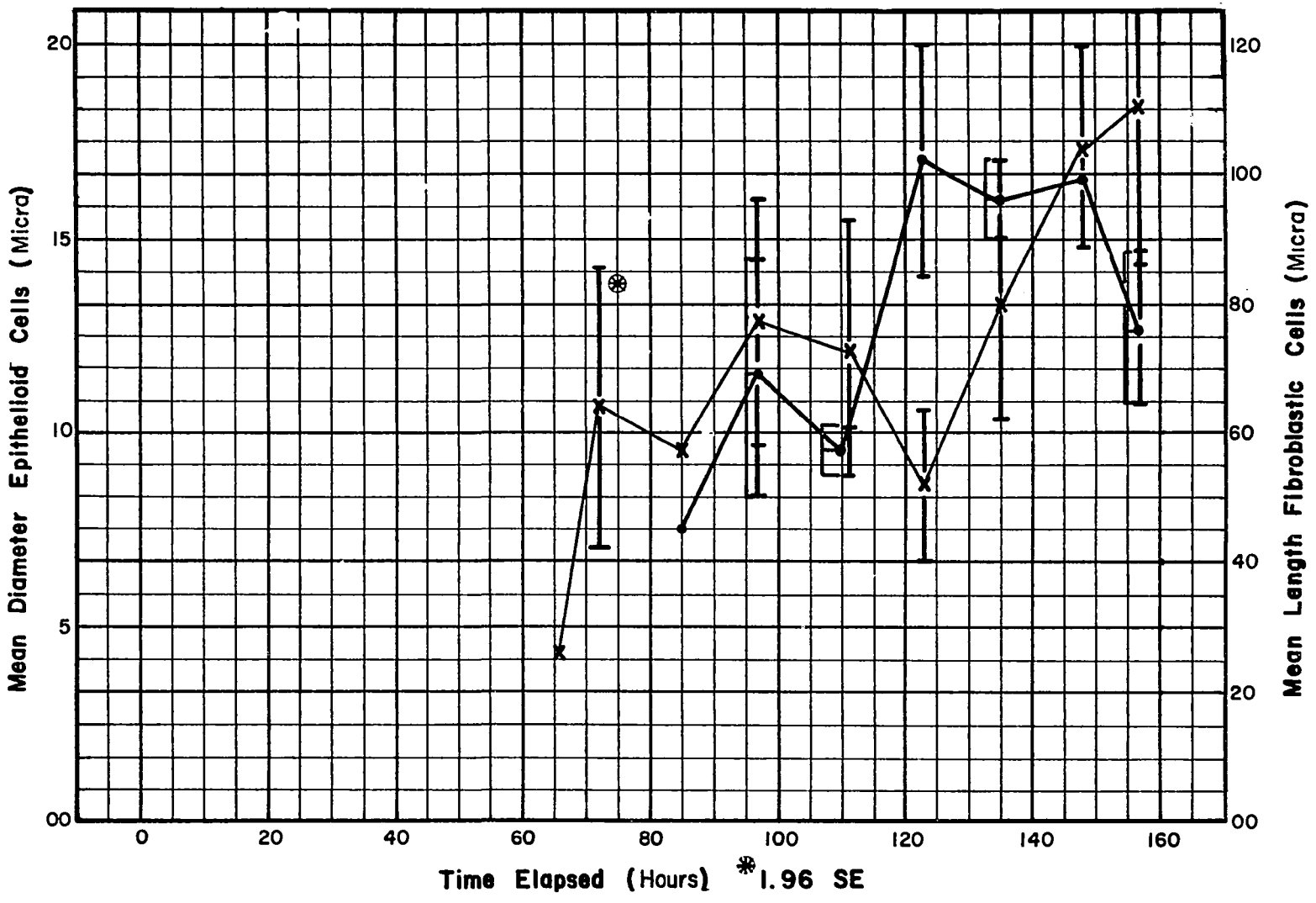
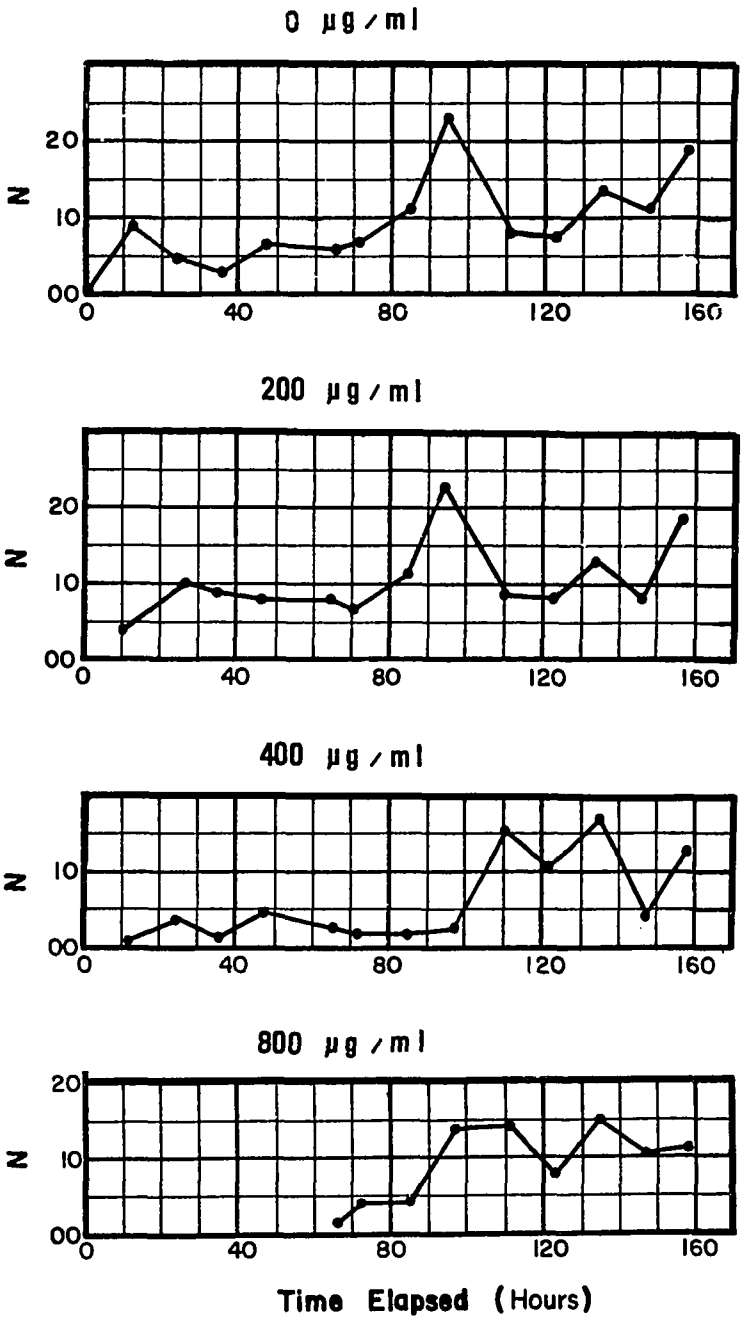


Figure 5

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish regressed testis tissue prepared 6/12/67, and chronically treated with 800 micrograms of saline pituitary extract (ADWP) per ml of medium o = Epithelioid
X = Fibroblastic

Figure 6



Total number of "in focus" cells with respect to elapsed time in cultures of goldfish regressed testis tissue prepared 6/12/67, and chronically treated with various amounts of saline pituitary extract (ADWP) per ml medium

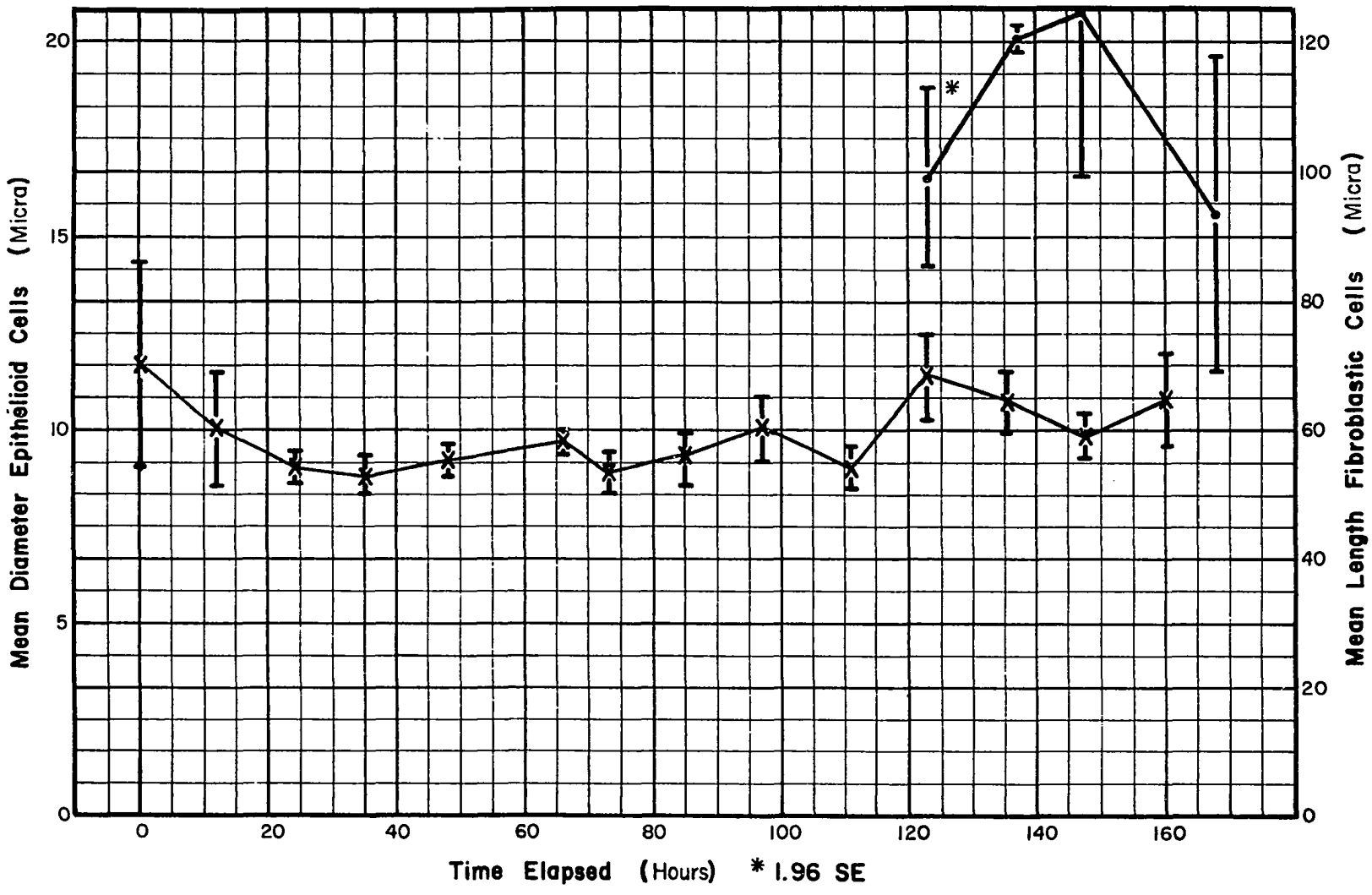


Figure 7

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish kidney tissue prepared 6/12/67 o = Epithelioid X = Fibroblastic

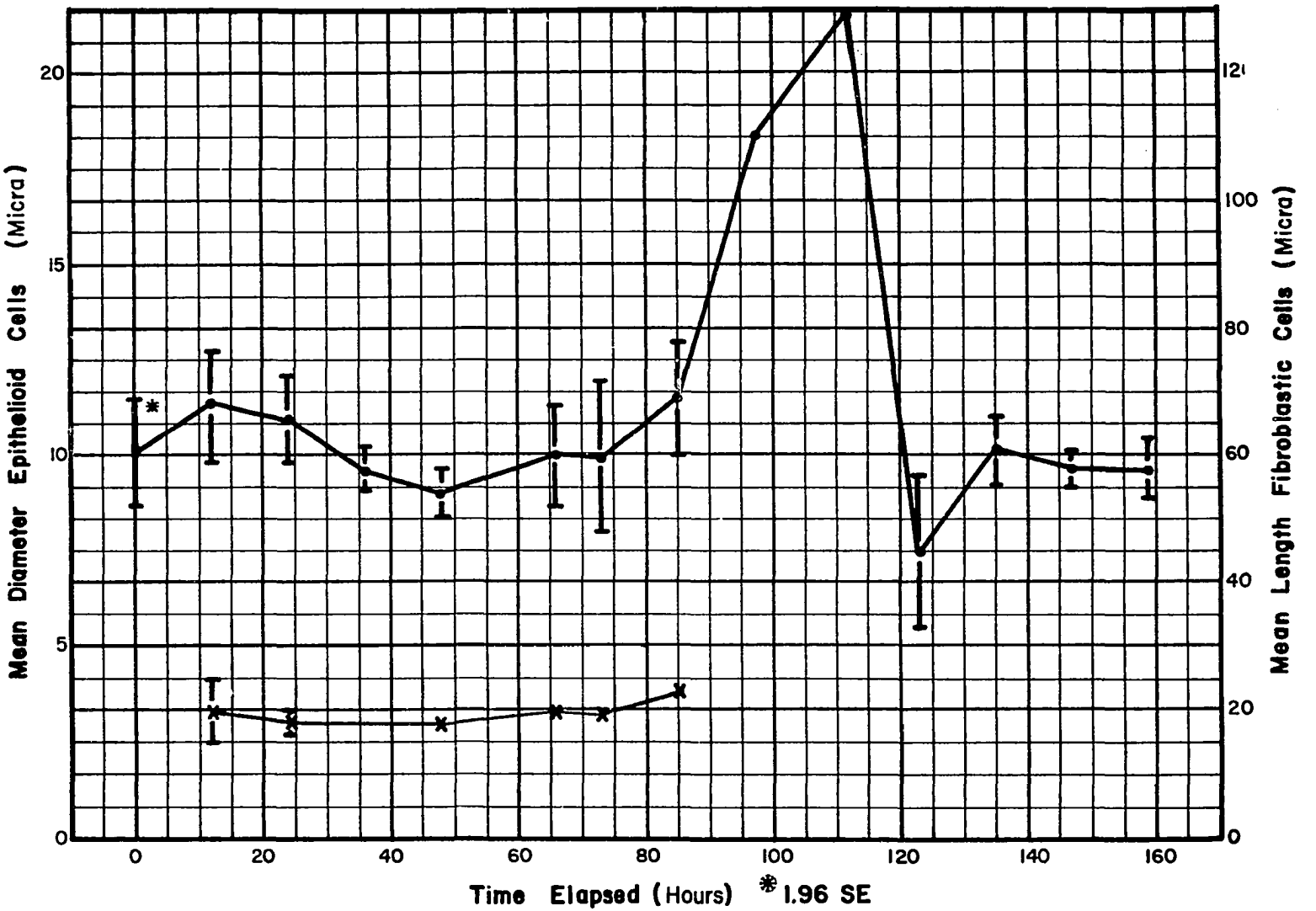
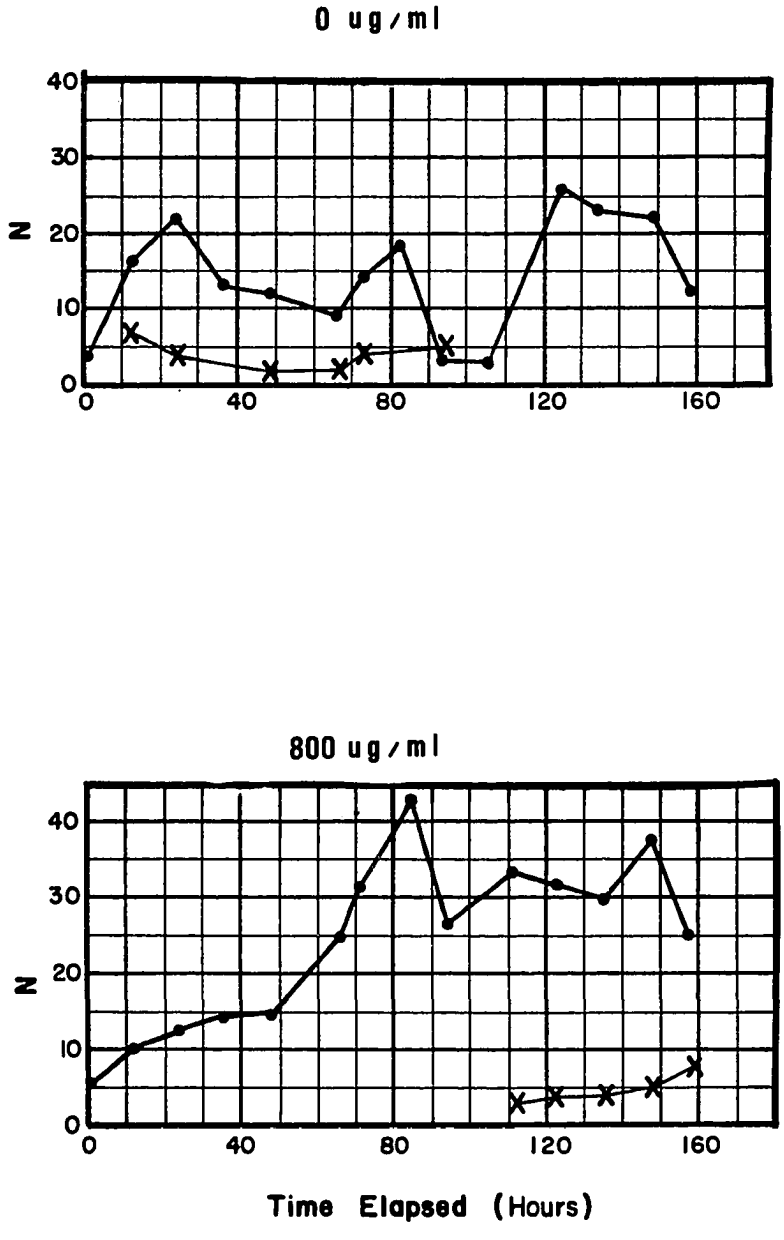


Figure 8

Mean diameter and length of "in focus" epithelioid and fibroblastic cells with respect to elapsed time in cultures of goldfish kidney tissue prepared 6/12/67, and chronically treated with 800 micrograms of saline pituitary extract (ADWP) per ml medium o = Epithelioid X = Fibroblastic

Figure 9



Total number of "in focus" cells with respect to time in cultures of goldfish kidney tissue prepared, 6/12/67 and chronically treated with 0 and 800 micrograms of saline pituitary extract (ADWP) per ml of medium

TABLE 4

Summary of Values of Resting Potentials and Specific Membrane Resistances of Cultured Goldfish Regressed Testis Tissue

Date	Age of Culture (hours)	Mean Resting Potential (mV)	N	Mean Specific Membrane Resistance (ohms·cm^2)	N
6/21/65	264	13.0±2.7*	27	--	--
7/18/65	96	2.0	9	44	3
7/22/65	192	2.1±0.7	16	267	2
8/ 7/65	576	5.4±0.3	23	615	6
11/28/66	216	9.0	1	134	1
5/ 9/67	72	59.7±8.6	29	--	--
5/11/67	120	45.0±4.6	102	262+78*	9
7/25/67	168	6.3±1.6	40	--	--
7/26/67	192	10.9±1.4	85	44	3
8/22/67	432	6.4±1.9	28	206+63	20

*1.96 SE. Tissues were in Simms' saline at pH 7.4 at 21° C.

TABLE 5

Summary of Values of Resting Potential and Input Membrane Resistance of Cells in Goldfish Back Muscle Strips Suspended in Simms' Saline pH 7.4 at 21°C.

Date	Mean Membrane Potential (mV)	N	Mean Input Membrane Resistance (M ohms)	N
5/15/67	57.0± 2.9*	45	112± 9*	9
7/ 6/67	71.7±13.9	58	265± 51	42
7/17/67	102.0±29.0	14	60	3
7/19/67	71.0± 8.0	159	680±140	12
7/29/67	90.0	1	530	3
8/13/67	53.8±12.3	23	305± 71	19
8/21/67a	178.0±36.0	20	238± 34	8
8/21/67b	131.0±20.0	39	328± 66	15
8/23/67	106.0±22.0	19	454± 67	23

*1.96 SE.

TABLE 6

Results of the Measurement of Membrane Potentials of Cultured Goldfish Regressed Testis Tissue Cells in the Presence of Various Substances 5/3/67

Time (min)	N	Mean Resting Potential (mV)	Range (mV)
a	102	45.00±4.64*	15-95
b			
1	12	37.08±7.54	20-60
2	12	32.08±5.60	15-50
3	14	23.21±5.22	10-40
4	17	27.65±6.77	10-50
5	12	28.33±6.04	15-55
6	12	37.08±7.28	20-60
7	19	22.63±7.33	10-80
c			
8	11	25.91±6.64	10-50
9	29	16.72±2.54	10-30
10	15	17.67±4.51	10-40
d			
11	22	27.73±7.47	10-90
12	17	17.94±4.01	10-35
13	42	22.14±3.25	10-65
e			
14	24	19.38±3.08	10-40
15	31	27.10±4.46	10-65
16	29	26.03±4.86	10-60
f			
17	18	15.56±3.50	8-30
18	25	20.20±5.78	10-60
19	14	32.14±5.92	10-50
20	29	20.34±4.74	10-65
22	21	23.33±5.70	10-45
23	21	14.28±2.50	10-30

Note: The tissue was placed in culture 5/3/67 and incubated for 144 hours. At (a) the potentials have been measured for several minutes in Simms' solution, pH 7.4. At (b) 34 ug/ml purified pituitary extract was added. At (c) the tissue was rinsed with 2 changes of Simms' solution. At (d) the KCl in the medium was increased 10x. At (e) the tissue was rinsed with 2 changes of Simms' solution. At (f) 1 mg/ml of NaCN was added to the chamber medium.

*1.96 SE.

TABLE 7

Results of the Measurement of Membrane Potentials of Cultured Goldfish Regressed Testis Tissue Cells in the Presence of a Saline Pituitary Extract 8/3/67

Time (min)	N	Mean Resting Potential (mV)	Range (mV)
a	22	8.45± 2.48*	4- 20
b			
1- 8	12	7.33± 3.44	4- 26
9-12	14	13.29± 4.38	5- 29
13-14	18	6.56± 2.02	2- 16
15-16	11	9.73± 1.98	6- 16
17-18	16	18.88± 7.40	4- 50
19-20	17	8.35± 4.52	3- 32
21-23	12	14.17± 6.85	5- 31
24-27	16	15.50± 3.54	5- 30
c			
28-36	19	62.53±22.05	10-135

*1.96 SE.

Note: The tissue was placed in culture 8/3/67 and incubated 456 hours. At (a) the potentials have been measured for several minutes in Simms' saline pH 7.4. At (b) 200 ug/ml saline pituitary extract was added. At (c) the tissue was rinsed with 2 changes of Simms' saline.

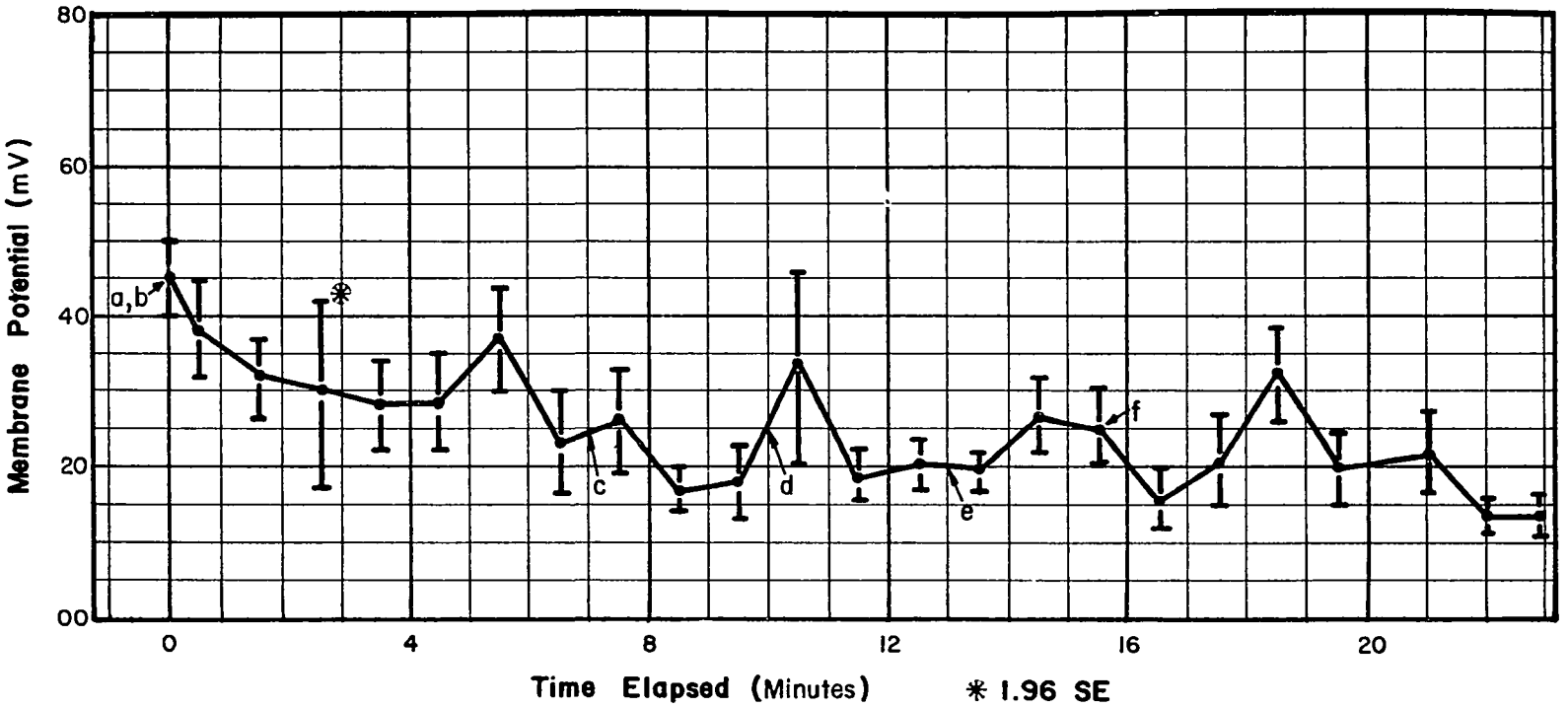


Figure 10

Membrane potentials of goldfish regressed testis tissue cells 144 hours in culture and acutely treated with a. control (Simms' Saline) b. 3.4 micrograms/ml purified hydration fraction (equal to 700 ug/ml of ADWP) c. 2 rinses Simms' Saline d. 10X increase in KCl concentration e. 2 rinses Simms' Saline f. 1000 micrograms/ml NaCN 5/9/67

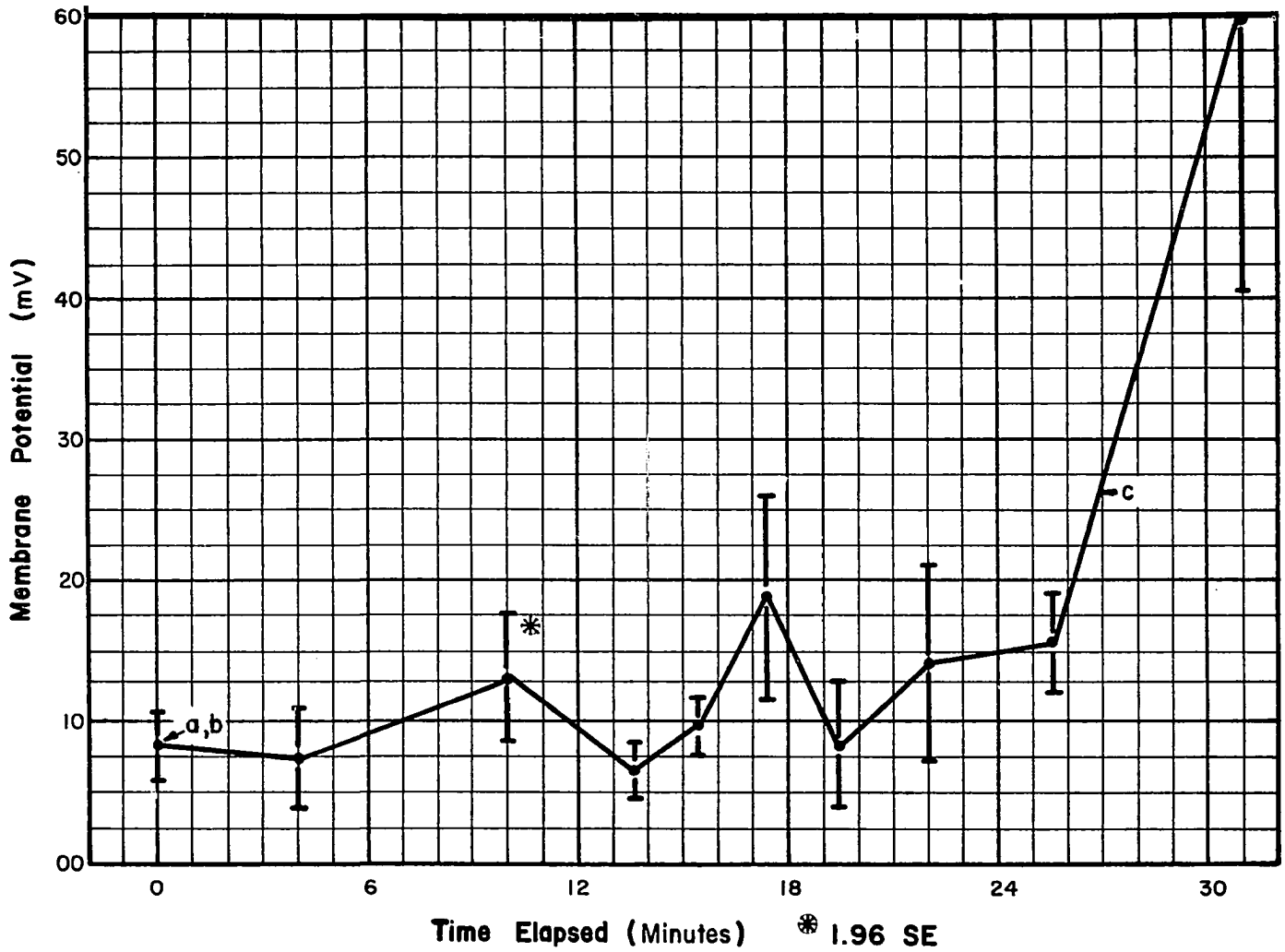


Figure 11

Membrane potentials of goldfish regressed testis tissue cells 456 hours in culture and acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 8/21/67 c. 2 rinses Simms' Saline

TABLE 8

Results of the Measurements of Specific Membrane Resistances of Cultured Goldfish Regressed Testis Tissue Cells in the Presence of a Saline Pituitary Extract 8/3/67

Time (min)	N	Mean Specific Membrane Resistance (ohms·cm^2)	Range (ohms·cm^2)
a	22	328.3±174*	54-1605
b 0 - 5.0	9	154.6± 35	107- 214
15	9	725.2±366	214-1926
15.5-17.0	10	1337.5±648	214-2889
18.0-19.3	12	240.8±132	54- 865
19.4-20.7	13	690.2±301	214-1926
24.5-27.3	8	635.3±244	268-1338
c 27.7-36.3	19	1633.1±599	268-3478

*1.96 SE.

Note: The tissue was placed in culture 8/3/67 and incubated for 456 hours. At (a) the potentials have been measured in Simms' saline, pH 7.4. At (b) 200 ug/ml saline pituitary extract was added. At (c) the tissue was rinsed with 2 changes of Simms' saline.

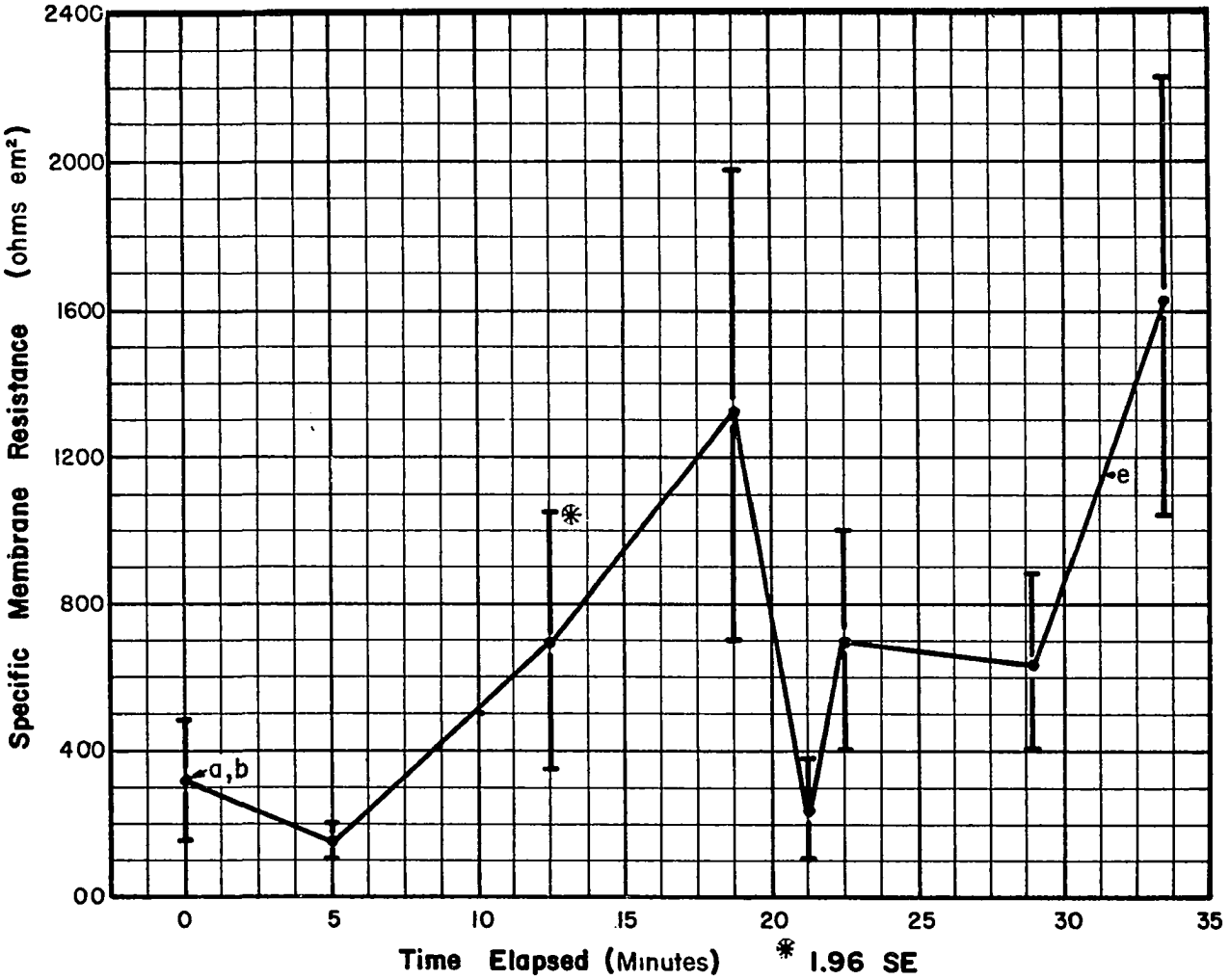


Figure 12

Specific membrane resistance of goldfish regressed testis tissue cells 456 hours in culture and acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract c. 2 rinses in Simms' Saline 8/22/67

TABLE 9

Results of the Measurement of the Membrane Potentials and Input Membrane Resistances of Cells in Goldfish Back Muscle Strips in the Presence of a Purified Pituitary Extract and Other Substances 5/15/67

Treatment and Time	Mean Resting Potential (mV)	N	Mean Input Membrane Resistance (M ohms)	N
a	56.9±2.9*	45	112.4±9.0*	9
b				
5	54.6±4.7	26	100	2
10	50.0±3.5	50	--	-
15	47.4±1.3	27	--	-
20	50.7±2.1	23	--	-
25	46.3±2.1	41	12.2	3
30	40.8±3.7	20	--	-
c				
d				
35	38.1±3.8	51	--	-
40	41.6±5.4	29	--	-
45	12.5±4.9	8	--	-
50	17.4±2.0	44	--	-
e				
53	17.7±2.3	48	--	-

*1.96 SE.

Note: The tissue was measured on 5/15/67. At (a) the potentials and resistances have been measured in Simms' saline, pH 7.4. At (b) 34ug/ml purified pituitary extract was added. At (c) the tissue was rinsed with 2 changes of Simms saline. At (d) 1.0 mg/ml NaCN was added. At (e) the tissue was rinsed with 2 changes of Simms' saline.

TABLE 10

Results of the Measurement of the Membrane Potentials and Input Membrane Resistances of Cells in Goldfish Back Muscle Strips in the Presence of a Saline Pituitary Extract 7/6/67

Treatment and Time	Mean Resting Potential (mV)	N	Mean Membrane Resistance (M ohms)	N
a	71.7±13.9*	58	265±51*	42
b				
2	--	--	252±76	9
4	12.2± 5.6	15	--	--
5	17.4± 4.3	22	--	--
12	50.2±11.0	9	--	--
17	57.5	4	--	--

*1.96 SE.

Note: The tissue was measured on 7/6/67. At (a) the potentials and resistances have been measured in Simms' solution, pH 7.4. At (b) 200 ug/ml saline pituitary extract was added.

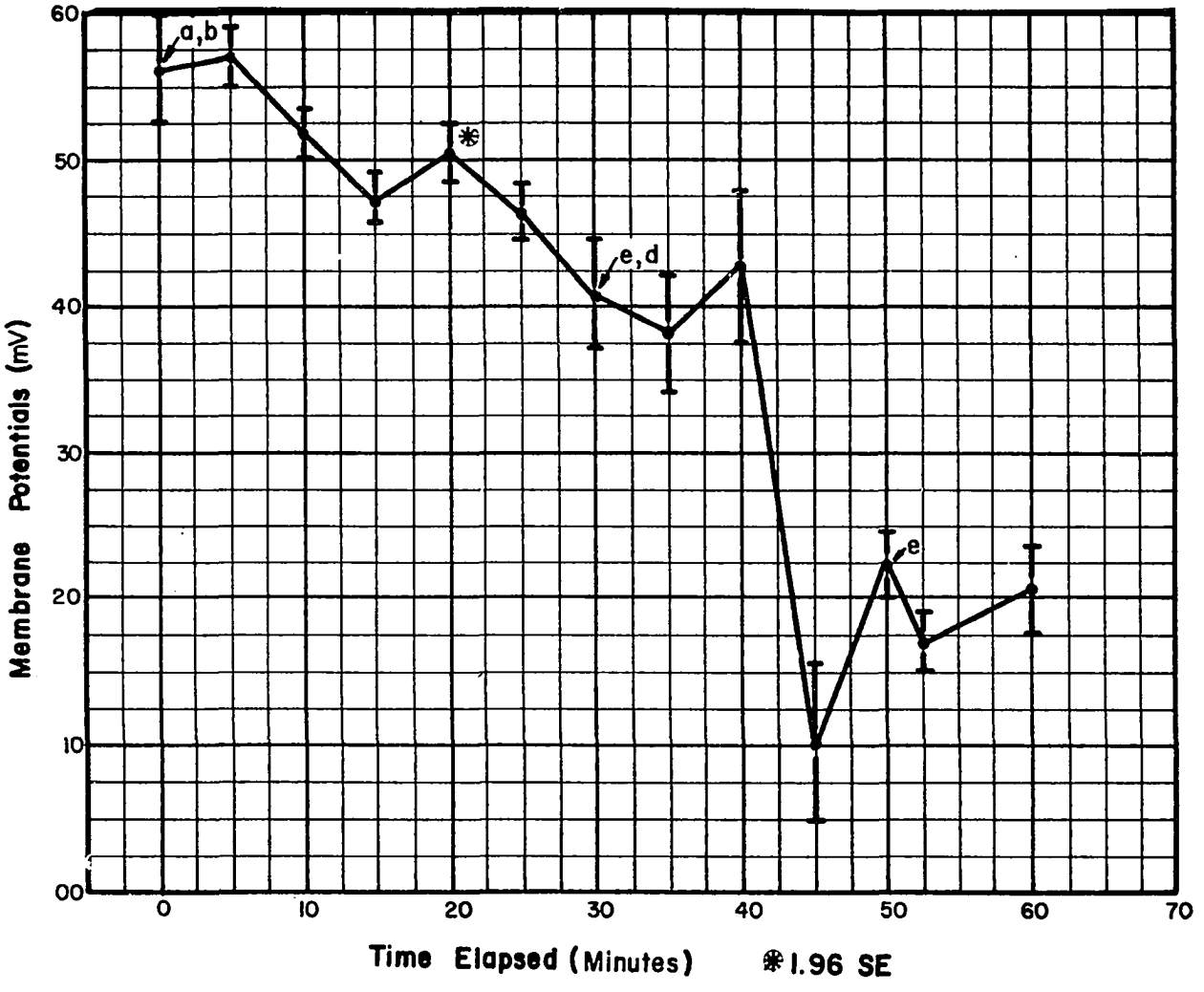


Figure 13

Membrane potentials of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 34 micrograms/ml purified hydration fraction (equal to 700 ug/ml of ADWP) c. 2 rinses in Simms' Saline d. 1000 micrograms/ml NaCN e. 2 rinses in Simms' Saline 5/15/67

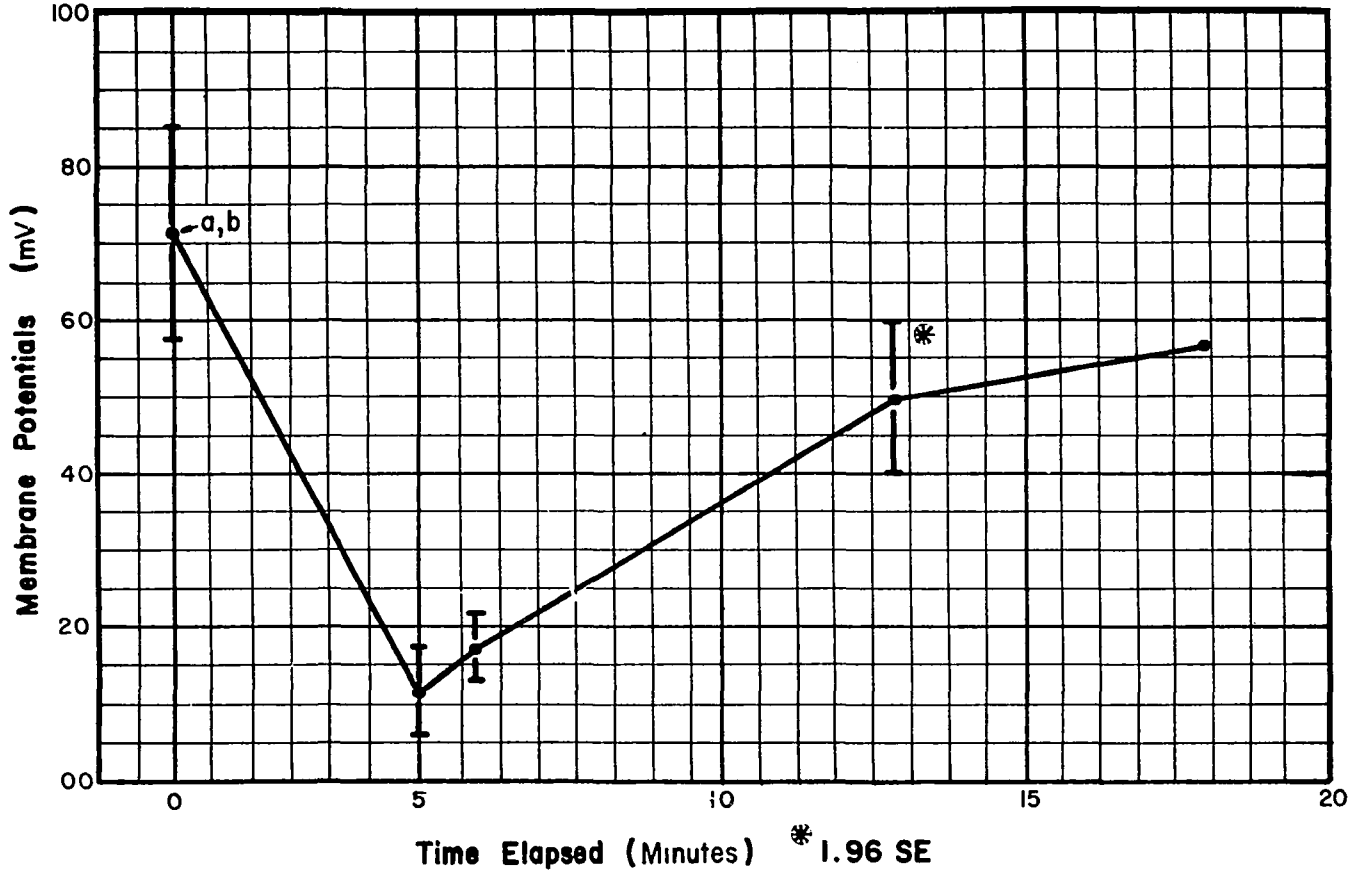


Figure 14

Membrane potentials of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 7/6/67

TABLE 11

Results of the Measurement of the Membrane Potentials and Input Membrane Resistances of Cells in Goldfish Back Muscle Strips in the Presence of a Saline Pituitary Extract 8/21/67a

Treatment and Time (min)	Mean Resting Potential (mV)	N	Mean Membrane Resistance (M ohms)	N
a	131.0±20*	39	378.00±66*	15
b				
2	92.0±22	22	60.08±43	14
10	158.0±23	33	138.00±36	28
20	38.0±16	20	1000	2
23	58	5	467	3
25	25.5±2.5	9	--	--

*1.96 SE.

Note: The tissue was measured on 8/21/67a. At (a) the potentials and resistances have been measured in Simms' solution, pH 7.4. At (b) 200 ug/ml saline pituitary extract was added.

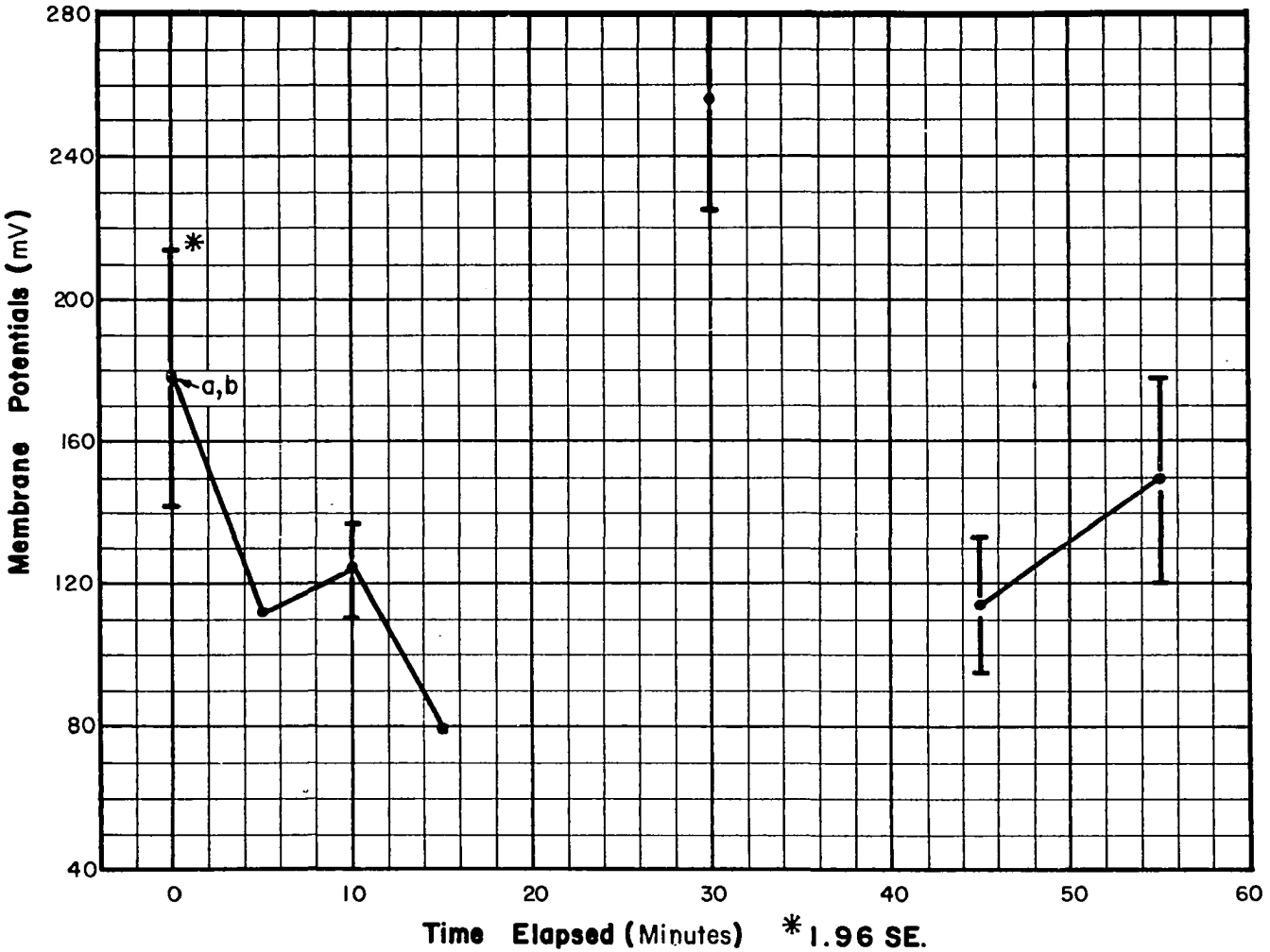


Figure 15

Membrane potentials of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 8/21/67a

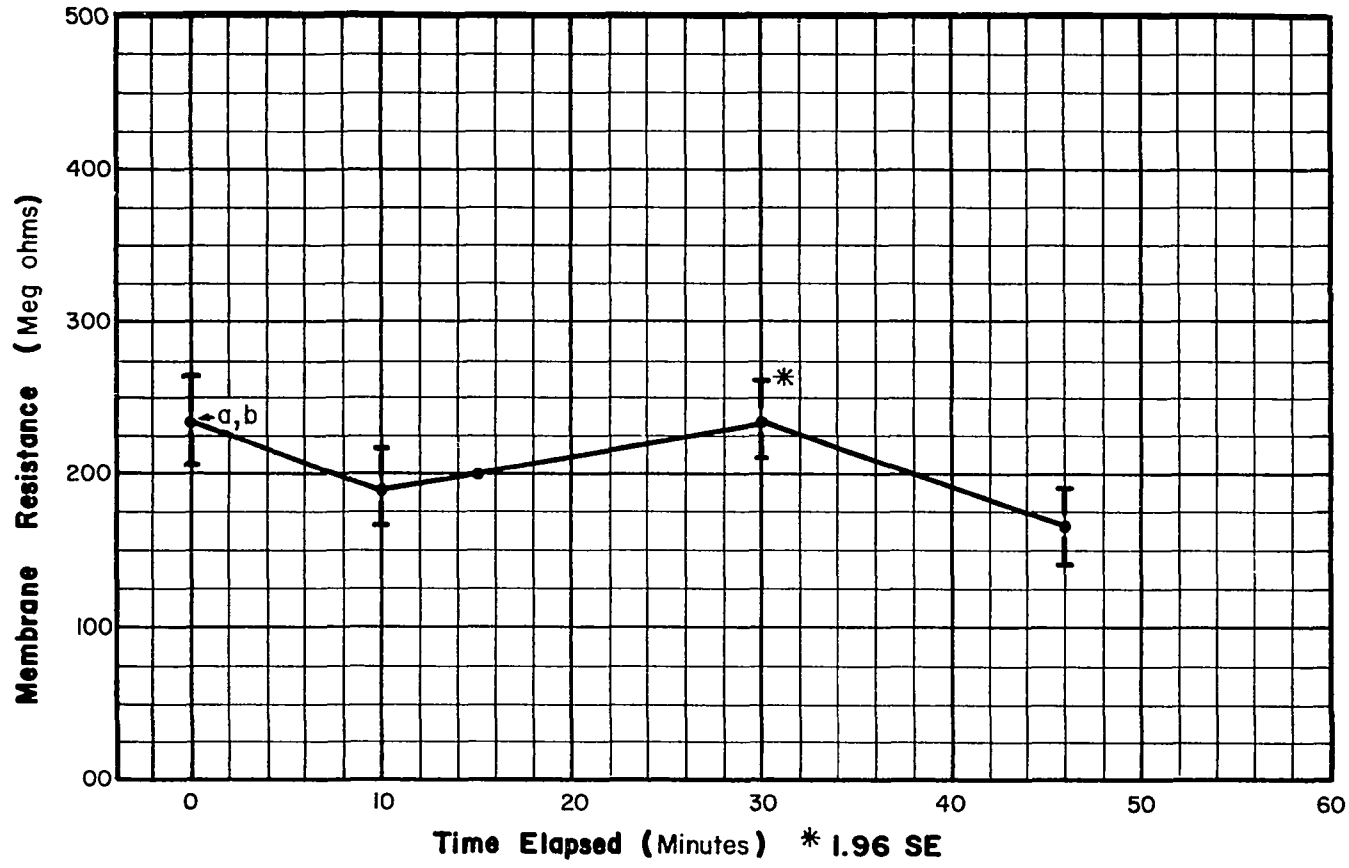


Figure 16

Membrane resistance of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 8/21/67a

TABLE 12

Results of the Measurement of the Membrane Potentials and Input Membrane Resistances of Cells in Goldfish Back Muscle Strips in the Presence of a Saline Pituitary Extract 8/21/67b

Treatment and Time	Mean Resting Potential (mV)	N	Mean Membrane Resistance (M ohms)	N
a	178±36*	20	328±34*	8
b				
5	113	4	--	--
10	124±13	35	189±23	32
15	81	5	200	2
30	256±33	29	237±26	19
45	115±19	28	165±23	23
55	140±29	7	347±34	5

*1.96 SE.

Note: The tissue was measured on 8/21/67b. At (a) the potentials and resistances have been measured in Simms' solution, pH 7.4. At (b) 200 ug/ml saline pituitary extract was added.

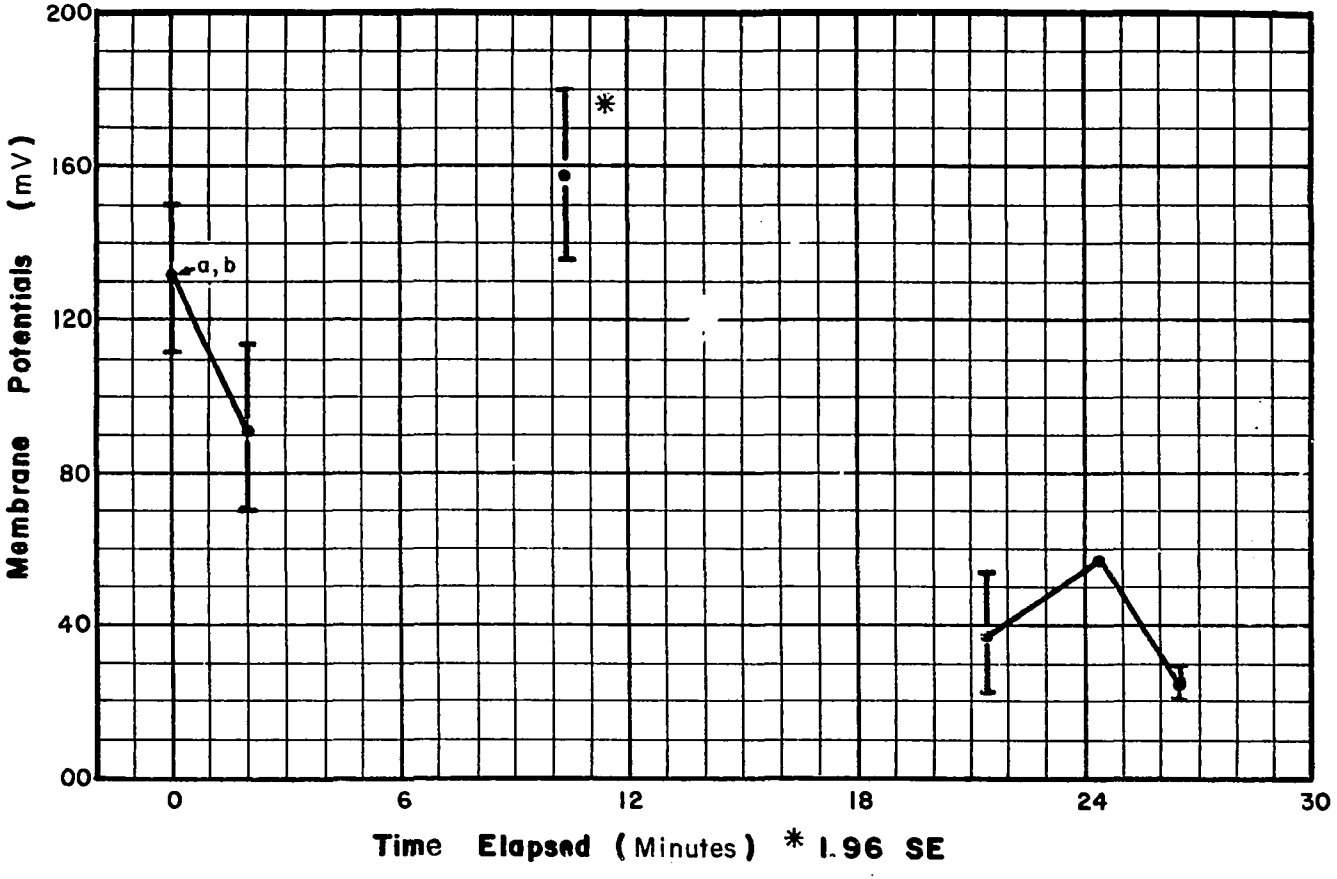


Figure 17

Membrane potentials of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 8/21/67b

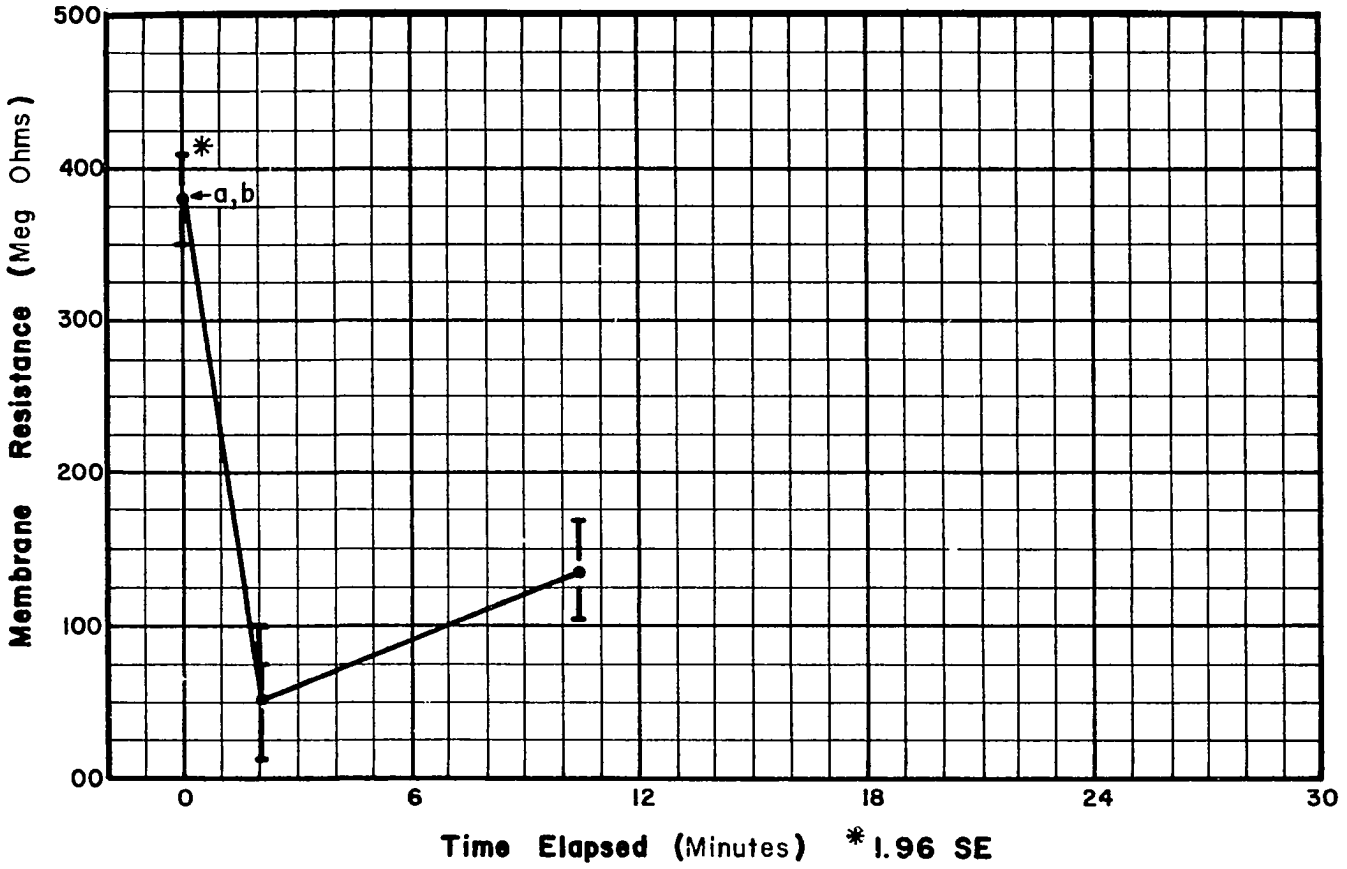


Figure 18

Membrane resistance of goldfish back muscle cells acutely treated with a. control (Simms' Saline) b. 200 micrograms/ml saline pituitary (ADWP) extract 8/21/67b.

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A P P E N D I X

TABLE 13

Summary of Sizes of the "in Focus" Cells around Explants Prepared 6/12/67 and 6/16/67* and Maintained in Tissue Culture for Varying Lengths of Time

Time in Hours	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
* 0	1	20.0				
0	5	8.4±0.7**	8-10			
* 12	9	11.8±2.1	10-20			
24	5	15.0±1.7	12-18			
* 25	7	10.1±2.2	8-17			
36	3	11.0±0.9	10-12			
* 47	3	9.3±1.1	8-10			
48	7	10.9±1.6	6-12			
66	6	10.5±1.6	8-15			
73	3	12.7±5.1	9-19	4	77.0±22.2	40- 99
* 73	7	11.9±1.8	10-16			
85	9	16.3±0.9	15-18	2	60.0	80- 40
97	12	15.9±1.8	10-19	11	52.6	20- 92
111	5	16.4±1.1	15-18	4	39.5± 1.6	38- 42
123	1	17.0		7	43.4± 8.9	29- 63
*126	111	9.5±1.8	77-10	12	84.5±15.2	52-132
135	8	12.5±2.6	10-20	6	41.6± 9.6	28- 58
*146	16	16.0±2.2	9-20	5	81.6± 7.8	68- 94
147	5	10.4±2.2	8-15	4	50.0±23.0	27- 89
159	14	12.8±1.9	8-20	13	54.2±11.0	25-100
*168	4	17.0±6.4	11-24	9	96.8±17.8	48-130

*6/16/67.

**1.96 SE.

TABLE 14

Summary of Sizes of the "in Focus" Cells around Explants Prepared 6/12/67 and 6/16/67* and Maintained in Tissue Culture for Varying Lengths of Time with 200 Micrograms per ml Medium of Saline Pituitary Extract

Time in Hours	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
0	0					
* 0	4	16.3±1.9**	13-18			
12	4	10.5±0.8	10-12			
24	10	11.2±1.1	10-16			
* 25	5	12.4±2.6	10-17			
36	9	14.7±2.1	10-20			
* 47	4	12.5±4.0	9-19			
48	7	11.0±1.5	10-17			
66	7	11.0±1.5	9-15			
* 73	Missing					
73	2	14.0	10-18	5	41.0±14.2	19- 60
85	5	11.8±2.8	7-16	8	40.1± 7.6	27- 58
97	4	11.5±2.7	9-16	17	47.5± 9.7	20-109
111	10	12.7±1.9	9-18	2	34.0	28- 40
123	9	15.1±3.3	10-26	1	49.0	
*126	14	14.6±2.6	8-29	12	92.3±20.3	26-150
135	4	16.0±4.1	9-20	3	34.7	19- 47
*146[a]	2	13.3	14-30	6	86.0±22.6	50-114
147	4	14.0±4.0	10-19			
159	10	10.7±1.6	9-16	2	16.5	16- 17
*168[a]	6	14.8±4.2	9-21	1	97.0	

*6/16/67

**1.96 SE.

[a]Most cells in sheets.

TABLE 15

Summary of Sizes of the "in Focus" Cells around Explants Prepared 6/12/67 and 6/16/67* and Maintained in Tissue Culture for Varying Lengths of Time with 400 Micrograms per ml Medium of Saline Pituitary Extract.

Time in Hours	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
0	2	8.0	7- 9			
* 12	2	16.5	15-18			
12	1	10.0				
24	4	9.5±0.5**	9-10			
36	2	9.5	9-10			
* 47				1	26.0	
48	5	12.6±3.2	8-17			
66	3	11.7	9-16			
* 73	6	11.5±2.4	8-16	1	58.0	
73	2	9.5	9-10			
85	1	19.0		1	22.0	
97				3	76.7	48- 88
111	12	11.3±1.3	8-16	4	75.8± 6.3	68- 85
123	9	18.6±1.7	15-20	2	66.0	31-101
*126	3	14.3	10-20	7	91.7±10.0	31-166
135	13	16.1±3.0	8-22	4	111.0±20.0	95-145
*146	13	13.8±1.9	8-20	7	103.3±31.5	31-166
147[a]				4	83.8±24.8	64-127
159	12	11.2±2.0	8-20	2	91.0	82-100
*168	13	14.8±1.9	8-18	11	87.4±23.0	27-162
255	19	17.9±1.2	9-21	2	69.0	68- 70

*6/16/67.

**1.96 SE.

[a]Sheet of Cells.

TABLE 16

Summary of Sizes of the "in Focus" Cells around Explants Prepared 6/12/67 and 6/16/67* and Maintained in Tissue Culture for Varying Lengths of Time with 800 Micrograms per ml Medium of Saline Pituitary Extract

Time in Hours	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
0	0					
* 0	5	17.3±1.0**	15-18			
12	0					
* 12	4	18.3±1.5	16-20			
24	2	10.0	9-11			
36	0					
* 47	0					
48	0					
66	0			1	27.0	
73	0			4	65.3±24.9	28- 96
* 73	5	11.2±2.1	10-16	0		
85	2	8.0	8	3	57.3	35- 79
97	10	12.5±2.2	9-18	4	75.8±19.1	58-108
111	6	9.8±0.7	8-11	5	73.4±18.9	46-110
123	2	19.5	18-21	7	52.6±11.3	40- 88
*126	4	16.7±3.3	11-19	11	154.6±21.4	110-216
135	8	15.9±0.8	14-18	70	80.0±17.7	40-110
*146	5	12.2±3.1	9-17	15	98.1±34.5	34-287
147	5	16.8±3.2	10-20	6	104.5±17.4	72-125
159	8	12.4±1.6	10-16	4	110.3±26.9	78-137
*168	18	15.9±1.2	10-18	11	85.8±16.8	38-119
183	2	15.0	9-18	2	162.0	159-165
255	16	17.3±2.0	10-20	7	136.0±33.9	60-210

*6/16/67.

**1.96 SE.

TABLE 17

Summary of Sizes of the "in Focus" Cells around Kidney Explants 6/12/67 and Maintained in Tissue Culture for Varying Lengths of Time

Time in min.	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
0	6	11.7±2.7*	9-19			
12	10	10.0±1.5	8-17			
24	13	9.0±0.4	8-10			
36	15	8.9±0.5	7-10			
48	15	9.1±0.4	8-11			
66	26	9.6±0.3	8-11			
73	31	8.9±0.7	7-16			
85	46	9.1±0.4	7-14			
97	27	10.0±0.5	7-18			
111	34	9.1±0.5	7-14	3	out of focus	
123	28	11.3±1.0	8-18	4	98.8±13.8	79-119
135	25	10.6±0.8	7-16	4	159.5± 2.0	158-163
147	39	9.8±0.6	8-16	5	163.8±24.9	130-196
159	21	10.7±1.1	8-17	7	132.7±24.5	97-200
255	22	11.6±1.3	8-18	6	167.5±43.6	117-270

*1.96 SE.

TABLE 18

Summary of Sizes of the "in Focus" Cells around Kidney Explants 6/12/67 and Maintained in Tissue Culture for Varying Lengths of Time with 800 Micrograms per ml of Culture Medium

Time in min.	Number of Epithelioid Cells	Mean Diameter (u)	Diameter Range (u)	Number of Fibroblastic Cells	Mean Length (u)	Range Length (u)
0	4	10.0±1.4*	8-12			
12	11	11.3±1.7	8-16	6	19.5±4.8	12-21
24	17	10.9±1.2	7-15	4	18.5±0.8	
36	14	9.6±0.7	7-12			
48	9	9.0±0.5	8-10	2	17.0	18-16
66	7	10.0±1.4	6-12	1	20.0	
73	12	9.8±1.5	7-17	2	19.5	20-19
85	17	11.5±1.5	7-18	2	23.0	20-26
97	3	18.3	10-25	Fibrocytes in sheets, not in focus		
111	2	22.0	14-30	Fibrocytes in sheets, not in focus		
123	26	10.2±1.9	6-18	Fibrocytes in sheets, not in focus		
135	23	10.1±1.0	6-18	Fibrocytes not in focus		
147	22	9.6±0.6	7-13	Fibrocytes not in focus		
159	12	9.7±0.8	8-13	Fibrocytes not in focus		
255	19	9.6±0.4	8-12	10	132.5+16.7	90-189

*1.96 SE.

TABLE 19

Membrane Potentials and Input Resistances of Cultured Goldfish Testis Tissue Cells

Culture Date	Age at Use (Hours)	Membrane Potential (mV)	N	Input Membrane Resistance (ohms X 10^6)	N
6/10/65-6/21/65	264	8.0 6.0 12.0 10.0 2.0 12.0 16.0 2.0 12.0 20.0 2.0 24.0 4.0 12.0 24.0 20.0 12.0 24.0 4.0 12.0 24.0 8.0 12.0 24.0 4.0 12.0 24.0 $\bar{X}$=13.00 SE=1.40	27		
7/14/65-7/18/65	96	2.0 2.0 1.0 2.0 2.0 2.0 2.0 2.0 2.0 $\bar{X}$=2.00	9	8.3 8.3 8.3 $\bar{X}$=8.3	3
7/14/65-7/22/65	192	2.0 1.0 2.0 2.0 1.0 2.0 1.0 1.0 2.0 1.0 1.0 4.0 1.0 2.0 3.0 1.0 $\bar{X}$=2.14 SE=0.38	16	75.0 25.0 $\bar{X}$=50	2
7/14/65-8/7/65	576	5.0 6.0 4.5 4.0 6.0 5.0 5.5 2.5 3.5 7.5 3.5 5.0 5.5 8.5 5.0 2.5 8.0 7.5 6.0 2.5 6.5 6.0 3.5 $\bar{X}$=5.43 SE=0.281	22	220.0 120.0 30.0 170.0 120.0 10.0 $\bar{X}$=115	6
11/19/65-11/28/66	216	9.0	1	25.0	1

TABLE 20

Membrane Potentials and Input Resistances of Cultured Goldfish Testis Tissue Cells

Culture Date	Age at Use (Hours)	Membrane Potential (mV)					N	Input Membrane Resistance (ohms X 10^6)		N
5/3/67-5/9/67	144	90	60	60	70	70	29			
		100	55	70	60	70				
		30	30	70	70	70				
		60	20	100	100	40				
		30	35	60	60	20				
		55	85	60	60	100				
		30	80	50	50	90				
		50								
		$\bar{X}$ = 59.66 SE = 4.37								
5/6/67-5/11/67	120	75	60	25	55	45	102	50	50	9
		15	20	40	40	20		50	50	
		35	15	50	45	20		50	100	
		20	45	100	65	80		50	10	
		15	45	115	35	20		30		
		15	45	65	15	50				
		50	65	50	30	15				
		55	90	75	20	20				
		35	50	60	20	20				
		35	30	35	40	20				
		15	60	75	20	40				
		35	115	20	35	70				
		35	70	35	20	30				
		25	60	70	15	65				
		30	35	55	30	50				
		15	35	80	30	60				
		45	15	70	25	40				
		50	15	45	30	95				
		35	75	65	20	80				
		75	70	50	30	95				
		65	65							
		$\bar{X}$ = 45 SE = 2.37						$\bar{X}$ = 48.89 SE = 7.44		

TABLE 21

Membrane Potentials and Input Resistances of Cultured Goldfish Testis Tissue Cells

Culture Date	Age at Use (Hours)	Membrane Potential (mV)	N	Input Membrane Resistance (ohms X 10^6)	N
7/18/67- 7/25/67	168	2 8 3 2 3 2 6 13 2 14 2 3 2 5 13 8 2 16 2 3 3 12 3 17 3 2 3 5 2 12 8 9 3 4 2 3 4 12 4 8 $\bar{X}$ = 6.35 SE = 0.8206	40		
7/18/67- 7/26/67	192	6 21 12 3 14 4 19 27 23 55 6 7 6 27 22 10 4 3 6 24 14 12 5 7 6 9 12 2 5 5 20 9 9 5 2 14 20 10 9 10 8 16 14 27 14 14 5 16 11 28 4 11 2 14 16 20 21 9 4 15 11 12 13 9 7 10 16 12 15 9 5 6 24 2 9 3 10 10 6 6 6 7 3 2 $\bar{X}$ = 10.87 SE = 0.7253	85	14.9 5.0 5.0 $\bar{X}$ = 8.3	3
8/3/67- 8/22/67	456	20 6 2 6 20 8 2 2 4 4 4 12 4 2 4 16 4 6 6 16 4 4 2 4 4 6 2 4 $\bar{X}$ = 6.38 SE = 0.98	28	40 120 20 280 40 20 40 300 20 40 20 40 80 20 10 40 60 20 80 20 20 20 $\bar{X}$ = 38.50 SE = 6.01	20

TABLE 22

Membrane Potentials and Input Resistances of Cells in Goldfish Back Muscle Strips

Date	Membrane Potential (mV)					N	Input Membrane Resistance (ohms X 10^6)		N
5/15/67	60	60	60	55	65	45	117		9
	60	60	60	55	80		144		
	65	60	60	55	35		117		
	60	65	65	45	25		117		
	70	65	65	40	35		117		
	65	70	70	30	45		100		
	55	70	65	35	50		100		
	60	55	40	35	50		100		
	60	60	55	80	55		100		
	$\bar{X}$ = 56.87		SE = 1.5001				$\bar{X}$ = 112.4	SE = 4.58	
7/6/67	50	13*	12*	165		58	300	110	42
	135	16*	15*	175			200	320	
	140	9*	8*	80			590	200	
	210	21	13*	100			580	200	
	45	14*	8*	55			370	100	
	35	4*	17*	100			320	200	
	25	17*	19*	55			590	150	
	25	10*	7*	35			220	50	
	15*	3*	28	30			330	100	
	20	10*	19*	45			500	100	
	25	4*	25	50			100	150	
	21	19*	21	50			580	50	
	13*	16*	6*	40			280	250	
	14*	10*	5*	60			580	150	
	11*	27	4*	35			250	100	
	6*	8*	18*	30			140	150	
	6*	16*	125	40			550	50	
	12*	20	145	10*			220	200	
	16*	12*	120	50			550	150	
	21	40	170	60			470	150	
	19*	22	165	50			540	150	
	17*	43	180	75					
	20	35	130	60					
	22	35	150	120					
	$\bar{X}$ = 71.74		SE = 7.10				$\bar{X}$ = 265.24	SE = 26.19	
5/29/67			90				750		
							100		
							750		

*Not used in calculations.

TABLE 23

Membrane Potentials and Input Resistances of Cells in Goldfish Back Muscle Strips

Date	Membrane Potential (mV)					N	Input Membrane Resistance (ohms X 10^6)			N
7/6/67	50	17	22	10	40	98	200	320	280	42
	135	20	43	18	600		300	200	580	
	140	22	35	125	35		590	200		
	210	13	12	145	30		580	100		
	45	16	15	120	40		370	200		
	35	9	8	170	10		320	150		
	25	21	13	165	50		590	150		
	15	14	8	180	60		220	50		
	20	4	17	130	50		330	100		
	25	17	19	150	75		500	100		
	21	10	7	165	60		110	150		
	13	3	28	175	120		580	50		
	14	10	19	80			250	250		
	11	4	25	100			140	150		
	6	27	21	55			550	100		
	6	8	6	35			220	150		
	12	16	5	30			550	50		
	16	20	4	45			470	200		
	21	12	19	50			540	150		
	19	40	16	50			100	150		
	$\bar{X}$ = 52.36 SE = 7.26						$\bar{X}$ = 265.24 SE = 26.19			

TABLE 24

Membrane Potentials and Input Resistances of Cells in Goldfish Back Muscle Strips

Date	Membrane Potential (mV)						N	Input Membrane Resistance (ohms X 10^6)	N
7/19/67	50	150	40	70	20	20	159	400	12
	30	150	40	140	40	20		400	
	40	120	30	120	35	20		1000	
	60	40	40	170	60	90		950	
	40	100	25	20	175	70		1000	
	30	70	40	90	115	80		1000	
	60	80	20	170	100	40		850	
	70	180	30	170	60	60		650	
	150	60	20	100	100	30		520	
	60	70	40	175	100	30		500	
	20	70	20	175	160	60		400	
	20	30	20	155	100	20		500	
	30	40	30	150	60	140			
	40	40	20	110	140	180			
	40	35	30	150	30	170			
	110	50	35	120	20	50			
	20	25	20	120	180	30			
	40	30	25	160	40	70			
	30	30	25	165	170	40			
	50	25	30	175	120	50			
	30	35	35	160	100	50			
	30	50	30	175	60	40			
	30	80	30	90	20	160			
	30	30	25	35	40	18			
	20	40	125	20	90				
	20	40	35	35	50				
	50	40	20	50	60				
	$\bar{X}$ = 70.59 SE = 4.12							$\bar{X}$ = 680 SE = 71.46	
8/13/67	50	45	100	40	25		23	300 250 350 200	19
	30	30	63	50	40			600 200 100 150	
	65	110	60	20	85			500 400 100 500	
	115	90	30	20	40			450 250 100 250	
	50	90	55					600 200 300	
	$\bar{X}$ = 53.82 SE = 6.32							$\bar{X}$ = 305.26 SE = 36.25	

TABLE 25
Membrane Potentials and Input Resistances of Cells in Goldfish Back Muscle Strips

Date	Membrane Potential (mV)			N	Input Membrane Resistance (ohms X 10^6)			N
8/23/67	40	100	80	19	500	450	200	23
	10	170	30		100	550	500	
	10	20	130		300	500	650	
	115	40	120		700	600	600	
	130	40	160		500	750	200	
	30	80	150		300	550	450	
	10	150	80		600	400	300	
	190				400	350	200	
	$\bar{X}$ = 106.22 SE = 11.22				$\bar{X}$ = 454.64 SE = 34.05			
8/21/67	55	250	80	39	50*	450	200	15
	60	190	110		33*	450	200	
	55	60	120		50*	400	200	
	50	210	140		34*	500	200	
	70	190	120		50*	450	300	
	60	150	80		50*	400	300	
	100	150	110		66*	300	200	
	110	190	240		500			
	85	130	220					
	95	220	250					
	50	180	130					
	55	140	90					
	200	220	80					
	$\bar{X}$ = 130.64 SE = 10.04				$\bar{X}$ = 327.51 SE = 33.49			
8/21/67 9:45 p.m. (0)	220	110	360	250	20	300	300	8
	180	320	80	130		200	300	
	140	120	110	320		200	200	
	220	140	240	90		200		
	80	120	220	80		200		
	$\bar{X}$ = 178.42 SE = 18.25				$\bar{X}$ = 237.50 SE = 17.11			
7/17/67	30	165	235	125	14	85		
	100	220	40	85		75		
	55	100	20	60		30		
	160	230						
	$\bar{X}$ = 101.78 SE = 14.92							

*Not included in calculations.