STUDIES ON

THE EFFECTS OF EQUILENIN ON THE EMBRYOLOGICAL DEVELOPMENT OF THE ZEBRA FISH, *Brachydanio rerio* (Hamilton)

By

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THE EFFECTS OF EQUILENIN ON THE EMBRYOLOGICAL DEVELOPMENT

OF THE ZEBRA FISH, Brachydanio rerio (Hamilton)

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INTRODUCTION

The purpose of this study was to determine the effects of equilenin on the embryonic development of the Zebra Fish, Brachydanio rerio (Hamilton). The investigations in this paper were concerned with the cytological, and gross effects of the chemical.

Equilenin is a naturally occuring female hormone as reported by Beall and Grant (1954). It, along with other natural steroids, was tested for its effects on cell division and embryonic development in screening tests (Jones and Huffman, 1957). The chemical produces its own characteristic effects on the fish embryos, which include swollen pericardium, circulatory disorders, swollen intestinal area, and growth abnormalities of the tail and other body parts. The effects of equilenin and a possible explanation of their cause are herein described.

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ing the chemicals and financial assistance.

LITERATURE REVIEW

Equilenin is an estrogenic steroid which can be extracted from the urine of a pregnant mare (Beall and Grant, 1954). A United States patent was obtained in 1948 for this compound. The compound was synthesized from d-desoxyequilenin which was obtained from the neutral fraction of the urine of a pregnant mare by Bachmann and Drieding (1950). Djerassi, Rosenkranz, Rome, Pataski, and Kaufmann (1951) and Mondon (1952) reported new methods for the synthesis of equilenin. Courvier, Horeau, and Jacques (1951) synthesized the compound with no ring, and Horeau (1952) described a recent synthesis of new estrogens which included equilenin.

Preparation or synthesis of a new compound may involve the synthesis of several new intermediate or related compounds. Bachmann and Dreiding (1950) synthesized d-isoequilenin and its methyl ether by use of palladium. Bachmann and Holmes (1951) reported the synthesis of 14,15-dehydroequilenin methyl ether. Johnson, Gutsche, Hirschman, and Stromberg (1951) prepared by Strobbe-condensation many valuable intermediate compounds. Grant and Glen (1952) also obtained a United States Patent for other equilenin derivatives. Habgood (1952) while making studies of the A ring of equilenin synthesized a valuable intermediate compound 4-carbomethoxyequilenin. Eglinton, Nevenzel, Newman, and Scott (1953) and Johnson, Peterson, and Gutsche (1950) reported intermediates occuring during the synthesis of equilenin. Djerassi, Rosenkranz, Kaufmann, Pataski, and Romo (1954) obtained a United

States Patent for the synthesis of 1-methyl-Δ^6-dehydroestrogen.

One of the valuable intermediate compounds obtained with 2-cyano-1-keto-7-methoxy-2-methyl-1,2,3,4,-tetrahydrophenanthrene is 14,15-dehydroequilenin. The compound is readily converted to the final product equilenin. 1-Methyl isoequilenin was prepared by dehydrogenation of 1-methylestrone thus providing the proof of the structure of 1-methylestrone (Dreiding and Pommer, 1953).

The configuration and functional groups of the steroids have been discussed by various authors. Stork and Singh (1950) discussed the resemblance of the c/d ring of equilenin and other steroids that are very similiar to equilenin in structure. Jones, Humphries, Herling, and Dobriner (1951) recognized the principle functional groups in steroids by infrared spectrometry.

Estrogenic steroids occur in mixtures and several reports have been made on methods of separation of such closely related epimeric estradiols as equilenin and equilin. By microanalysis, phenolic estrogens were separated from other phenols, as reported by Boscott (1949). Infrared spectrophotometry can be utilized for the identification of the compounds as reported by Banes (1950). Hoftmann (1951) and Grausberg (1952), by use of paper chromatography, removed equilenin and equilin from an estradiol mixture. Separation of ketosteroids by benzilic acid hydrazide was accomplished by Velluz, Petit, Racine, Amiard, and Joly (1953).

Derivatives of equilenin may be important because they may lead to the synthesis of other important estrogenic steroids. Bachmann, Dreiding, and Stevenson (1951) recorded that the products of hydrogenation of dl-equilenin yielded, 1,2,3,4,-tetrahydroequilenin. When

equilenin was reacted with nitrosodisulfonate other intermediate compounds were obtained by Teuber (1953). McNiven (1954) reported products obtained from the oxidation of equilenin.

Some authors have reported results of the action of estrogens on various tissues and cells. Chang and Witschi (1953) reported on tests involving the action of equilenin and equilin on frog larva. They produced extensive hyperplasia and modified sexual differentation. The optimum conditions for the intravaginal administration of estrogens were obtained by Biggers and Claringbold (1954) and they concluded that certain estrogens reduced growth while others increased growth. Huggins and Jensen (1955) observed the effects of phenolic estrogens on the growth of the uterus and concluded that uterine growth is related to the dosage until a maximum is reached.

Through personal communication, the Biological Screening Office of The Upjohn Company reported that when equilenin was administered subcutaneously to male and female rats, the typical effects of an estrogen; adrenal hypertrophy, thymolysis, and loss of body weight were evident. They also reported that female rats treated with equilenin and estrone gave evidence that equilenin has about one-tenth the estrogenic potency of estrone.

As stated by the Council on Pharmacy and Chemistry of the American Medical Association, the estrogens are the most potent regulators of growth and cellular division of the female genital tract. They produce hypertrophy of the muscle layers and endometrium. An increased water content of organs follows administration. The effects on the vagina seem to be keratization of the superficial cells of the vaginal mucosa.

They also cause extensive growth of the mammary glands.

Several authors have reported the effects of various chemicals on the developmental pattern of Brachydanio rerio. Some of the most recent work has been done by Jones and Huffman (1957, 1958). They discuss the various uses of the embryos in the rapid detection of chemicals which affect cell division and differentation (1957). Jones, Huffman, and Katzberg (1957) noted that some steroids have a much higher cytostatic action than others. The various steroids they investigated were all different in their effects on cell division depending on the steroid and the concentration. Jones and Huffman (1958) reported some new cytostatic steroids possibly useful in the treatment of human cancer of the breast.

Jones (1949) also observed that the toxic effect of antuitrin (growth hormone) was proportional to the concentration of the hormone. Podophyllotoxin was referred to as a mitotic poison because it completely stopped cell division (Jones, 1951).

Ethyl carbamate was tested in six different developmental stages of Brachydanio rerio by Battle and Hisaoka (1953) who found that the chemical elicited a retardation of growth and embryonic structure. Jones, Gibson, and Nichols (1951) noted that the degree of retardation was proportional to the thyroxine concentration and that morphogenesis and pigment formation were greatly retarded while metabolism was accelerated. Gibson (1954) concluded that exposure of the embryos to thyroxine produced marked growth retardation.

Observations have been made on the effects of various physical factors on the development of Brachydanio rerio. Goff (1940) stated

that increased atmospheric pressure caused retardation for a period of time but the embryos were able to regulate themselves. Anderson and Jones (1950) reported on exposure of the embryos to ultra-sonic vibrations. They found that the ability of the embryos to survive exposure to ultra-sonic vibrations increased as morphogenesis occurred. The effects of variations of light and dark background on chromatophore development were observed by Whittington (1952) and he also observed the migration pattern followed by the chromatophores.

Battle (1940), Budd (1940), Harrington (1947), and Moore (1944) reported observations on fish embryology and larval development in connection with life history studies. Some of these papers give the chronological time of organ development.

Ingersol (1949), Price (1934, 1935), Solberg (1938) observed that embryonic differentation in fish could be described chronologically when the oxygen supply and the temperature were kept constant. Hubbs (1943) designated precise terminology for the stages of the early development of fishes.

Oppenheimer (1937) and Balinsky (1948) used a stage-naming system in which development was expressed in terms of the degree of organ and tissue differentation instead of a chronological age. They concluded that the chronological age is not a satisfactory method of expressing the stage of embryonic development because the rate of growth varies with oxygen and environmental temperatures. Balinsky (1948) describes the 46 developmental stages of Cyprinid fishes and exactly which morphological data are associated with a certain stage.

Wilson (1889) reported the complete gross and histological descriptions of the derivatives and the fates of the primary germ layers.

He also summarized the previous work done in fish embryology and compared teleost development with that of higher vertebrates. Wilson's paper is considered a classic in the field of fish embryology.

Various authors have discussed the development of the Zebra Fish, Brachydanio rerio. Roosen-Runge (1938) described the cytological and protoplasmic events occuring in the living egg. He also observed differentation of the egg without cleavage and described cell division and mitotic rate during development.

Creaser (1934) described a technique for collecting and handling eggs of Brachydanio rerio for laboratory use. Goff (1940), Battle and Hisaoka (1951), and Jones (1957) have described their methods for the collection of the embryos.

Jones (1939) and Self (1937) used the "mitotic index" to determine the relationship between cell division and differentation of Fundulus and Gambusia respectively. Oppenheimer (1947) gave an account of the general organization of the teleost blastoderm.

Goodrich and Nichols (1931) working with the regeneration of the portions of the fin of the adult Zebra Fish studied the action of the chromatophores in newly formed appendages. They found that the chromatophores increased in a region of future dark stripes while the chromatophores decreased in the region of future light stripes.

Ingersol and Jones (1949) compared Brachydanio rerio with other exotic fish suitable for classroom work, and discussed the usefulness of various forms of exotic fish in the laboratory. Orton (1954) discussed the use of developmental stages of fish as a rich source of material that is useful in embryological research.

METHODS AND MATERIALS

Many workers have recognized that the Zebra Fish is a useful material for experimental study. Several different cultural techniques have been suggested, e. g., Battle and Hisaoka (1951), Creaser (1934), Goff (1940), Jones (1957), and Roosen-Runge (1938, 1939).

The Zebra Fish, a cyprinid originally imported from India, is easily kept under laboratory conditions and is very popular in tropical fish aquaria. It is small (maximum length, 2.5 inches, average, 1.5 inches), highly colored, active and hardy. It feeds readily on both dry and live food. It becomes sexually mature when about six months of age. Longevity is approximately two years. Spawning is not seasonal and occurs in healthy females about every three or four weeks.

In these studies, the adult fish were kept in a battery of nine, five-gallon, aerated aquaria, each containing some 30 fish in the ratio of two males to one female. The bottom of each tank was covered with glass marbles which protected the freshly laid eggs from the carnivorous adults. Each tank had a filter, which served as an aerator, and a thermostatically controlled heater. The laboratory was air-conditioned and maintained at a year around temperature of 70-80^{0} F. The tanks were maintained at 26$\pm$ 2^{o} C (79^{o}F). The water depth level was maintained at six inches. Fresh tap-water was added daily to replace that lost by evaporation and siphoning for eggs.

The adult fish were fed two types of commercial fish food, plank-

ton, Daphnia major, dwarf white worms of the family Enchytraeidae, brine shrimp of the genus Artemia, and a baby food mixture. The mixture was prepared by mixing; one can of strained liver, one can of cooked egg yolk, and two cans of strained spinach with Pablum; thicking to a paste and cooking for one hour. Creaser (1934) reported that Drosophila (vestigial wing) and plankton, consisting namely of Cyclops served well for food.

The aquaria were arranged in a covered cabinet so that the light could be controlled easily, because a strong light after a period of complete darkness seems to stimulate ovulation (Jones, 1957). This enabled us to control somewhat the time of ovulation and to secure embryos in relatively uniform stages of development.

Breeding activity was usually observed soon after the lights were turned on in the morning and the eggs were then siphoned from the tanks using rubber tubing and a tea strainer. The eggs were separated from the debris by washing and decantation. The debris was usually much lighter than the eggs and was poured off during the decanting process. The eggs were removed with a pipette and were sorted and counted out into culture dishes. During each test, a certain developmental stage was used. In the process of sorting, the eggs were all sorted according to the stage desired. They were usually secured around stages 3-5 (Balinsky, 1948).

Zebra Fish eggs are about 0.5 mm. in diameter, transparent, and can be seen with the unaided eye. The first cleavage usually occurs 35-40 minutes after fertilization and subsequent cleavages occur approximately every 18-20 minutes at 26°C (Roosen-Runge, 1938). The

embryos undergo rapid cleavage and morphogenesis. The animal is completely transparent during the formation of the major organs. The embryos develop into recognizable baby fish in the first 24 hours and usually hatch during the third day, which would be in the later part of the prolarval stage (Hubbs, 1943). The embryos were usually in the 2-64 cell stages at the time of exposure to the chemical. This corresponds to stages 3-8 as reported by Balinsky (1948). The embryos were usually collected within two hours after the time of fertilization.

The number of embryos in the culture dishes varied from 10 to 25 and these were incubated in 50-100 ml. of aerated tap water at 80°F. Into each of these culture dishes were placed certain materials. In one dish, only aerated tap water was present. In another dish propylene glycol was added, while in the other culture dishes certain amounts of equilenin dissolved in propylene glycol were added. The concentration of the stock solution provided by Dr. Huffman, was one mg. of equilenin dissolved in one ml. of propylene glycol.

In the dilutions used in these tests, the propylene glycol produced no increase in the number of deaths. The propylene glycol was used as a control along with aerated tap water because the equilenin was dissolved in the propylene glycol. Cultures were rated on the number of parts per million of the test chemical in the culture solutions. Test concentrations of 0.18, 0.32, 0.56, 1.0, 2.0, 3.0, 5.0, 10.0, 20.0 ppm were used.

Observations and notes of the effects of the chemical on the embryos were made at 6, 12, and 24 hours after exposure and then every 24 hours. The test usually was closed after the 4th day (96 hours).

By the eighth hour of development at 80°F, the embryo is usually approaching the closure of the blastopore (Blumenkrantz, 1956). This corresponds to stage 15 (Balinsky, 1948). If there is any interference with mitosis and cell movements by a chemical, this can be observed readily at this time. Approximately 12-14 hours after fertilization, the main body axis is organized and the process of organogenesis is starting. The purpose of recording observations at this time is to observe any abnormalities in organ formation. The organs have been formed by 24-26 hours after fertilization and if there are any abnormal or retarded structures they can readily be seen at this time. The effects of the equilenin on development of the embryos can be seen very well 24 hours after they were exposed to the chemical.

A wide field binocular Stereomicroscope with eye pieces of 10 or 15X and lens objectives of 1.0 to 7.5X was used for observations of gross anatomical features. This gave a three-dimensional aspect to the observations. The Stereomicroscope was utilized during the tests also for the taking of photographs, and for the removal of chorions from some specimens.

Photographs were made of the embryos with a 35 mm. Leica camera attached to a standard Spencer microscope fitted with a Micro-Ipso. This is an attachment for viewing specimens while photographing them. Kohler illumination with a ribbon-filament lamp was used throughout. Also some pictures were taken with the phase microscope when a particular area was to be studied in detail.

In this study, pictures were made every 24 hours during the first 4 or 5 days of embryonic development. At various times, embryos were

removed and fixed in Bouin's Fluid. Some pictures were made with the chorion present while others were made after the chorion had been removed. The chorion was punctured with microdissecting needles and then torn open, releasing the embryo. The most important reason for removing the chorion is that it becomes adhesive and picks up dust particles which obstruct the view. The chorion of the Zebra Fish is freely permeable to equilenin as evidenced by the fact that there was no significant difference in susceptability when the chorion was or was not punctured. Therefore, it was not considered necessary to remove the chorion of the embryo during the tests.

Other pictures were taken of another series of tests which were designed to test the rate of recovery of the embryo. The embryos were put into the culture dishes containing the chemical for 24 hours and then the equilenin was removed and replaced with freshly aerated tap water. Pictures were made every 24 hours. The pictures were taken of the control specimens, of the embryos from culture dishes in which the chemical was still present, and of the embryos undergoing recovery. Some of the pictures taken during this study are shown in the plates at the end of this paper. They are arranged according to photographic density rather than according to stages or sequence. The purpose of this arrangement is for better photographic reproduction.

Some of the embryos were preserved for future study at various stages of development. They were fixed in Bouin's fluid, and stored in 60-70 percent isopropyl alcohol. All chorions were removed after fixation and the embryos were transferred to pieces of glass tubing which were plugged at both ends with absorbent cotton. A small piece

of paper recording the date of fixation, and the age of the embryos were inserted in the tubes. The tubes were immersed in vials within a larger jar containing 60-70 percent isopropyl alcohol and stored. This made fixed material of all stages available for use or reference at any desired time.

The fixed material was utilized in two different ways. Some of the material was put on a clean slide with a drop of aceto-carmine stain and a smear of the embryo was made and covered with a cover slip. This method is excellent for studying cell structure and size under the microscope.

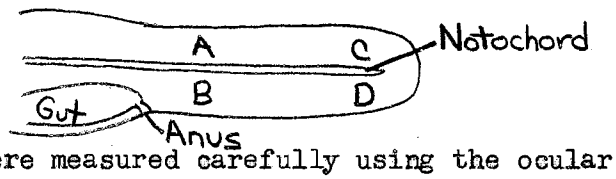
Using these prepared slides it was observed, that the nuclear diameters of the cells of the treated specimens appeared to be of a larger size than those of the control specimens. This led us to make measurements to determine if there were significant differences. In the preliminary tests, measurements were made using an ocular micrometer to measure the diameter of selected nuclei of the treated and of the control embryos. It was observed that there were statistically significant differences between the nuclei of normal and of treated specimens. With the results obtained and with the aid of the Statistical Laboratory a test design was developed.

The fish were collected and divided in three classes. One class was cultured in a two ppm. solution of equilenin. The second class was cultured in a two ppm. solution of propylene glycol as a control. The third class was a group of untreated embryos placed in aerated tap water. All of the embryos were approximately six hours old at the time of exposure, or in the early cap stage. They were allowed to develop for 24 hours before the test counts were started. All of these

embryos were somewhat retarded in expected development probably due to the fact that they were kept at a lower temperature (74°F) during the six hours prior to exposure.

The three cultures or classes were labled I, II, and III. The equilenin treated culture was III. The propylene glycol treated culture was II. Culture I consisted of embryos in aerated tap water. A scheme was set up to draw by lot the embryos from the dishes. The individual eggs were removed in a sequence that was not patternized and which assisted in keeping the time element or degree of development constant in the three classes. Each individual embryo was taken from the culture and treated as follows. The chorion was removed, and the posterior end (tail area) of the embryo was severed and placed on a slide. A cover slip was gently applied so as not to disturb the relative positions of the cells and a squash was made of the tail. The cellular structure and nuclear diameters were studied in vivo under an oil-immersion objective(bright contrast medium) on the Spencer phase microscope.

Certain areas of cells were selected and these areas were designed loci A, B, C, and D, see diagram below. Locus A consisted of cells anterior and dorsal. Locus B consisted of cells anterior and ventral. Locus C was made up of cells posterior and dorsal. Locus D consisted of cells ventral and posterior.



In each locus, ten nuclei were measured carefully using the ocular micrometer. Diameters of the nuclei were estimated to $\frac{1}{4}$ of an ocular unit. The loci were measured in a sequence determined by lot so as to

avoid bias as to location in the embryo. The nuclear measurements were made at three different times. The first was approximately 24 hours after exposure to the chemical. The second was approximately 29-30 hours after exposure. The third was 34 hours after exposure to the chemical.

The ocular counts of the nuclei were then treated statistically to see if there was a significant difference between the diameters of the cell nuclei in the three classes, the four regions or loci, and in the measurements taken at different stages of development.

Some of the fixed material was also dehydrated in isopropyl alcohol. The embryos were then cleared in xylene. The embryos were embedded in soft paraffin (52-54°C), mounted in paraffin blocks. A rotary microtome was used to cut serial sections of the embryos. The sections were mounted on a slide and stained in Harris' hematoxylin followed by an eosine counterstain. Some were stained in Heidenhain's iron-hematoxylin. Poor adhesion of the sections occurred so that comparative histological studies were not feasible. Some studies of the cytological structure were made using these slides and those prepared by the squash technique.

A special experiment sheet was used for each separate test and contained such needed information as: date of the experiment, developmental stages of the eggs at the time of exposure to the chemical, number of hours exposure to the chemical, parts per million of the chemical in each culture dish, and the number of eggs in each culture dish. Readings or observations were made after certain time periods and the information that was recorded with each observation period

consisted of: the number dead, the number dying, the number abnormal, the number retarded, the amount of pigmentation at the time of the reading, and any other unusual observation which might indicate the effects of the treatment on the normal embryonic differentation. Each culture was replicated twice in each test. A sample data sheet is included in the appendix.

EXPERIMENTAL DATA AND OBSERVATIONS

General observations of effects

The embryos used in the experiments demonstrated retardation in direct proportion to the concentrations of the chemical used and they showed the effects in solutions as low as 0.32 ppm. A concentration of 1.8 ppm caused death in approximately 72 hours after fertilization, (stage 30-Balinsky, 1948). The embryos that were exposed to the chemical were unable to hatch except in the lowest concentration(0.18 ppm). All of the embryos used in these tests demonstrated certain abnormal and retarded structures after exposure to equilenin. These abnormalities will now be discussed in more detail.

(A) Pigmentation

In all concentrations, there was a tendancy toward abnormal distribution of melanophores of both the eyes and the body. At the higher concentrations the pigment was absent or very sparse (Plate 7, Figures 2 and 3).

In general, the development of pigmentation in the Zebra Fish follows that described by Balinsky for cyprinid fishes (1948). The first sign of pigmentation appears in the eye in the antero-cranial portion of the optic cup around 25 hours. The area becomes darker and wider, covering more and more of the optic cup (27-28 hours). The melanophores are distributed on the body and tail of the embryo in four longitudinal lines. The dorsal line, consisting of one or more

rows of cells, is located along the dorsal edges of the myotomes. The lateral pigment line, consisting of one row of cells, runs along the middle of each side. The ventro-visceral line consists of several rows of cells in the trunk region lining the upper part of the body cavity. In the tail region this line is continued along the ventral edge of the myotomes. In addition to these paired lines, there is also a fourth unpaired line, the midventral line, running along the ventral border of the gut in the trunk region.

Pigment cells are also located in the interior of the body and they can be separated into three areas: (1) The melanophores located in the walls of the pericardium and yolk sac. The heart itself is entirely free of pigmentation. (2) The melanophores of the horizontal myoseptum. This is the connective tissue membrane that separates the dorsal from the ventral longitudinal muscles of the body. (3) The melanophores of the nasal region. They adhere to the wall of the nasal pit.

The pigmentation of the eye did not appear in the treated specimens until approximately 28-30 hours after fertilization. There was a large reduction of pigmentation in the treated specimens compared to the normal embryos.

After 48 hours of development the melanophores were in great abundance in the normal embryos (Plate VII, Figure 1). In the treated specimens, there were few melanophores present on the yolk sac and around the area of the pericardium (Plate VII, Figure 2). There was sparse pigmentation in the optic cup of the eye. The three main paired lines along the body had some melanophores present but they were very much reduced in size and number.

In general, the reduction in pigmentation was in direct proportion to the concentration of equilenin used. However, some of the reduction may be due to propylene glycol rather than the equilenin. A propylene glycol solution of 2.0 ppm inhibits pigmentation on the body but does not seem to affect the eye pigment. However, in a 1.0 ppm solution of propylene glycol there are not any visible effects of reduction of pigment. In a 1.0 ppm solution of equilenin the pigment development is greatly retarded.

(B) Eye

The eye was also affected by the 1.8 ppm concentration of equilenin. It was not deformed or abnormal in any way but appeared to be retarded in growth by the presence of the chemical. In the normal embryo, the optic vesicles appear as outpocketings from the posterior part of the prosencephalon around 9 hours. An inpocketing at the center of each vesicle is seen to be formed as a longitudinal slit which deepens in the spherical surface of the oval vessicles. The ectoderm thickens above the cup and separates from the rest of the ectoderm to become the primitive crystalline lens. The optic stalk formation takes place by constriction of the basal portion of the optic cup. The iris of the eye is formed around 24 hours on the outer border of the optic cup. The eye contains both gold and black pigment. The gold pigment was only visible (48 hours) when the black pigment failed to develop. The equilenin did not appear to keep any of the structures of the eye from developing, but it did slow down or retard the development of these structures. In some cases, in the stronger concentrations, the optic cup had just developed 48 hours after fertilization.

(C) Pericardium, heart area, and circulation

The pericardium, heart and the circulation seemed to be especially sensitive to equilenin. The pericardial sac appears as a thin flat sac upon the yolk. It extends in a perpendicular plane from beneath the eyes to the ventral side of the yolk sac. The heart appears as a simple tube within the pericardium. A constriction of the tubular heart separates the atrium from the ventricle. Circulation begins about 30 minutes after the heart starts to beat. This is approximately 28-30 hours after fertilization. The weaker concentrations of equilenin did not appear to affect the development of the heart and circulation, but it caused the pericardial sac to swell to approximately 2-3 times its normal size.

The stronger concentrations of the chemical caused a large swelling of the pericardium (Plate 1, Figure 8 and Plate IV, Figure 9). The heart appeared to be developed normally but the beat was very erratic. At first there would be a series of strong rapid beats followed by a series of very weak beats. The circulation appeared to be conjested in areas of the yolk sac and in some of the tail areas while other areas of circulation appeared normal.

(D) Tail and gut

The development of the tail begins anterior to the point of blastoporal closure (dorsal tip). Here there is formed an undifferentiated mass of cells, which comprises the future tail bud. The first major change is the constriction of the tail away from the yolk (18-19 hours). As this separation from the yolk continues, the yolk sac which was ovoid in shape, begins to constrict in the region ventral and anterior to the tail bud. This constriction forms the hindgut which is located ventral to the anterior portion of the tail. The tail is formed

concurrently with the hind gut by the posterior elongation. The hind gut continues to lengthen by the continual constriction from the yolk mass, and elongation. The tail continues to lengthen and eventually becomes free from the yolk. The anus appears as an invagination of the ectoderm which connects the hind gut to the exterior.

In all the tests that were run in the laboratory, the gut and tail area were by far the most sensitive to equilenin. The whole area of the gut is retarded in structure to about one-half the normal size. The very short area that is visible from the yolk sac posterior to the anus is swollen from 1 to 4 times its usual size depending on the concentration used. In the 3.0 ppm concentration the gut will be twice as thick as the tail area dorsal to the gut (Plate 4, Figure 9). In the normal embryo, the gut should be about one-fourth the thickness of the tail. The tail does not appear especially abnormal in structure but appears more to be retarded in growth. The tail of the normal embryos should be longer than the rest of the body area anterior to the yolk sac (Plate 1, Figure 1). In the 1.8 ppm concentration, the tail is reduced to approximately one-fifth the normal size and has become more thickened (Plate 4, Figure 9).

(E) Pectoral appendages

The pectoral appendages appear as bud-like growths on each side of the median body axis near the myelencephalon around 30 hours. As development continues, the fin bud becomes larger and extends farther from the body axis. The distal end becomes serrated and will develop into a functional fin before hatching. Equilenin definitely stops the development of these fins. The pectoral appendages did not appear in

any of the embryos that were exposed to 0.32 ppm of equilenin or higher. In the normal 72 hour embryos the fins were evident and well developed (Plate V, Figure 2).

The above were some of the typical equilenin effects that occurred. They were never produced until after the embryos had developed for 20-24 hours. This tends to indicate that the embryos must reach a certain stage of differentiation before the equilenin can produce its effects.

The only effect produced during the early hours of exposure was a normal fish kill. In Table 4, it can be seen that at the 6th and 12 th hour readings there were no visible effects except that the death rate in the treated specimens was greater. The normal death rate during the first 24 hours of life in the Zebra Fish is about 10 percent. This usually occurs during the first 12 hours. In Table 4, the 24 hour reading corresponds to approximately 26-27 hours after fertilization.

Since all of the body organs are usually formed in the first 24 hours, observations or readings were made daily thereafter; i. e., at 48, 72, and 96 hours. Equilenin affects the embryos much more the second day than it does the first. In the 0.32 ppm concentration, all of the embryos were either dead or abnormal after 48 hours exposure. At the higher concentrations, the effects were much more severe with approximately half of the embryos dead in 48 hours. At the 72 hour observations period, it was noted that the control embryos were hatching while the other treated embryos were not approaching the hatching stage. An increase in fish kill because of the long exposure to the chemical was observed at the end of the 96th hour of exposure (Table 4). The 0.32 ppm concentration embryos were greatly retarded and abnormal while development of the embryos in the stronger concentrat-

ions was completely arrested. The embryos in the propylene glycol culture and the 0.18 ppm equilenin culture were hatched and appeared normal except for the partial absence of pigmentation in the specimens in propylene glycol.

Exposure during early cleavage

From February 8, 1957 to January 3, 1958 several series of tests were run and data compiled (Table 4). The embryos used during these tests were in early cleavage corresponding to stages 3-4 (Balinsky, 1948). They were exposed to equilenin in concentrations of 0.18, 0.32, 0.56, 1.0, and 1.8 ppm. Again the typical equilenin effects were not observed until after 24 hours of exposure. After 48 hours exposure, the effects were very evident in the 0.32 ppm culture but there were no apparently abnormal embryos in the 0.18 ppm culture. In the 1.8 ppm, development was completely arrested.

The 72 hour observations were approximately the same as the 48 hour except the long exposure to the chemical was becoming evident at this period. A larger number of embryos were dead in the solutions of higher (1.0 and 1.8 ppm) concentrations. At the time of the 96 hour observations, a still greater number of embryos were dead. At the higher concentrations of 1.0 and 1.8 ppm some of the embryos were so arrested that it was difficult to tell the posterior body areas from the anterior. At this time, the embryos of the 0.18 ppm culture and the controls were hatched.

In Table 4, the percentage of the number dead, normal, and abnormal and retarded is readily seen. Therefore, at any given concentration and at any given time, the observational data on the effects are shown.

Exposure during late cleavage

In the same period from February 8, 1957 to January 3, 1958, several other series of tests were run and the data are compiled in Table 5. This table shows in graphic form the percentages of normal, abnormal and retarded, and dead embryos. The embryos used during these tests were exposed to equilenin in the various concentrations during late cleavage, stages 5-8 (Balinsky, 1948). During these tests, the same results were obtained as when the embryos were exposed while in early cleavage. There seemed to be a greater fish kill when the embryos were exposed during early cleavage. At the time of the 96 hour observation period, all of the embryos in the 0.32, 0.56, 1.0, and 1.8 ppm concentration cultures were affected. The control embryos and the 0.18 ppm culture dish embryos were hatched and normal.

Recovery ability of the embryos

From September 25, 1957 to March 25, 1958 another series of tests was run and the data compiled (Table 6). Results of these tests are presented in Plates 2, 3, 5, and 6. These tests were designed to test the rate of recovery of the embryo after the equilenin was removed. In these experiments, the tests were set up as shown in Table I. One half of the embryos were exposed to the equilenin and allowed to stay in this chemical for 96 hours. In the other half of the test, the embryos were exposed to the chemical for 24 hours. The embryos were then removed from the chemical and washed in fresh water and put back in culture dishes containing only fresh aerated tap water. The 24 hour observation period showed there was a fish kill of approximately 40% in the 1.0 and 1.8 ppm cultures. The other 60% of the embryos of

these cultures was abnormal and retarded with some of the embryos completely arrested.

At the time of the 48 hour observations, or 24 hours after the removal of the chemical, some of the 0.32 ppm culture embryos were abnormal but others had begun to recover. At the higher concentrations (1.0 and 1.8 ppm), the embryos were still very abnormal. However, 24 hours later at the 72 hour observations, all of the 0.32 ppm culture was fully recovered and appeared normal. Approximately 30% of the 1.0 ppm and 1.8 ppm cultures appeared to have recovered while 30% were still abnormal and retarded. When the recovery test was terminated 72 hours after removal from the chemical, there were only 10% of the embryos in the concentrations of 1.0 and 1.8 ppm that did not appear to have completely recovered.

Table 6 shows in graphic form the data on the recovery test. Part (a) is set up to show percentage recovery of the embryos. It was found that as the observation time increased or the time in which the embryos were removed from the chemical, the percentage of fully-recovered embryos increased. Part (b) consists of the half of the test where the chemical was not removed. The results of this part of the test show that at the higher concentrations, 100% of the embryos were dead or abnormal and retarded.

It was observed during the recovery tests that pigment cells appeared to be very quick to recover from the effects of the hormone. The eye, heart, and circulation also appeared to be able to completely recover in a short time. The pericardium and the tail area took a much longer time to show the signs of recovery. The gut regained

normal size and shape much more quickly than the tail. In some cases the tail never did completely recover from the effects of the equilenin. In some cases, the tail demonstrated a very typical curving (Plate V, Figure 6 and Plate VI, Figure 6).

Severity of the effects on the embryonic development

Tables 7 and 8 show the severity of the effects with the different concentrations and lengths of exposure. In the 1.8 ppm culture, after 24 hours of exposure the embryos were completely arrested, and development had been stopped. In the 0.56 and 1.0 ppm cultures the embryos were very abnormal but development was still progressing. The 0.18 and 0.32 ppm cultures produced no detectable effects in the first 24 hours. At the 48 hour observation period, it was noted that in the 1.0 ppm culture the growth had stopped and now the lower concentration of 0.32 ppm also showed the typical effects. When the 96 hour observations were made, the embryos in the lower concentrations were abnormal while in the higher concentrations the embryos had completely stopped development.

Table 9 shows the severity of the effects for another group of tests. In this group of tests, the embryos were allowed to develop for 24 hours and then were exposed to the hormone. It was noted that six hours after exposure to the equilenin, the effects were very apparent and all the embryos were abnormal. Again in these tests, as exposure time progressed the severity of the effects became more intense. At the 96 hour observation time, the embryos in the lower concentrations were abnormal while in the higher concentrations embryonic development was completely arrested.

In Table 10, the severity of the effects for the recovery test was noted. Observations were made 6 and 12 hours after the removal of the chemical. In the 1.8 ppm culture the development of the embryos was arrested. In the 0.56 and 1.0 ppm cultures the embryos were starting to develop. Recovery seemed to be progressing very well in the cultures from the concentrations(lower) 72 hours after removal. However, in the 1.0 and 1.8 ppm cultures, the embryos still showed retardation. At the time of the 96 hour observations (four days after removal of the hormone), all of the embryos were recovered except in the 1.8 ppm culture. Almost all of these were recovered except for a few that appeared still slightly retarded in the region of the tail.

Important steroids and estrogenic steroids

Plate VIII in the appendix shows the structural formulae of some important steroids and some of the estrogenic steroids that have been tested in this laboratory. It was found that although some of these compounds differ only in the presence or absence of a methyl group, hydroxyl group, or the location of a double bond, they differ very much in their effects on the development of the fish embryo.

Other test observations

Variations of the concentrations of the chemical and of length of exposure time were made in another series of tests. One group of tests consisted of increasing the concentrations to 3.0, 5.0, 10.0, and 20.0 ppm. Similiar results were obtained in these tests. However, the effects were much more intense than in the previous high concentration of 1.8 ppm. Another group of tests was run to test the time for a total fish kill. In these tests it was found that none of

the fish in the 0.56, 1.0, and 1.8 ppm concentrations were able to live more than seven days in the presence of the chemical while approximately 95% were dead within six days after administration of the hormone.

Cytological observations

The study of the cells in the sectioned embryos and in the squashed embryos stained with aceto-carmine gave the following information. In those areas where the effects of the equilenin seemed to be most severe, it was noted that some of the intracellular elements were absent. The nuclei of the cells in the embryos treated with equilenin seemed to be larger than they were in those cultured in propylene glycol and water. In the hormone-treated cells, the chromosomes appeared to be larger and thicker than in the controls. The chromatin threads and network seemed to be denser and more heavily stained in the cells from equilenin-treated embryos.

The results from the statistical study of the diameter of the nuclei of treated and untreated cells confirmed at least one of the above impressions. The size of the cells, based on the nuclear diameters, of the embryos cultured in 2.0 ppm of equilenin were significantly larger than were the cells of the controls.

The raw data and measurements of the diameter of the randomly selected cells are given in Table 12 in the appendix. The statistical data based on these measurements are presented in the following tables:

TABLE 1
ANALYSIS OF THE VARIATIONS IN THE DIAMETER OF CELL NUCLEI

Source	df	ss	ms	f
Total	1079	4,200.028		
Replications	8	8.610	1.076	
Treatments	2			
(I & II) vs. III	(1)	3,672.535	3,672.535	13,335.00 P<.001
I vs. II	(1)	0.735	0.735	2.67 P<.05
Error (a)	16	4.400	0.275	
Location	3	0.378	0.126	< 1.00
Location X Treatment	6	12.632	2.105	6.05 P<.01
Error (b)	72	25.023	0.348	
Between cells within location	972	475.715	0.489	

df- degrees of freedom
ss- sum of squares
p- probability
ms- mean of squares
f- ratio

TABLE 2
LOCATIONS X TREATMENTS

Loc / Treat	A	B	C	D	Totals
I	483.00	473.50	473.50	483.00	1913.00
II	487.75	487.00	479.75	481.50	1936.00
III	831.25	838.25	836.75	826.50	3332.75
Total	1802.00	1798.75	1790.00	1791.00	7181.75

Treatment SS: 3673.27

GF: 47756.975

Location SS: 0.378

Location X Treatment: 12.632

TABLE 3
REPLICATIONS X TREATMENTS SS

1	219.50	219.50	383.75	822.75
2	209.25	221.75	376.50	807.50
3	213.75	212.00	369.00	794.75
4	213.75	212.25	364.00	790.00
5	211.50	213.75	368.50	793.75
6	213.00	214.50	368.50	796.00
7	213.75	213.75	369.00	796.50
8	210.25	219.00	368.50	797.75
9	208.25	209.50	365.00	782.75

Treatment SS: 3673.27

Rep. SS: 8.61

Rep. X Treatment SS: 4.4

DISCUSSION

Equilenin has been shown to affect the embryonic development of the Zebra Fish in concentrations as low as 0.32 ppm. These effects have been shown to be characteristic of the hormone and to be directly proportional to the concentration of the chemical used. That the effects are primarily due to retardation in the process of organogenesis seems evident by the fact that recovery occurred when the chemical was removed. This would perhaps indicate that the hormone interfers with the metabolism of the cell and the process of morphogenesis.

The cytological and histological studies were not complete enough to justify any conclusions concerning the selective effects of equilenin upon a particular tissue. There was some morphological evidence that certain tissues were affected more than others but the recovery data would seem to contradict this.

It was found that the equilenin caused certain abnormalities, for example, a vast reduction of pigment. This lack of pigment formation could be the result of several factors. Equilenin could prevent the formation of melanin, or it could prevent the formation of melanophores. However, it seems more likely that the equilenin actually retards the process of mitosis and morphogenesis so much that the cells that produce pigment are not developed. Propylene glycol in a 2.0 ppm concentration may actually stop the production of melanin in

the body area while no visible effect is detectable in the eyes. The eyes are also sensitive to equilenin. They did not appear abnormal in structure, but the equilenin seemed to have retarded development.

The gut and pericardium show a very typical abnormal shape after exposure to the hormone. This may also be the result of the vast retardation of body processes. These areas show signs of swelling while other affected areas have not done this. The Council on Pharmacy and Chemistry of the American Medical Association reveals that estrogens produce an increase in water content of the tissues after administration. This could be an explanation for the large swollen areas. The gut, while very much swollen, is also very much retarded in length. The tail area is not particularly abnormal in shape but it is very much retarded in development by the chemical, possibly because the chemical affects the cells in such a way that they can not undergo mitosis at the usual rate.

It was found that when the embryos were exposed to the hormone the pectoral appendages did not develop, while in the recovery specimens they were formed. This was due probably to the fact that the cells that organize and produce these fins have been so retarded that they are not fully produced or developed while in the presence of the chemical.

It was found that when the embryos were exposed in early cleavage, late cleavage, or 24 hours of age the same results were obtained. Thus, the chemical probably becomes affective in its action at a certain period of development. The effect is probably not a disruption and disorganization of the tissues but merely a stoppage of develop-

ment.

Through the observations of the cytological detail of the embryos, it was found that the cell size and nuclear diameters had increased in the treated specimens. The increase in cell size could cause part of the swelling of the different areas.

"The size of the nucleus is variable, but in general it bears some relation to that of the cytoplasm. This may be expressed numerically in the so-called nucleoplasmic index (NP) (R. Hertwig).

$$NP = \frac{Vn}{Vc-Vn}$$

Vn being the nuclear volume and Vc the volume of the cell."

"This NP index states that a relationship exists between the volume of the cytoplasm and that of the nucleus, of such a nature that when the former increases, the second also should increase. The lack of maintenance of the NP ratio would seem to act as a stimulus to cell division. In general, younger cells have larger nuclei, but this rule is not constant." (DeRobertis, Nowinski, and Saez, 1954).

The increase in the nuclear diameter of the cell could result from two major factors. First a polyploid condition, which is a duplication of chromosomes without cell division. This does not seem as likely as the second factor, but one point that could be very significant is the increased thickness and density of the chromosomes of the treated specimens. The second possibility might be that mitosis has been retarded or stopped and the tissues are therefore not formed and differentiated. This would explain the vast retardation of the different affected areas.

It is not known just how the chemical would affect mitosis but

the most probable explanation is that it interfers with the actual metabolism of the cells. It could interupt certain enzyme chains that break down certain food and release energy that would normally be utilized so that mitosis and differentation could occur in the usual fashion. The undividing cells might, however, increase in size instead of dividing and differentiating. Since equilenin does seem to have some affect on the process of mitosis, it is very possible that this drug could be used in the future in treatment of certain types of cancers.

It would appear very desirable for further studies in this area to see if the nuclear diameters decrease more and more with age. Also another study could be made to test the nuclear diameters of the cells of the recovered embryos to see if they return to normal size. Studies of the relative numbers of cells in the various stages of mitosis in treated and untreated material might be indicative (mitotic index). No abnormal or amitotic cells were observed.

By analysis of the results of the statistical data, some very interesting factors were observed. As the age of the embryos in all classes increased, the diameters of the nuclei decreased slightly. These results are shown in Replication X Treatment (Table 3). There did not appear to be a significant difference between the nuclear diameters of the cells in different loci as can be seen in Locations X Treatment (Table 2). The large mean square, 0.489, between the cells within the loci when compared with error (a) indicates that different types of cells might have been measured. The location x treatment mean square might be false due to the excessive size of the

treatment square. The mean square for replications might arise from three sources: namely, fatigue in making measurements, maturity of the embryos, or just random effects. Further study on this is advised.

SUMMARY

A study of 1,460 embryos of the Zebra Fish, Brachydanio rerio, reveals that these embryos are sensitive to equilenin concentrations as low as 0.32:1,000,000 (ppm) and that stronger concentrations of 3.0:1,000,000 (ppm) are not immediately lethal. Three developmental stages of the teleost were used during these tests. Early cleavage, late cleavage, and day-old embryos were exposed to various concentrations and exposure periods varying from 96 hours to seven days.

It was found that the severity of the effects produced was directly proportional to the concentrations of the solution and the length of exposure. The results of the tests indicated that the chemical was specific in its action because all of the embryos reacting to to the chemical showed typical equilenin effects (Plate IV, Figure 9).

Several hundred embryos were given a recovery test and only about 10 percent of these embryos were unable to recover. It was found that the recovery rate was in direct proportion to the concentration of equilenin and the length of recovery time.

These typical equilenin effects consist of the following:

1. Equilenin in concentrations as low as 0.32 ppm retards the production of melanophores and pigmentation.
2. Heart and extra-embryonic circulation seemed to be very sensitive to equilenin.
3. The gut and pericardium were extensively swollen.
4. The eyes are undeveloped at some of the higher equilenin concentrations.

5. The body and tail area were greatly retarded and development was stopped in the stronger solutions.

6. The nuclear diameters of the cells were increased in the presence of the chemical.

7. Certain intracellular elements were absent in the areas of extreme effect, and much of the tissue had a granular appearance.

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APPENDIX

Plate I. . . . . .

Figure 1. Normal Brachydanio rerio embryo, 26 hours after fertilization.

2. Normal Brachydanio rerio embryo, 50 hours after fertilization.

3. Brachydanio rerio embryo, 50 hours after fertilization and 48 hours after exposure to 1.8 ppm of propylene glycol.

4. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 0.18 ppm of equilenin.

5. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 0.32 ppm of equilenin.

6. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 0.56 ppm of equilenin.

7. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.0 ppm of equilenin.

8. Brachydanio rerio embryo, 74 hours after fertilization and 72 hours after exposure to 1.0 ppm of equilenin.

9. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.8 ppm of equilenin.

Plate I

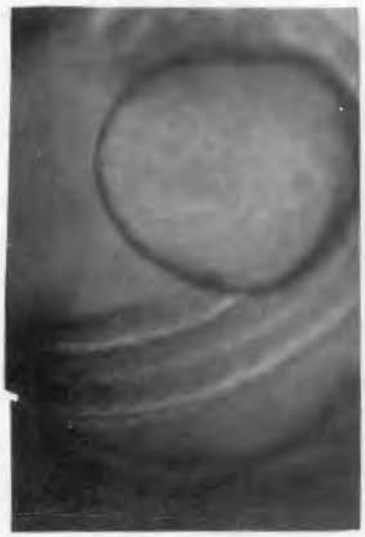


Figure 1.

Figure 2.

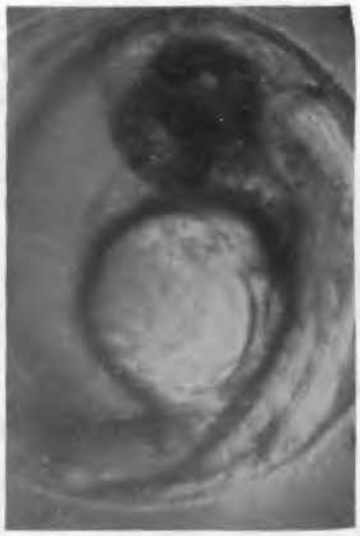
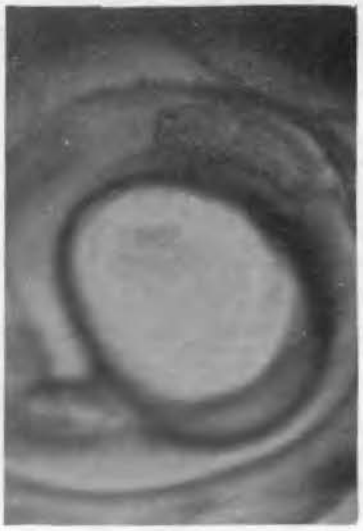


Figure 3.

Figure 4.

Figure 5.



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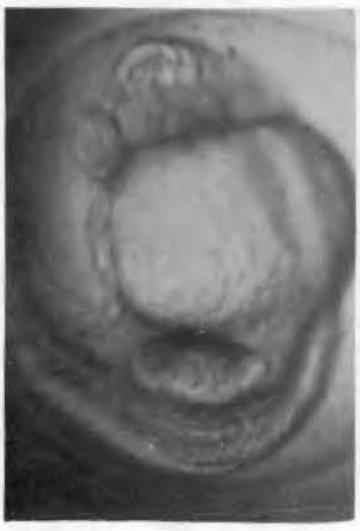

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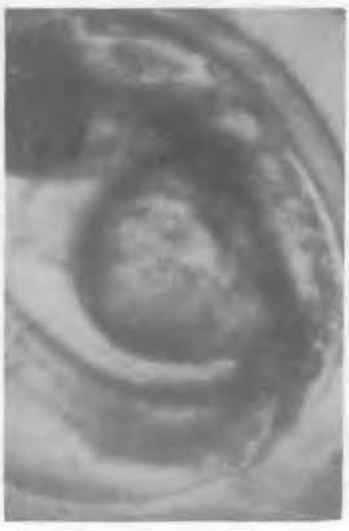


Figure 8.

Figure 9.

Plate II. . . . . .

Figure 1. Brachydanio rerio embryo, 24 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-50 hours.

2. Brachydanio rerio embryo, 48 hours after removal from 0.18 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

3. Brachydanio rerio embryo, 48 hours after removal from 0.18 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

4. Brachydanio rerio embryo, 48 hours after removal from 0.32 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

5. Brachydanio rerio embryo, 24 hours after removal from 0.32 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-50 hours.

6. Brachydanio rerio embryo, 24 hours after removal from 1.0 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-50 hours.

7. Brachydanio rerio embryo, 24 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-50 hours.

8. Brachydanio rerio embryo, 24 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-50 hours.

9. Brachydanio rerio embryo, 48 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

Plate II

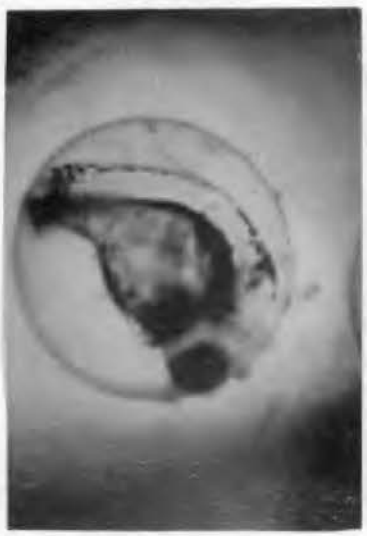
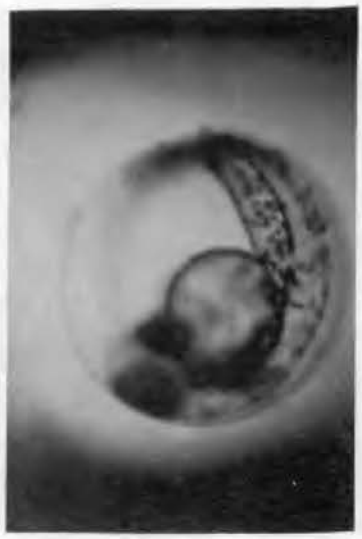


Figure 1.



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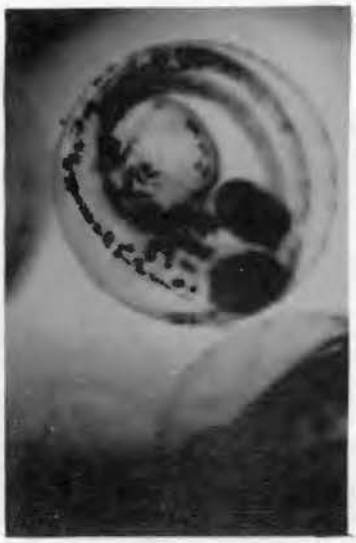

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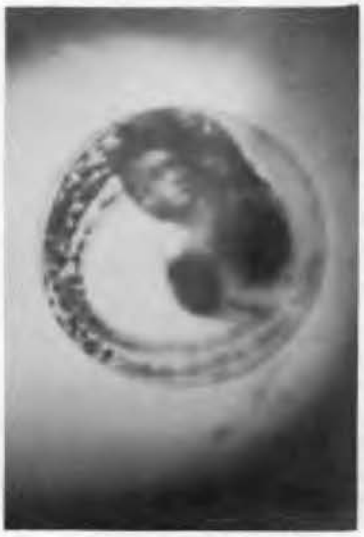

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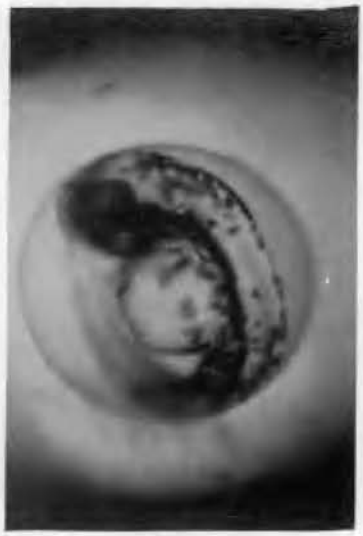

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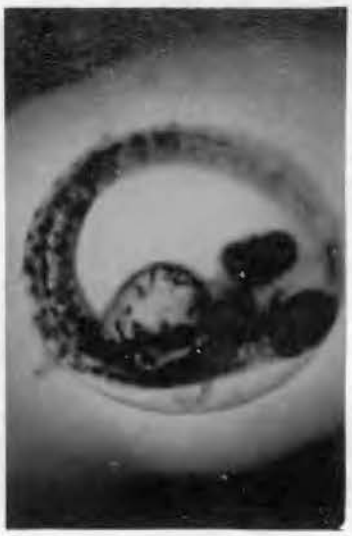
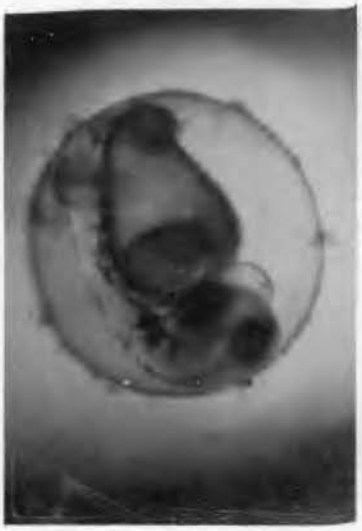


Figure 6



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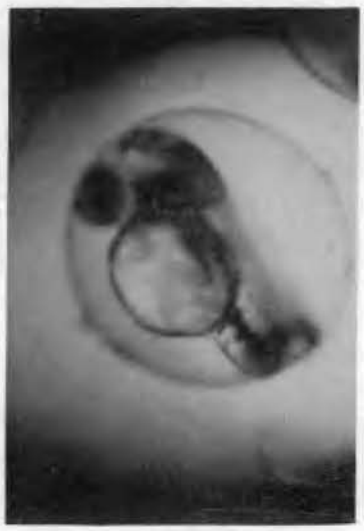

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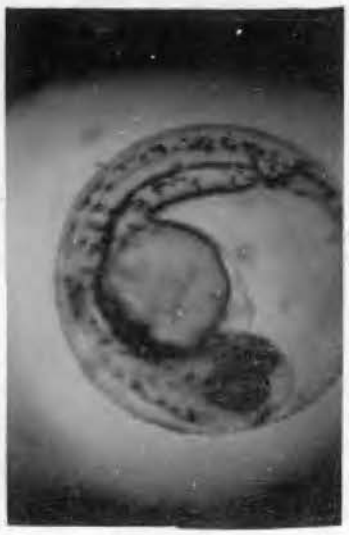


Figure 9.

Plate III. . . . . .

Figure 1. Brachydanio rerio embryo, 72 hours after removal from 0.32 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-98 hours.

2. Brachydanio rerio embryo, 48 hours after removal from 1.0 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

3. Brachydanio rerio embryo, 48 hours after removal from 1.0 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

4. Brachydanio rerio embryo, 48 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

5. Brachydanio rerio embryo, 72 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-98 hours.

6. Brachydanio rerio embryo, 48 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-74 hours.

Plate III

Figure 1

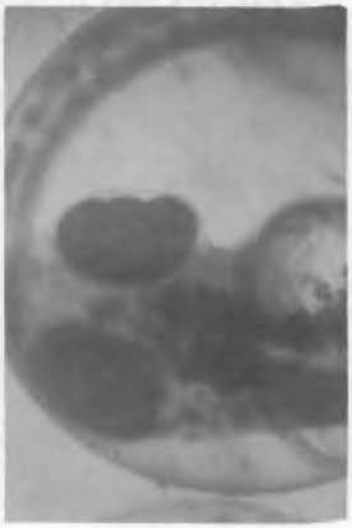


Figure 2

Figure 3

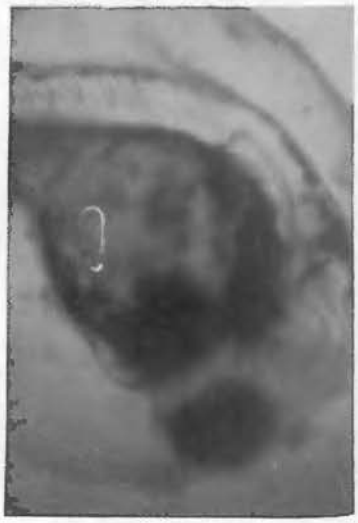


Figure 4

Figure 5.

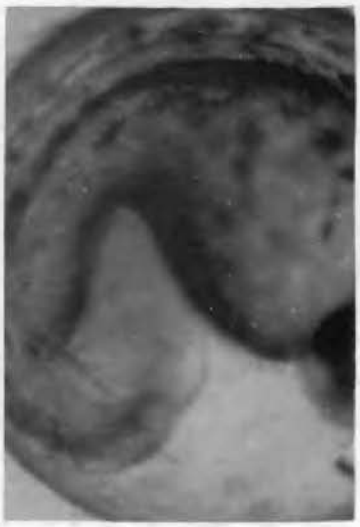


Figure 6

Plate IV. . . . . .

Figure 1. Brachydanio rerio embryo, 98 hours after fertilization and 96 hours after exposure to 1.8 ppm of equilenin.
2. Normal Brachydanio rerio embryo, 26 hours after fertilization.
3. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 0.18 ppm of equilenin.
4. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.0 ppm of equilenin.
5. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.0 ppm of equilenin.
6. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.8 ppm of equilenin.
7. Brachydanio rerio embryo, 26 hours after fertilization and 24 hours after exposure to 1.8 ppm of equilenin.
8. Brachydanio rerio embryo, 50 hours after fertilization and 48 hours after exposure to 1.8 ppm of equilenin.
9. Brachydanio rerio embryo, 50 hours after fertilization and 48 hours after exposure to 3.0 ppm of equilenin.

Plate IV

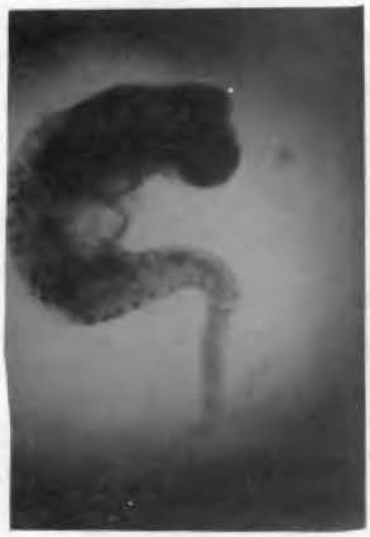
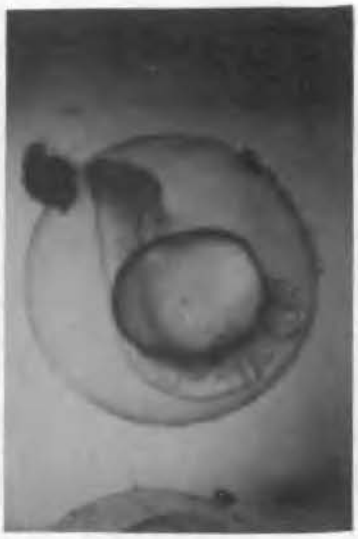


Figure 1.



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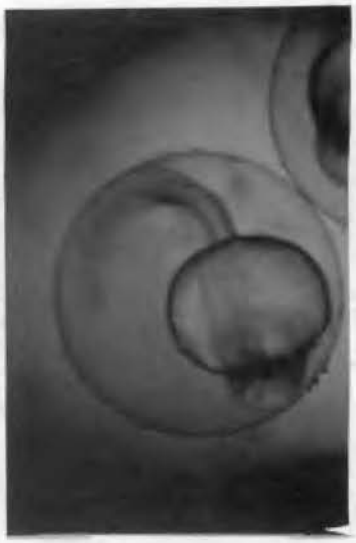

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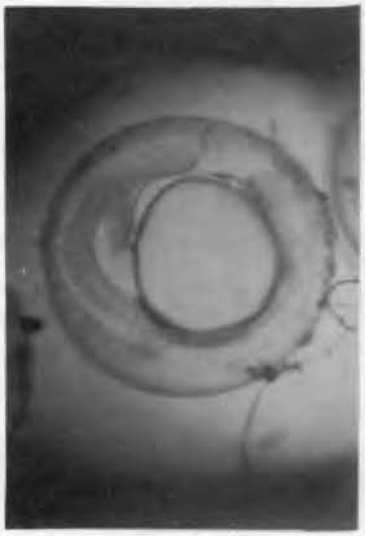

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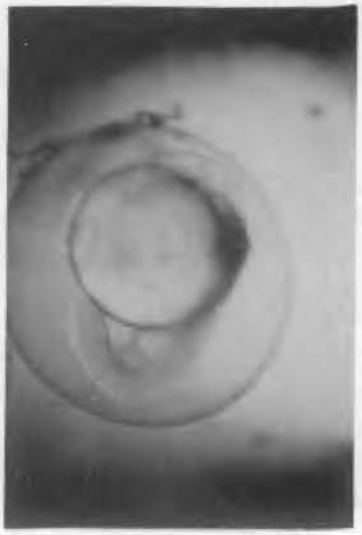
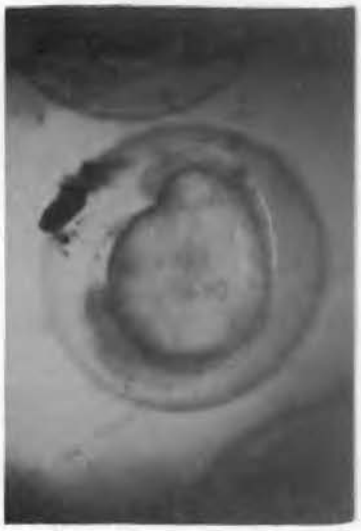


Figure 5.

Figure 6.



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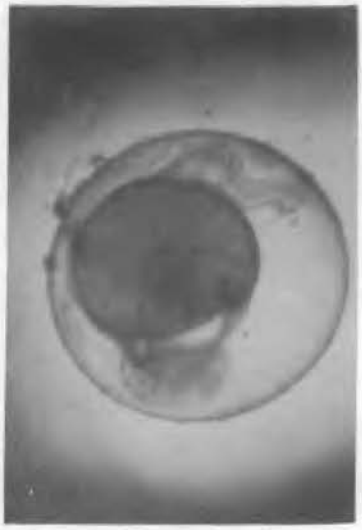

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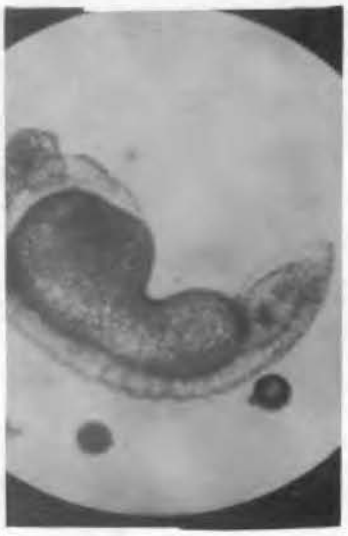


Figure 9.

Plate V. . . . . .

Figure 1. Brachydanio rerio embryo, 72 hours after removal from 1.0 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-98 hours.

2. Normal Brachydanio rerio embryo, 74 hours after fertilization.

3. Normal Brachydanio rerio embryo, 98 hours after fertilization.

4. Brachydanio rerio embryo, 96 hours after removal from 0.18 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

5. Brachydanio rerio embryo, 72 hours after removal from 0.32 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-98 hours.

6. Brachydanio rerio embryo, 96 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

Plate V

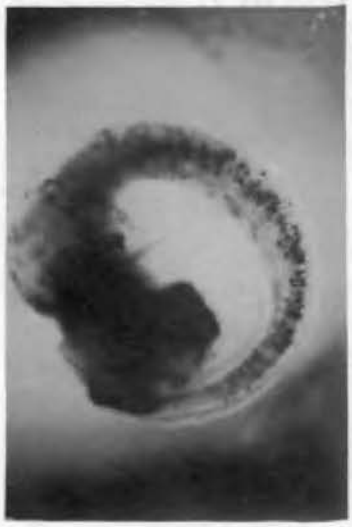
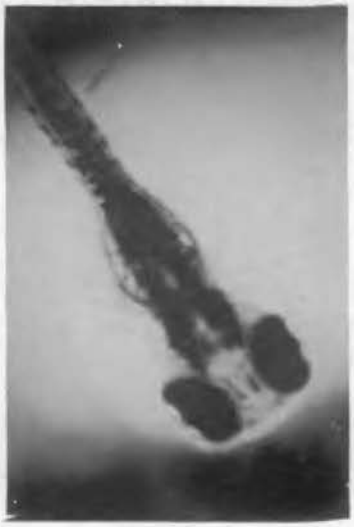


Figure 1.



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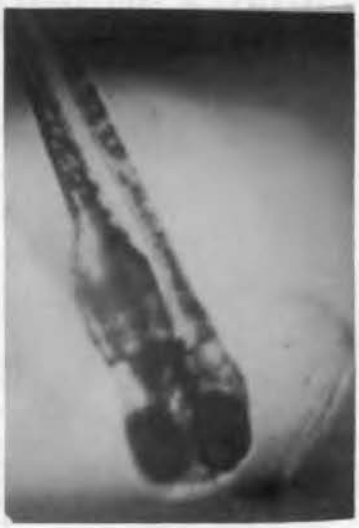


Figure 3.

Figure 4.

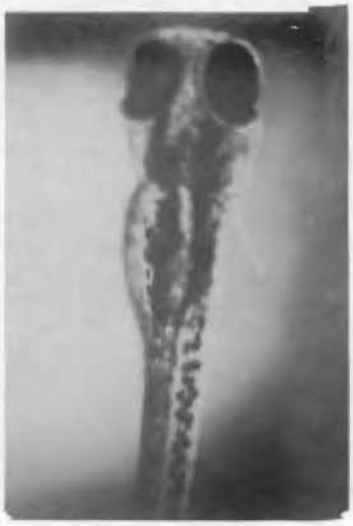


Figure 5.

Figure 6.

Plate VI. . . . . .

Figure 1. Normal Brachydanio rerio embryo, 74 hours after fertilization.

2. Normal Brachydanio rerio embryo, 74 hours after fertilization and fixed in Bouin's Fluid.

3. Brachydanio rerio embryo, 96 hours after removal from 0.56ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

4. Brachydanio rerio embryo, 96 hours after removal from 1.0 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

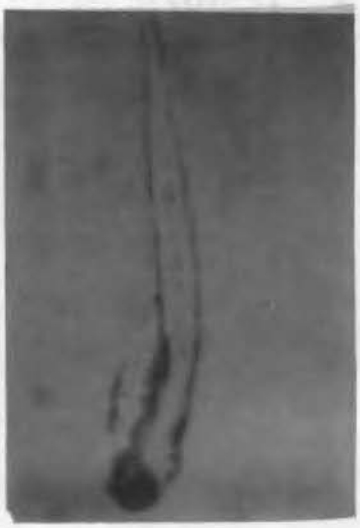
5. Brachydanio rerio embryo, 96 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

6. Brachydanio rerio embryo, 96 hours after removal from 1.8 ppm of equilenin. Was exposed for 24 hours. Total age of embryo-120 hours.

Plate VI

Figure 1

Figure 2.



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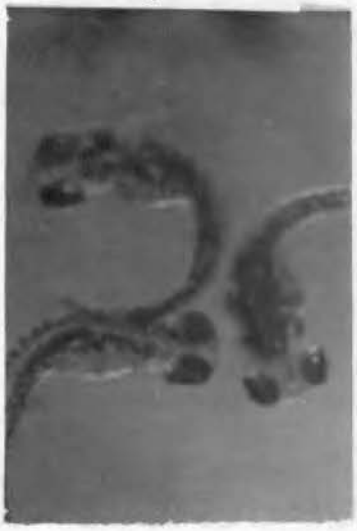

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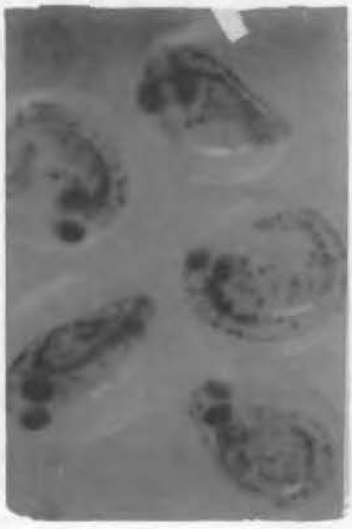


Figure 5.

Figure 6.

Plate VII. . . . . .

Figure 1. Normal Brachydanio rerio embryo, 50 hours after fertilization and fixed in Bouin's Fluid.

2. Brachydanio rerio embryo, 50 hours after fertilization and 48 hours after exposure to 1.0 ppm of equilenin. Fixed in Bouin's Fluid.

3. Brachydanio rerio embryo, 50 hours after fertilization and 48 hours after exposure to 1.8 ppm of equilenin. Fixed in Bouin's Fluid.

4. Cells and nuclei of normal Brachydanio rerio embryo 26 hours old. Fixed in Bouin's Fluid and stained in aceto-carmine.

5. Cells and nuclei of Brachydanio rerio embryo exposed to 2.0 ppm of equilenin for 24 hours. Fixed in Bouin's Fluid and stained in aceto-carmine.

6. Cells and nuclei of Brachydanio rerio embryo exposed to 2.0 ppm of equilenin for 24 hours. Fixed in Bouin's Fluid and stained in aceto-carmine.

Plate VII

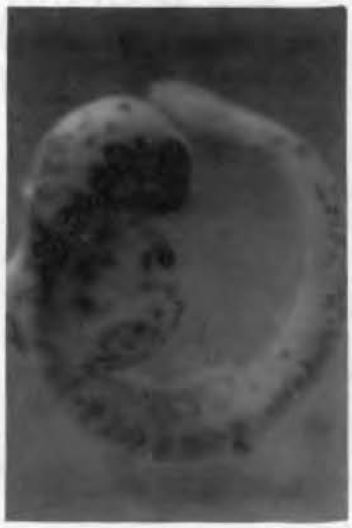
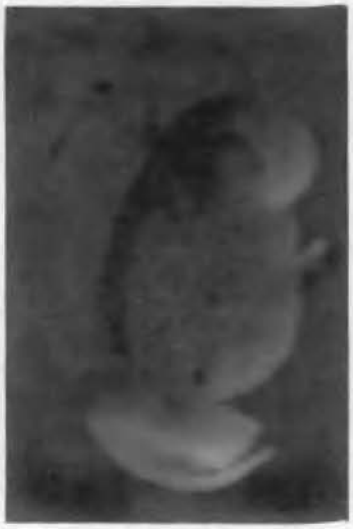


Figure 1.



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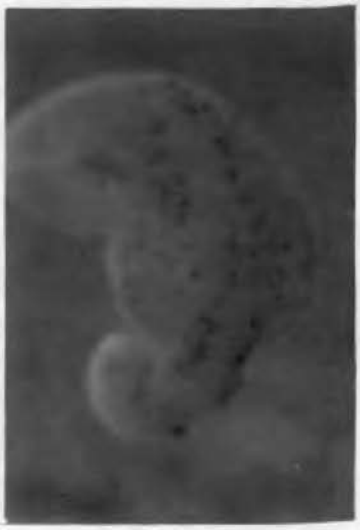

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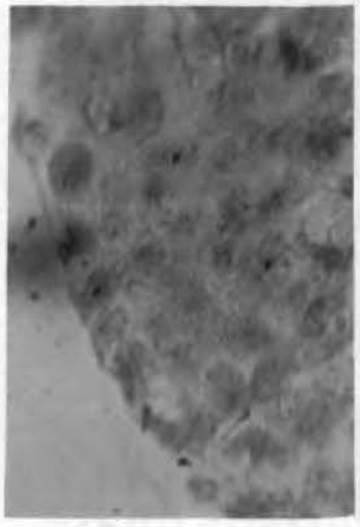

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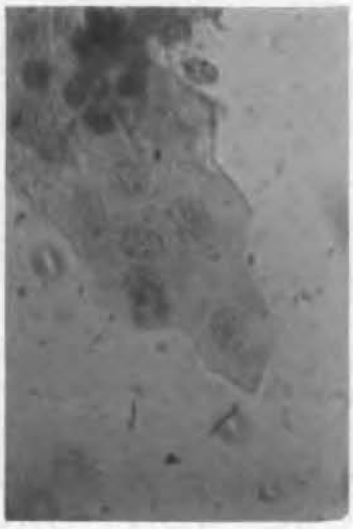
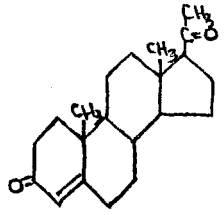


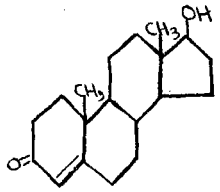
Figure 5.

Figure 6.

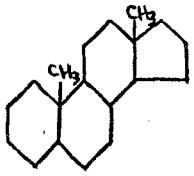
Plate VIII



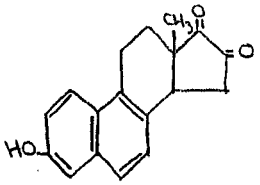
Progesterone



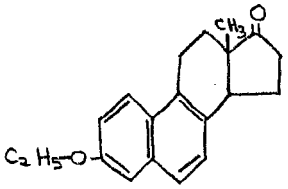
Testosterone



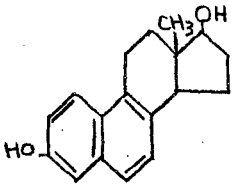
Androstane



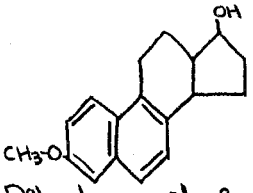
16-Keto Equilenin



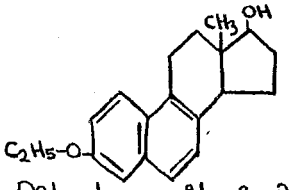
Equilenin Ethyl Ether



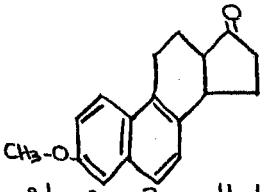
Dihydroequilenin-17β



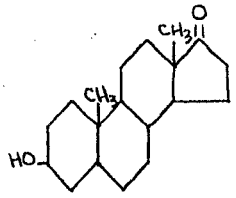
Dihydroequilenin 3-methyl Ether



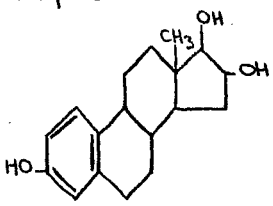
Dihydroequilenin-3-ethyl Ether



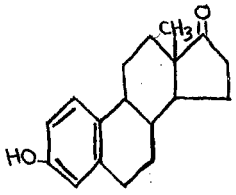
Equilenin -3-methyl Ether



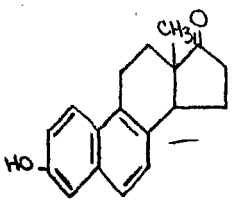
Androsterone



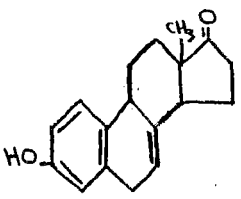
Estriol



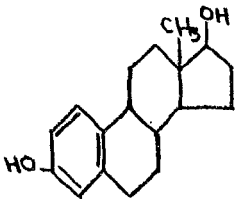
Estrone



Equilenin

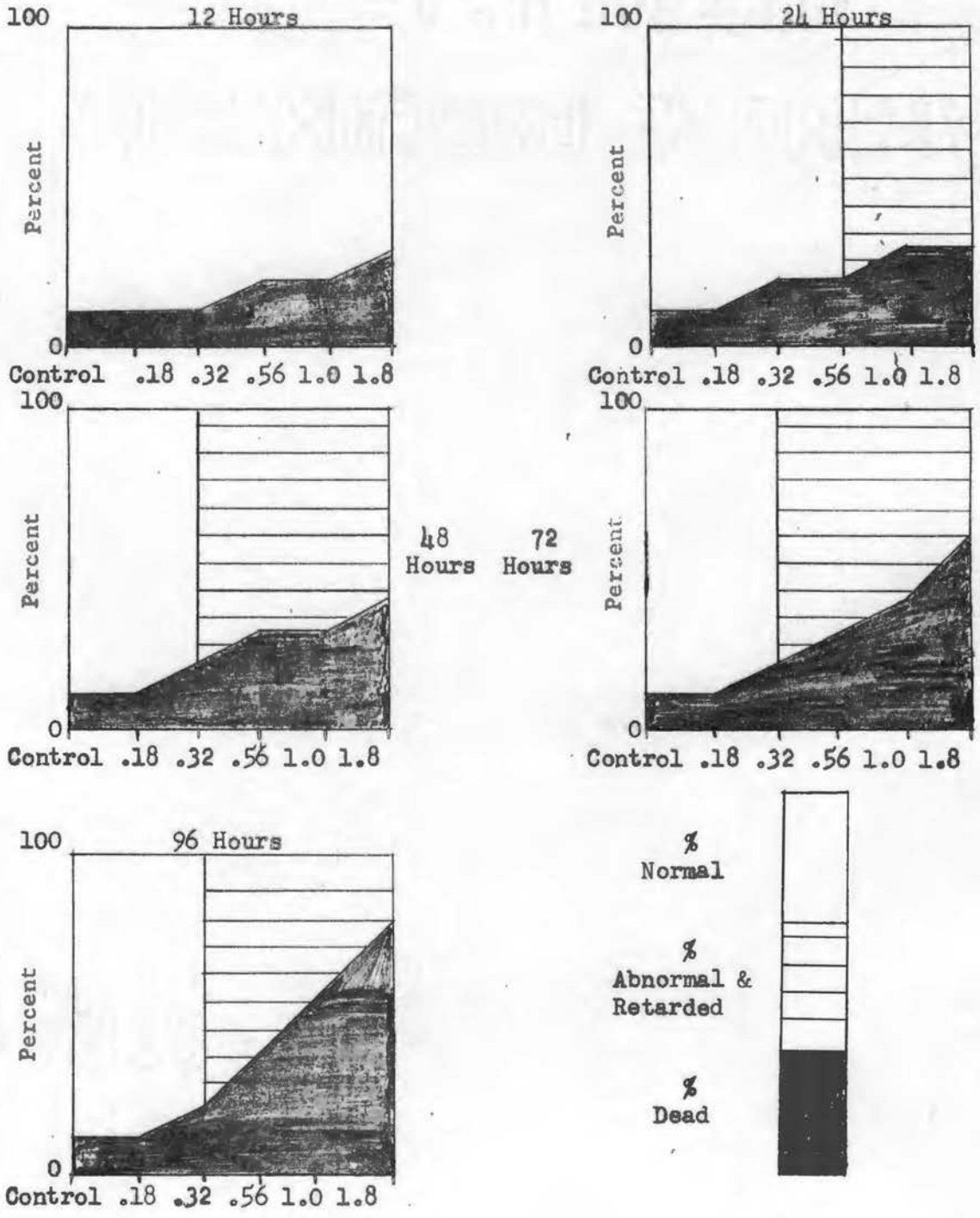


Equilin



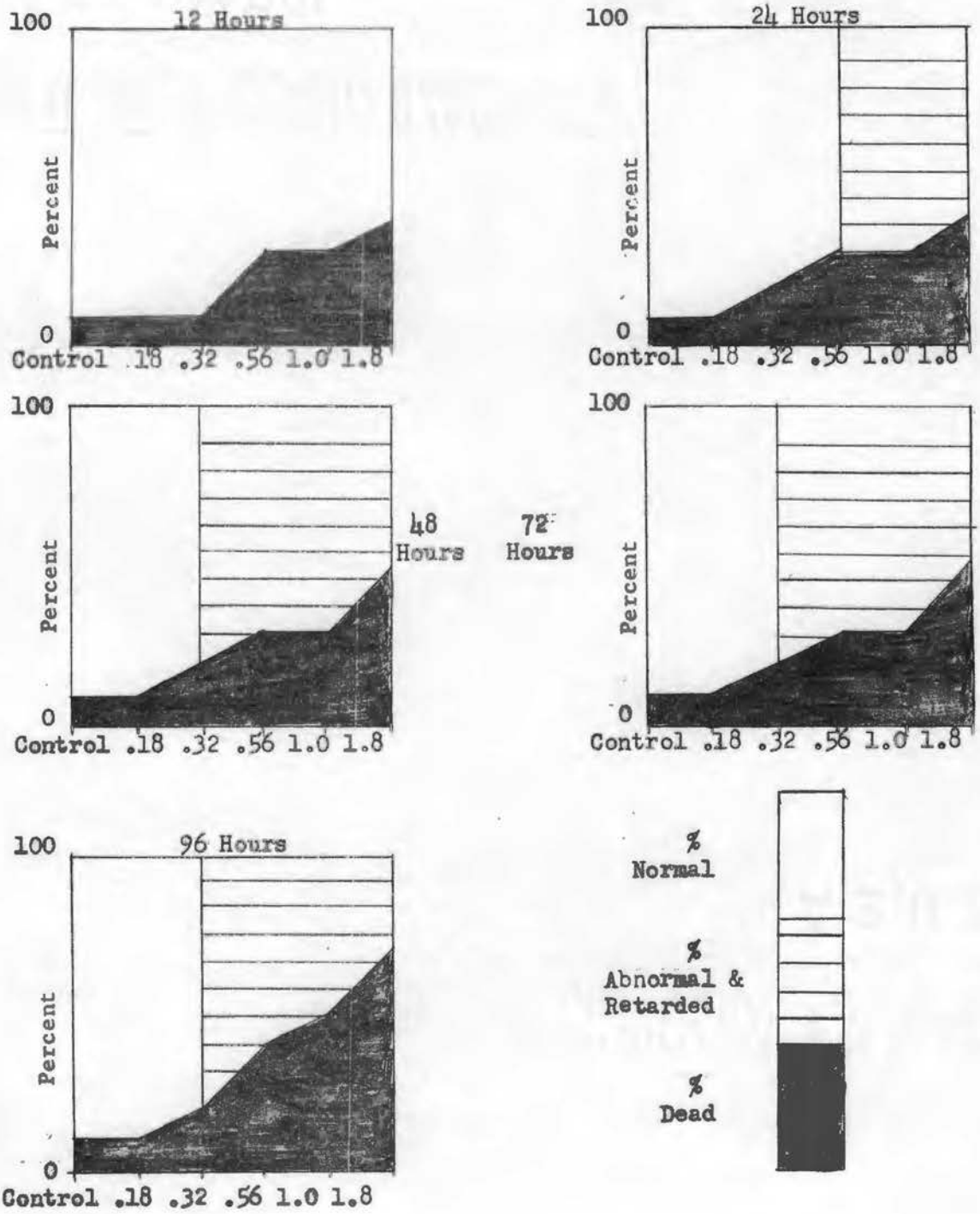
α-Estradiol

TABLE 4
EXPOSURE DURING EARLY CLEAVAGE



Ten tests consisting of 10 embryos in each culture

TABLE 5
EXPOSURE DURING LATE CLEAVAGE

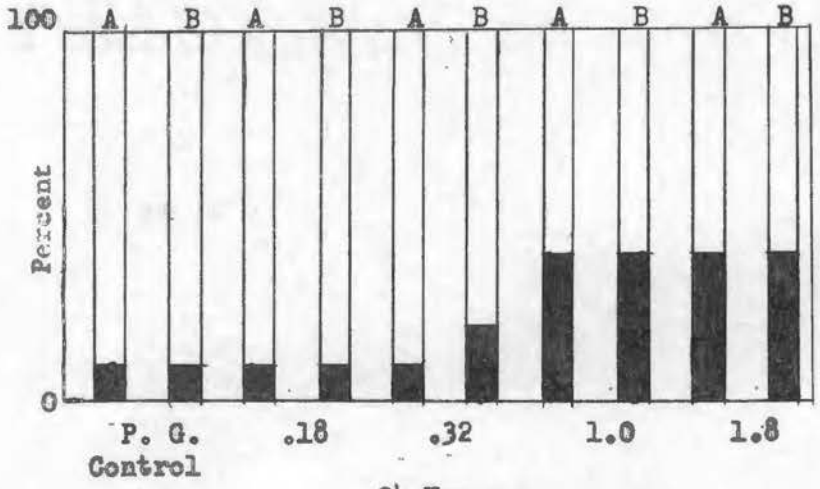


Ten tests consisting of 10 embryos in each culture

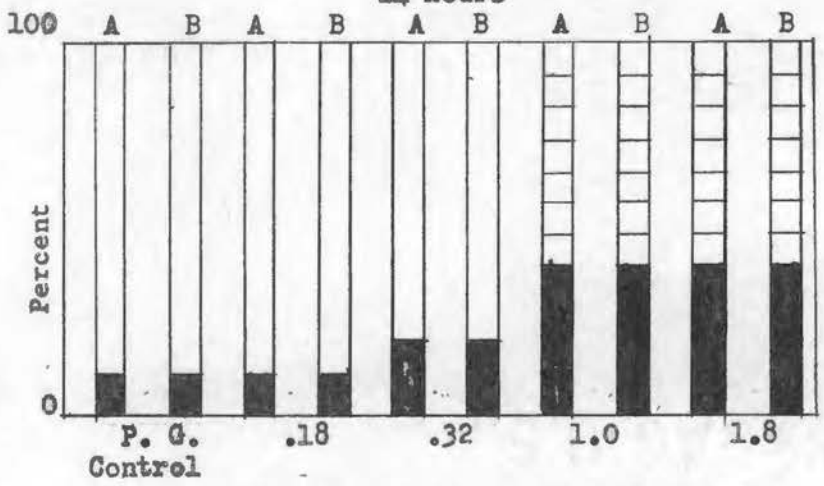
TABLE 6
RECOVERY TEST

(A) Chemical removed. (B) Chemical not removed.

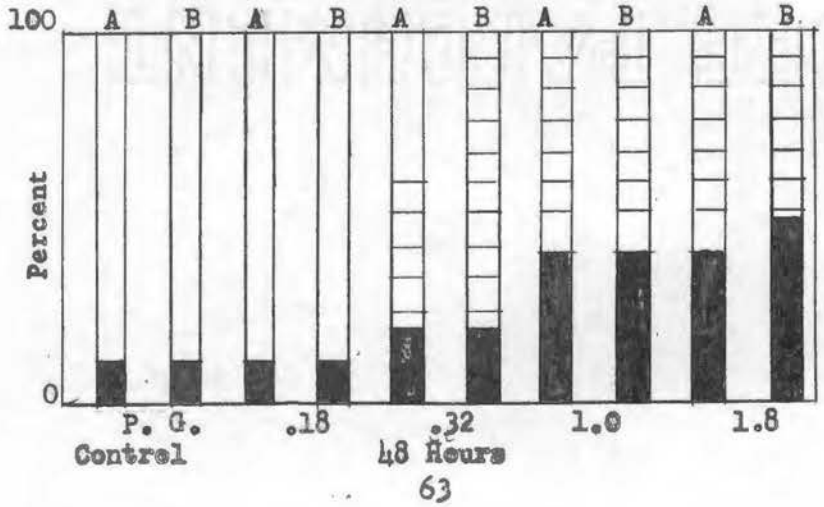
12 Hours



24 Hours



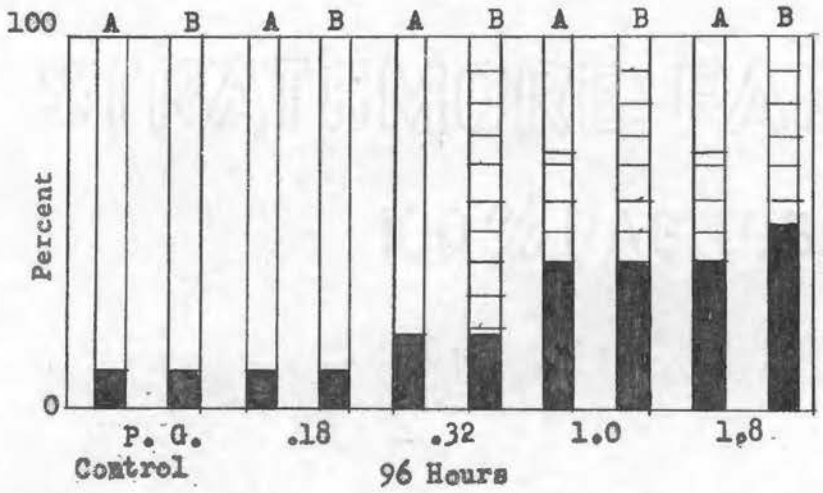
Chemical was removed after 24 hours



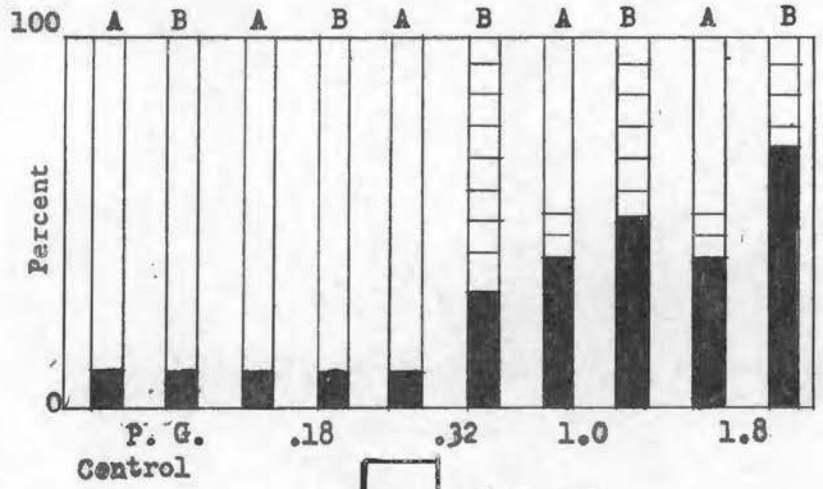
48 Hours

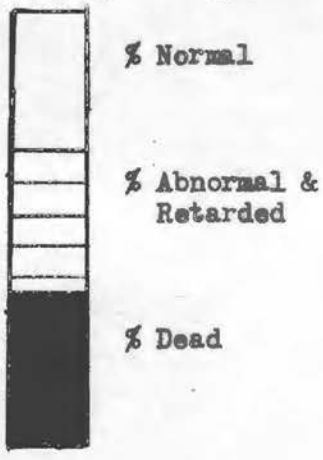
TABLE 6
RECOVERY TEST (continued)

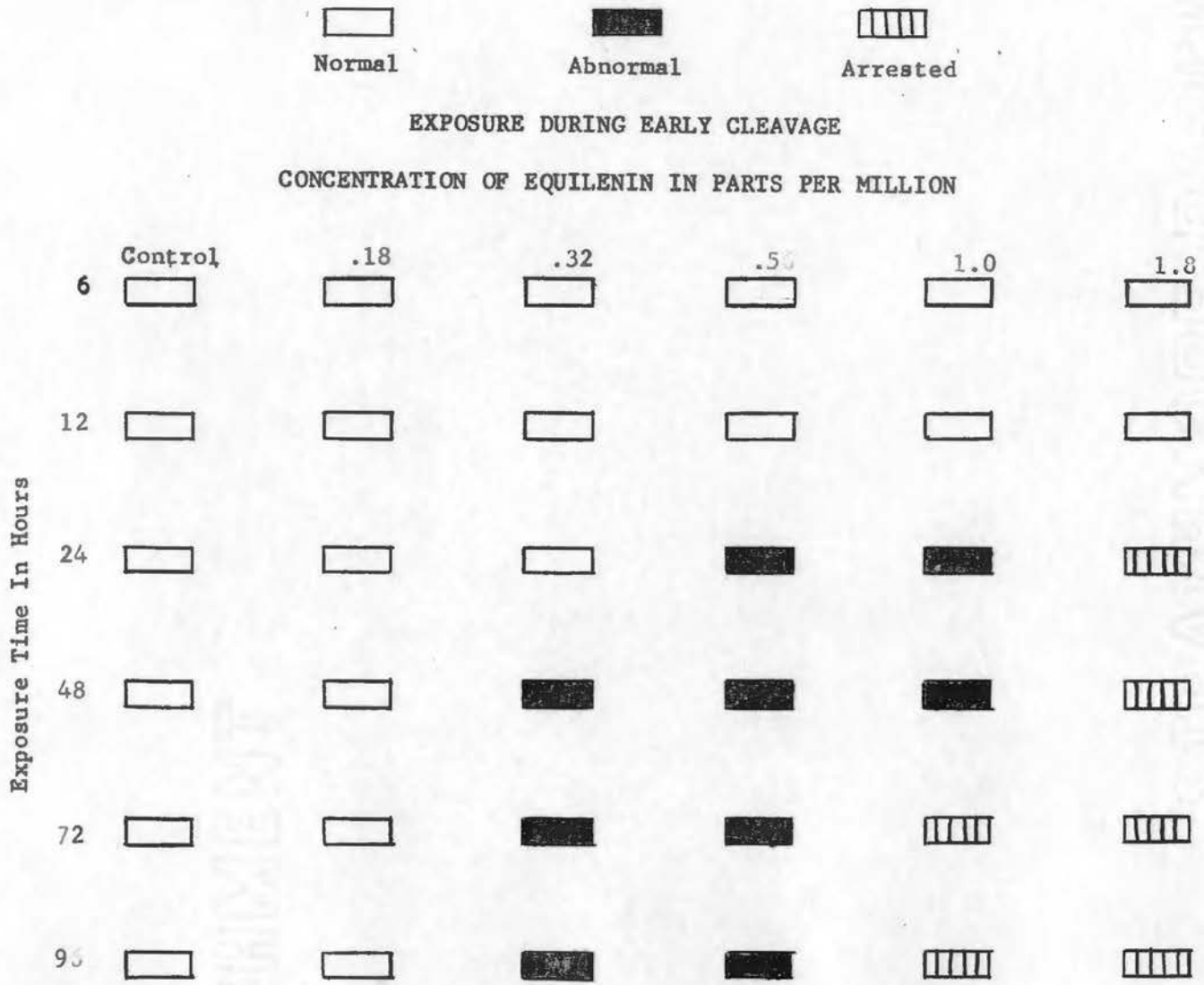
72 Hours

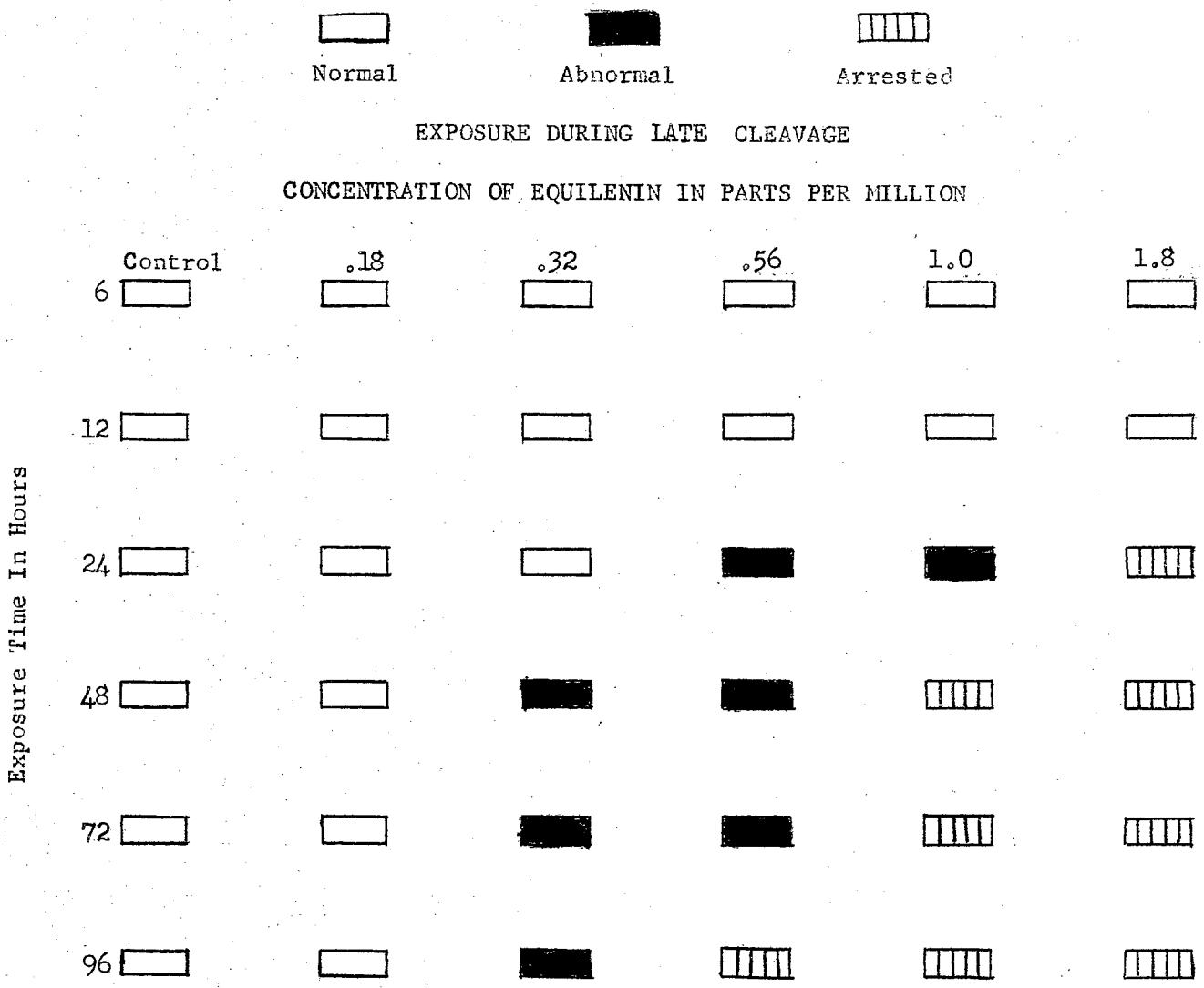


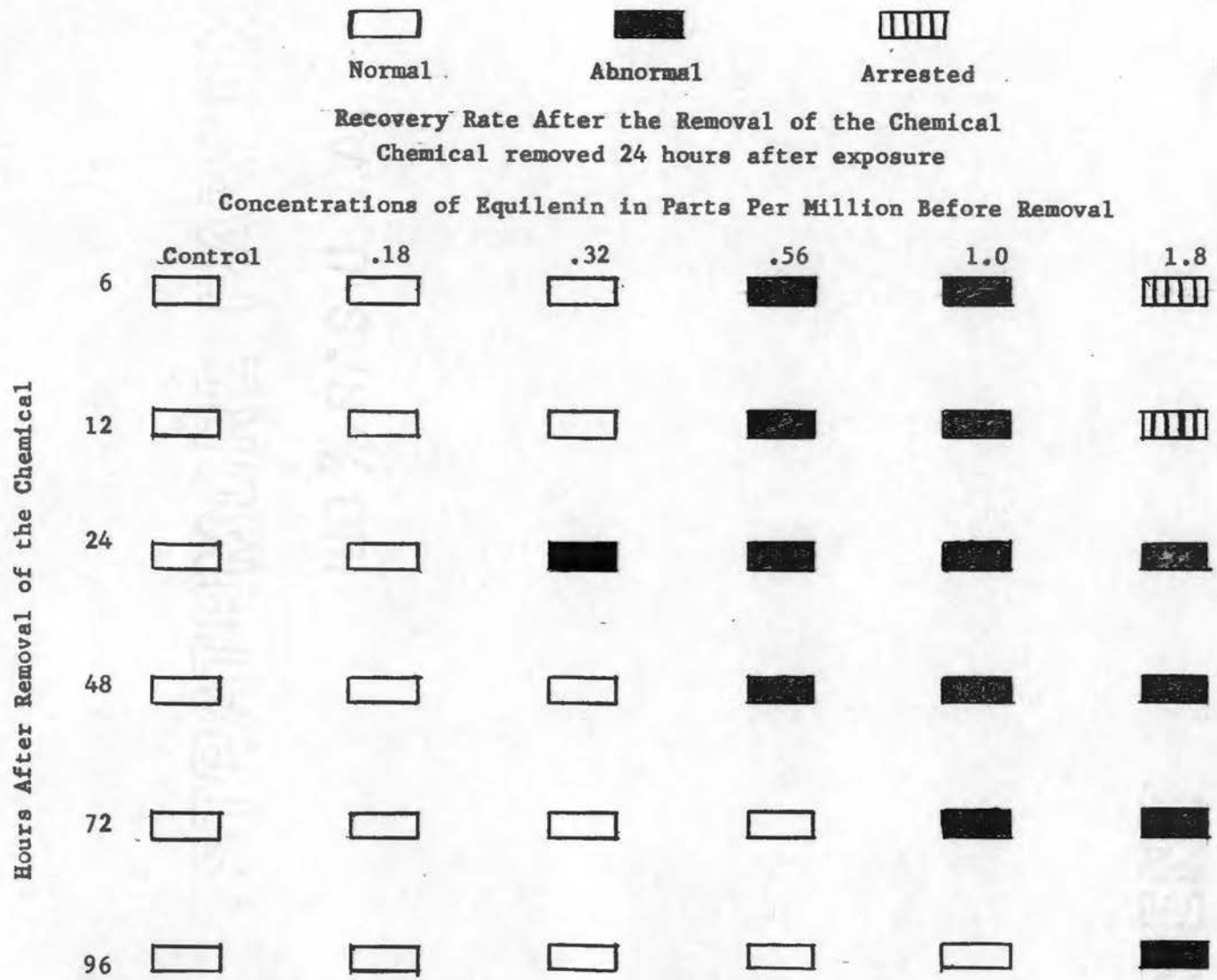
96 Hours






Normal
Abnormal
Arrested
EXPOSURE DURING EARLY CLEAVAGE
CONCENTRATION OF EQUILENIN IN PARTS PER MILLION
Control
.18
.32
.5
1.0
1.8
Exposure Time In Hours
6
12
24
48
72
96


Normal
Abnormal
Arrested
EXPOSURE DURING LATE CLEAVAGE
CONCENTRATION OF EQUILENIN IN PARTS PER MILLION
Control
.18
.32
.56
1.0
1.8
Exposure Time In Hours
6
12
24
48
72
96


Normal
Abnormal
Arrested
Recovery Rate After the Removal of the Chemical
Chemical removed 24 hours after exposure
Concentrations of Equilenin in Parts Per Million Before Removal
Control
.18
.32
.56
1.0
1.8
Hours After Removal of the Chemical
6
12
24
48
72
96

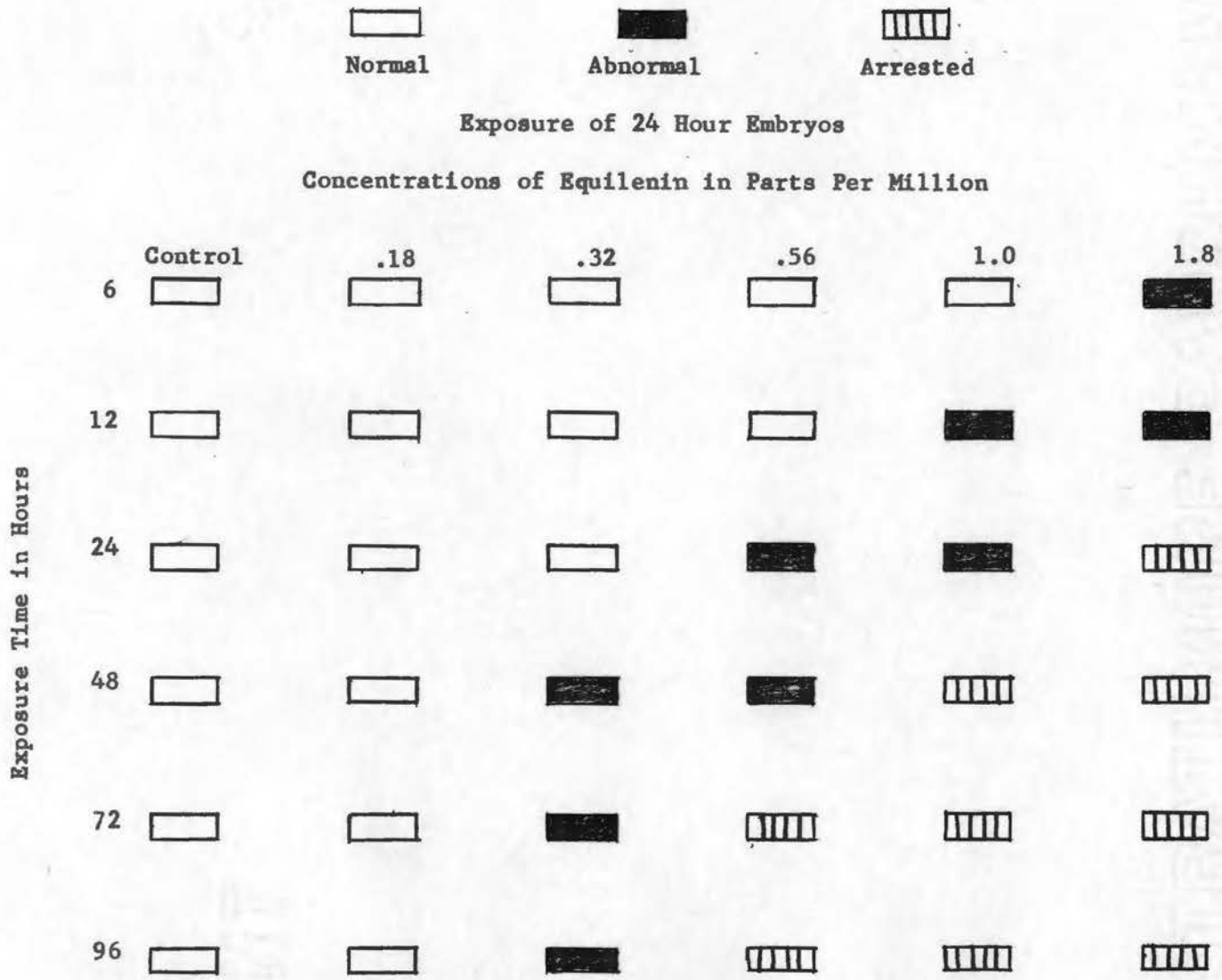

Normal
Abnormal
Arrested
Exposure of 24 Hour Embryos
Concentrations of Equilenin in Parts Per Million
Control
.18
.32
.56
1.0
1.8
Exposure Time in Hours
6
12
24
48
72
96

TABLE 11

TYPICAL LABORATORY EXPERIMENT

Date started 2/8/58 Time 0930 Date closed 2/12/58 Time 0930

Experiment No. 602

Developmental stage of eggs at time of exposure 4-64 cell

		Control P. G. 1.8ppm.	0.18 ppm.	0.32 ppm.	0.56 ppm.	1.00 ppm.	1.80 ppm.	0.18 ppm.A	0.32 ppm.A	0.56 ppm.A	1.00 ppm.A	1.80 ppm.A	Control(H2O)
No. of eggs		10	10	10	10	10	10	10	10	10	10	10	10
1st Reading 6 & 12 hrs. Exposure	No. Dead & dying	1	1	0	3	3	3	2	3	3	4	5	1
	No. abnormal	0	0	0	0	0	0	0	0	0	0	0	0
	No. retarded	0	0	0	0	0	0	0	0	0	0	0	0
	Stage of Devel.	9	9	10	7	7	7	8	7	7	6	5	9
	C-stage	Ok	Ok	Ok	Ok	Ok	Ok	Ok	Ok	Ok	Ok	Ok	Ok
	Comments	No abnormal effects except for a few dead individuals											
2nd. Read. 24 hrs. Exposure	No. dead & dying	1	1	0	3	3	3	2	3	3	4	5	1
	No. abnormal	0	0	0	7	7	7	0	0	7	6	5	0
	No. retarded	0	0	0				0	0				0
	Comments	Pigmentation is not developed in 0.56, 1.0, and 1.8ppm. Pericardium swollen, conjested circulation, tail not developed, gut swollen, eyes appear abnormal in structure. Heart (erratic).											
3rd. Read. 48 hrs. Exposure	No. dead & dying	1	1	2	3	3	3	2	3	3	5	5	1
	No. abnormal	0	0	8	7	7	7	0	7	7	5	5	0
	No. retarded	0	0					0					0
	Comments	Effects apparent in all concentrations except the 0.18 ppm. Development in the 1.0 and 1.8 is arrested. Pericardium and gut are swollen, conjested circulation, tail retarded, heart has an irregular beat. Eyes are retarded in growth.											
4th. Read. 72 hrs. Exposure	No. dead & dying	1	1	2	3	3	4	2	3	3	5	5	1
	No. abnormal	0	0	8	7	7	6	0	7	7	5	5	0
	No. retarded	0	0					0					0
	Comments	Same effects present as in 48 hour reading, except the effects are more severe at this period. Some of the embryos of the controls are hatching.											
5th. Read. 96 hrs. Exposure	No. dead & dying	1	1	2	6	6	7	2	5	6	5	8	1
	No. abnormal	0	0	8	4	4	3	0	5	4	5	2	0
	No. retarded	0	0					0					0
	Comments	The 0.32 embryos are very abnormal. The 0.56, 1.0, 1.8 are completely arrested and will soon die. They have the same typical equilenin effects. Some of the embryos in 1.0 and 1.8 ppm it is hard to tell the tail area from the head area.											

Additional notes or observations

Examples: Date of fixation of material
Date of pictures
Age of the embryos used in pictures

TABLE 12
RAW DATA FROM MEASUREMENTS OF NUCLEAR DIAMETERS

Class		I				II				III			
Locus		A	B	C	D	A	B	C	D	A	B	C	D
	1.	6.75	5.00	5.00	4.75	6.00	5.00	5.00	5.50	9.50	9.00	8.50	9.50
C	2.	4.00	5.50	4.75	6.50	6.50	5.75	4.75	6.00	10.00	10.00	10.50	9.00
e	3.	6.00	4.00	6.50	6.00	4.75	6.00	5.00	5.00	9.50	11.50	8.50	10.00
l	4.	4.50	6.50	5.00	5.50	7.00	5.00	4.50	5.50	8.50	9.00	11.00	9.50
l	5.	7.50	4.00	5.00	6.00	4.75	5.00	5.00	4.50	11.00	9.50	8.00	9.00
s	6.	6.50	4.50	5.50	5.50	5.75	5.50	5.75	6.00	9.75	8.50	10.50	10.00
	7.	4.00	6.00	5.50	4.50	5.00	6.50	6.00	4.50	9.00	8.50	10.50	10.50
	8.	5.75	5.50	6.00	6.00	5.00	6.00	5.75	5.00	10.50	9.00	9.50	8.50
	9.	5.00	6.00	4.75	6.50	6.50	5.50	5.75	4.50	10.00	9.00	10.00	10.50
	10.	5.50	5.00	6.00	6.75	7.50	6.00	5.50	5.50	10.00	10.50	9.00	9.50
Totals		55.50	52.00	54.00	58.00	56.25	56.25	53.00	52.00	97.75	94.00	96.00	96.00
	1.	6.00	6.75	4.75	4.50	4.75	4.50	5.00	6.50	9.75	9.50	8.50	9.00
	2.	4.75	5.50	5.50	6.00	5.50	5.50	5.00	4.75	10.00	8.50	10.00	8.50
	3.	5.50	4.00	5.00	4.50	4.50	7.00	5.50	6.00	9.50	9.00	9.50	9.50
	4.	5.00	5.50	5.00	6.00	6.00	6.50	7.75	4.50	9.50	10.50	10.00	10.50
	5.	5.75	6.00	4.50	5.50	4.50	6.00	5.00	7.00	8.50	8.50	10.00	10.50
	6.	6.00	4.50	4.75	5.00	5.50	4.50	5.75	4.75	10.00	9.50	10.50	10.50
	7.	5.00	4.75	6.00	4.50	6.50	7.00	6.50	5.50	9.50	9.00	8.50	9.50
	8.	6.00	5.50	4.50	6.50	6.50	6.50	6.00	4.75	8.50	9.50	10.50	9.00
	9.	4.50	5.50	5.50	4.50	5.50	4.75	5.50	4.75	9.75	10.00	8.75	8.50
	10.	4.50	5.75	5.00	5.00	5.50	5.25	6.50	5.00	10.00	9.75	9.50	8.50
Totals		53.00	53.75	50.50	52.00	54.75	58.00	55.50	53.50	95.00	93.75	95.75	92.00
	1.	6.00	5.00	6.50	5.00	6.00	5.00	6.00	6.00	8.00	9.00	9.00	8.50
	2.	5.50	5.50	5.00	6.50	5.00	4.50	4.50	5.00	9.00	10.00	8.50	9.00
	3.	5.00	4.75	5.50	4.75	5.00	4.75	5.00	5.00	10.00	10.00	10.00	10.00
	4.	4.75	4.50	6.50	5.00	5.50	5.00	5.50	5.50	9.00	8.00	9.50	9.00
	5.	5.00	5.00	4.50	5.00	4.50	5.50	6.50	4.50	9.50	9.50	10.00	8.00
	6.	6.00	4.75	4.75	4.75	6.50	5.00	4.50	5.00	8.00	9.00	8.50	9.50
	7.	5.00	6.50	6.50	5.50	5.75	4.75	5.50	4.50	9.50	9.00	9.50	9.00
	8.	6.50	5.00	5.00	5.00	5.50	6.00	6.00	5.50	10.00	10.00	10.00	9.50
	9.	6.00	6.00	5.50	6.50	6.00	5.75	5.50	5.00	8.50	9.50	8.50	10.00
	10.	5.50	5.00	4.75	5.00	4.50	5.00	5.00	6.50	9.50	9.00	10.00	9.00
Totals		55.25	51.25	54.50	53.00	54.25	51.25	54.00	52.50	91.00	93.00	93.50	91.50

TABLE 12
(continued)

Class		I				II				III			
Locus		A	B	C	D	A	B	C	D	A	B	C	D
	1.	4.75	5.00	5.00	5.50	4.50	5.00	5.00	4.50	8.00	9.00	8.00	9.00
C	2.	5.00	5.50	4.50	4.50	5.00	6.00	4.50	5.00	9.00	8.50	9.50	8.50
e	3.	5.50	5.00	4.50	5.00	4.75	6.50	5.00	6.00	9.50	9.50	10.00	9.00
l	4.	6.00	5.50	4.50	5.50	5.50	4.50	5.00	4.75	9.50	8.00	9.00	8.00
l	5.	4.75	6.00	6.00	6.50	5.50	4.50	6.50	6.00	8.00	9.50	9.50	10.00
s	6.	6.00	5.50	5.00	4.50	5.50	5.00	5.50	5.00	9.50	9.00	9.00	9.50
	7.	5.00	6.00	5.50	6.50	4.75	6.00	5.50	5.00	9.50	9.00	8.00	10.50
	8.	6.50	4.50	5.50	5.00	5.50	6.00	4.50	6.00	8.00	9.50	9.50	10.00
	9.	5.00	5.50	6.00	4.75	5.00	5.00	5.00	5.00	10.00	9.00	10.00	8.00
	10.	5.50	5.50	5.50	6.00	6.00	5.50	5.50	6.00	9.50	8.00	9.50	10.00
Totals		54.00	54.00	52.00	53.75	52.00	54.00	53.00	53.75	90.50	89.00	92.00	92.50
	1.	4.75	6.00	5.00	4.75	3.50	4.75	5.00	5.50	8.00	9.00	8.50	8.00
	2.	6.00	5.00	5.50	5.50	6.00	6.50	5.00	4.75	10.00	9.50	9.00	9.50
	3.	5.00	4.75	6.00	5.00	5.00	5.00	5.50	6.50	9.50	10.00	8.00	9.00
	4.	6.00	5.50	4.50	6.50	5.00	5.50	4.75	5.50	10.50	8.50	10.00	10.00
	5.	6.00	5.00	5.50	5.50	4.75	5.50	6.00	4.50	8.00	9.50	8.00	9.00
	6.	5.00	5.00	5.00	5.00	6.50	5.50	5.50	6.00	10.50	9.50	10.00	8.50
	7.	5.00	4.75	6.00	6.00	5.00	4.50	4.75	5.00	9.00	10.00	9.50	8.00
	8.	5.50	5.50	4.50	6.50	6.00	5.00	5.50	6.00	10.00	8.00	10.00	10.50
	9.	4.50	5.00	5.50	4.75	5.50	5.00	5.00	5.50	9.50	10.00	8.50	9.00
	10.	4.75	5.00	5.00	5.50	4.75	5.50	6.00	4.75	8.50	10.50	9.50	8.00
Totals		52.50	51.50	52.50	55.00	54.00	52.75	53.00	54.00	93.50	94.50	91.00	89.50
	1.	5.00	5.00	5.50	4.50	5.00	5.50	5.00	5.00	8.50	9.00	8.00	9.00
	2.	5.50	4.75	5.00	5.50	6.00	4.50	5.50	4.75	10.00	9.50	9.50	8.50
	3.	6.00	5.50	5.00	6.00	5.50	5.50	5.00	6.00	9.00	8.00	10.00	9.50
	4.	5.00	5.50	4.50	4.75	4.50	5.00	5.50	4.50	10.50	8.50	9.00	8.00
	5.	6.00	4.75	6.50	5.50	6.50	5.50	4.75	6.50	8.00	10.00	10.00	8.50
	6.	5.50	6.00	5.00	5.00	5.00	6.00	5.75	5.00	8.50	9.50	8.00	10.00
	7.	4.75	5.50	4.75	6.00	5.50	4.75	6.50	4.50	9.50	10.50	9.50	8.50
	8.	5.00	5.00	6.00	5.00	5.00	5.75	5.50	6.00	9.00	8.50	10.50	10.50
	9.	6.50	6.50	5.00	6.50	5.00	6.00	6.50	5.00	10.00	10.50	10.00	9.00
	10.	4.50	4.75	5.00	5.00	4.75	5.75	4.75	5.50	8.50	9.50	8.00	9.50
Totals		53.75	53.75	52.25	53.75	52.75	54.25	54.75	52.75	91.50	93.50	92.50	91.00

TABLE 12
(continued)

Class	I				II				III			
Locus	A	B	C	D	A	B	C	D	A	B	C	D
1.	5.50	5.00	5.00	5.00	6.00	6.00	5.00	6.00	10.00	8.00	9.00	8.50
2.	5.00	4.75	6.00	4.75	4.50	5.00	4.50	5.00	8.00	9.50	10.50	10.50
3.	4.50	6.00	5.50	6.00	6.50	4.50	6.00	4.75	9.50	10.50	9.00	9.50
4.	5.00	5.50	4.50	5.00	5.00	5.00	6.00	5.00	9.00	9.50	8.00	8.00
5.	6.50	5.50	5.00	4.75	6.00	5.50	4.50	5.50	8.00	8.00	9.00	9.00
6.	4.75	4.50	5.50	5.50	4.75	6.50	5.00	6.00	9.50	9.50	10.50	8.00
7.	5.50	6.50	4.75	6.50	5.50	5.00	5.50	4.75	10.50	10.00	9.50	9.50
8.	6.00	5.00	5.50	5.00	5.00	4.75	6.00	5.50	9.50	9.50	8.00	10.00
9.	4.50	5.50	6.50	5.50	6.00	5.50	5.50	6.50	8.00	9.00	9.50	9.50
10.	6.00	5.00	6.00	5.50	5.00	5.00	4.75	5.00	9.50	10.50	10.00	8.00
Totals	53.25	53.25	59.25	53.00	54.25	52.75	52.75	54.00	91.50	94.00	93.00	90.50
1.	6.00	4.50	5.00	6.00	4.50	6.50	5.00	4.75	8.00	9.50	8.50	9.00
2.	5.50	5.50	4.50	5.00	5.00	6.00	5.50	5.50	9.50	10.00	10.00	10.50
3.	4.50	5.00	5.50	5.50	5.50	4.75	4.50	6.50	9.00	9.00	9.50	8.50
4.	5.00	5.00	6.00	5.00	6.00	5.50	6.00	4.75	10.00	8.50	9.00	9.50
5.	5.50	4.75	5.50	4.75	5.50	5.50	5.50	5.00	10.00	9.50	8.00	9.50
6.	4.75	5.50	4.75	5.50	5.00	6.50	6.50	5.50	9.00	9.50	9.50	10.50
7.	6.50	5.00	5.50	5.00	6.50	4.75	4.75	6.00	9.00	10.50	10.50	8.00
8.	5.50	4.50	5.50	6.00	5.50	5.50	5.50	5.50	8.50	8.00	9.00	9.00
9.	5.50	6.00	5.00	5.50	4.75	6.00	5.00	5.50	9.00	9.00	9.50	10.50
10.	4.75	5.00	6.00	4.50	5.50	5.00	5.50	6.50	8.00	10.50	8.00	8.00
Totals	53.50	50.75	53.25	52.75	53.75	56.00	53.75	55.00	90.00	94.00	91.50	93.00
1.	4.75	6.00	5.00	6.00	4.75	6.00	4.50	5.50	8.50	9.00	10.00	9.00
2.	5.50	5.00	4.75	5.50	5.50	5.50	5.50	4.75	9.00	10.50	9.00	8.50
3.	6.00	4.75	5.50	4.00	5.00	5.00	5.00	6.50	9.50	8.00	9.00	9.00
4.	5.00	5.50	5.00	5.00	6.50	4.75	4.50	5.00	10.00	9.00	9.50	10.00
5.	4.50	5.00	5.50	6.00	6.00	5.00	6.00	5.00	8.50	10.00	8.50	8.00
6.	5.50	6.50	4.50	4.00	5.00	6.00	5.00	6.50	9.00	9.00	9.00	10.50
7.	5.00	5.50	5.50	5.50	4.00	5.00	4.50	5.00	9.00	9.50	9.50	9.50
8.	6.50	4.75	5.00	5.00	5.00	5.00	5.00	4.75	10.00	8.00	9.00	9.00
9.	5.50	5.00	5.50	6.00	6.00	5.00	5.00	6.00	9.00	10.50	10.00	8.00
10.	4.00	6.00	4.00	4.75	5.50	4.50	5.00	5.00	8.00	9.00	8.00	9.00
Totals	52.25	54.00	50.25	51.75	53.75	51.75	50.00	54.00	90.50	92.50	91.50	90.50

Units of an ocular micrometer were used for measurements. Nuclear diameters were estimated to $\frac{1}{4}$ an ocular unit.

VITA

Donald Jay Dunn

Candidate for the Degree of

Master of Science

Thesis: STUDIES ON THE EFFECTS OF EQUILENIN ON THE EMBRYOLOGICAL DEVELOPMENT OF THE ZEBRA FISH, Brachydanio rerio (Hamilton).

Major Field: Zoology

Biographical:

Personal data: Born in Coweta, Oklahoma, July 30, 1934, the son of Marion I. and Ruby E. Dunn.

Education: Attended grade school and high school at Coweta, Oklahoma; graduated from Coweta High School in 1952: received the Bachelor of Science degree from Northeastern State College, with a double major in Chemistry and Biology in May, 1956. Completed the requirements for the Masters of Science degree in July, 1958.

Professional experience: One academic year as laboratory assistant in General Zoology, Invertebrate Zoology, and Agricultural Zoology, in 1956, 1957, and 1958. Two years as laboratory instructor in General Biological Science 1957 and 1958.