STUDIES ON

THE EFFECTS OF 17-METHYLESTRADIOL ON THE EMBRYOLOGICAL DEVELOPMENT

OF THE ZEBRA FISH Brachydanio rerio HAMILTON

Thesis Approved:

Roy W. Jones
Thesis Adviser

L. Herbert Bunneau

Robert J. Seray

Raben Nardecai
Dean of the Graduate School

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INTRODUCTION

The purpose of this project was to determine the effects of 17-methylestradiol on the embryological development of the Zebra fish, Brachydanio rerio (Hamilton). The study consisted of a gross morphological and cytological investigation of the effects resulting from the chemical.

17-Methylestradiol is a synthetic steroid (Plate 6) which produces a definite effect on cell division in the embryos of the Zebra fish. Treatment of early stages of cleavage with relatively high concentrations of the chemical brings about a complete stoppage of cell division. Lower concentrations produce definite abnormalities characteristic of the chemical. A description of these effects and a discussion of possible causes follow in the body of the paper.

The Zebra fish, a cyprinid native to India, is an excellent laboratory animal for experimental purposes. They are relatively inexpensive, hardy animals and fertilized eggs may be obtained in fairly large numbers. Cell division takes place approximately every 18-20 minutes during early development. This rapid rate of mitosis makes any abnormalities in cleavage more easily detected.

The animal is transparent and the formation of organs is easily observed during early embryonic development. The embryos develop into recognizable baby fish in the first 24 hours and usually hatch during the third day.

17-methylestradiol was chosen for this project because it proved to be active in a screening test carried out on newly-synthesized chemicals. The stoppage of cell division and early toxicity in higher concentrations as well as the characteristic abnormalities produced by lower concentrations designated it as being worthy of further research.

LITERATURE REVIEW

Estradiol is the most potent of all naturally occuring estrogens. Estradiol 17β was isolated from sows' ovaries by Doisy in 1935. It proved to be a potent estrogen whereas 17α is quite weak. Estradiol was first obtained artificially by reducing the ketone group of estrone to a secondary alcohol. It was subsequently isolated from follicular fluid, pregnancy urine, and the placenta (Grollman, 1947).

Wintersteiner characterized the diols in his paper (Pincus, 1955). The 17α hydroxy compound is readily precipitated with digitonin, a reaction which is unique in the estrogen series. Butenandt and Goergens reported preparation of α and β estradiol from estrone by catalytic reduction in the presence of a nickel catalyst. Other methods have been described, in every instance α estradiol is the predominant reduction product.

The synthesis of 17-methyl-C^{14}-estradiol was carried out by Bocklage, et al. (1953). The synthesis of the chemical used in this study was based on this procedure.

The effects of different chemicals on the embryological development of Brachydanio rerio have been pointed out be several authors. Jones and Huffman (1957-1958) used B. rerio for bioassay tests on various steroids. It proved to be a fast and reliable technique to determine the effect of chemicals on cell division and differentiation. Huffman, Jones and Katzburg (1957) reported the activity of a number of steroids whose

effect varied according to the concentration and the chemical used. Included in this was estradiol-3,16α and 3-Methylestradiol-3,16β both of which are strongly antimitotic.

Jones (1949) reported on the effects of antuitrin (growth hormone) which brought about a disturbance in morphogenesis causing retardation and abnormal morphogenesis. Jones, Gibson, Nickolls (1951), and Gibson (1954) reported that thyroxine showed no effect in early stages but interferred with melanophore formation and morphogenesis at 3-5 days. It also produced an acceleration in metabolism. Thioracil produced an opposite effect, it increased pigmentation, speeded up morphogenesis and slowed metabolism. Jones, Waller, and Nickolls (1951) reported that podophylotoxin inhibited cell division in concentrations as low as 0.01 parts per million and designated it as being a mitotic poison.

Ethyl carbamate produced retardation in growth and structure as reported by Battle and Hisaoka (1953). The relative ineffectiveness of barbituric acid was reported by Hisaoka (1957). It was thought that this was due to the lack of penetration of the chemical through the chorion. Diethylbarbituric acid differed though in that it produced abnormalities which were later overcome after transfer to fresh water.

Dunn (1958) in his work with equilinin found that it has a definite effect on morphogenesis which might be attributed to its pronounced effect on circulation. Stockard (1921) concluded that the action of a chemical must be expressed relatively early (8-32 cell stage) for the most effective results.

Most of the work done concerning the effects of physical conditions on Brachydanio rerio point to the fact that increased age lessens the severity of the effects produced by the inhibiting conditions

(Anderson and Jones, 1950). Goff (1940) in his work with increased atmospheric pressure reported the ability of B. rerio to regulate themselves to the surrounding conditions. Embryos showed slight retardation of development, fewer somites, reduced muscular activity and slower heartbeat, all of which were overcome leaving no structural abnormalities.

Oppenheimer (1937), and Balinsky (1948) used a stage naming system to express development. Instead of chronological age, which they considered to be an arbitrary means of expression, they used the degree of organ and tissue differentiation. Ingersol (1949), and Blumenkratz (1956) demonstrated that if oxygen supply and temperature were kept constant one could consistently identify the embryonic stage of differentiation of fish embryos in chronological terms.

The development of Brachydanio rerio has been worked on by several authors. Roosen-Runge (1938) gave a detailed description of the early cytological and protoplasmic events taking place within the living egg. He also gave a detailed account of cell division and mitotic rates during normal development. Blumenkratz (1956) gave a chronological description of development from the 16 cell stage until hatching some 72 hours later. Hisaoka and Battle (1958) described 25 developmental stages of the Zebra fish from the fertilized ovum to hatching. They included the characteristics of the particular stage discussed and illustrated it with photomicrographs.

Work on the chromatophore pattern in the Danio was undertaken by Whittington (1952) who also observed the effect of variations of light and dark background on development. Goodrich and Nichols (1931) worked with the development of chromatophores in newly-formed appendages of the Zebra fish.

The effect of different chemicals on cell division has involved much study. The action of the alkaloid colchicine as a mitotic poison has brought about wide spread investigation. Colchicine produces a metaphasic explosion which slows mitotic rate and brings about a reversion of cell division (Eigsti, 1955). This slowing of the mitotic rate is opposite to that of the naturally-occuring estrogens which promote tissue growth and stimulate cell division (Turner, 1940).

Keppel (1939) studied the effect of colchicine on the eggs of Rana pipiens and found that in concentrations as low as 1.0 ppm. unequal cell division took place resulting in death to some embryos. Waterman (1940) reported that much higher concentrations were necessary to bring about structural abnormalities in Oryzias embryos.

Tests conducted on derivitives of colchicine such as octahydro-colchicin, N-acetylcolchinol and four of its derivitives showed that they produce mitotic effects similar to colchicine but require much higher concentrations in order to bring about the effect. Di-and tri-methyl-colchicine acids were found to be ineffective. (Brues, 1936).

METHODS AND MATERIALS

Brachydanio rerio is a small (average length 1.5 inches) active fish very suitable for laboratory work. Spawning is not seasonal but occurs every three to four weeks, thus assuring a continuous egg supply. Longevity is about two years with the most productive age being 9-18 months.

Creaser (1934) formulated a technique for laboratory care and egg collection which yielded maximum egg production of the Zebra fish. This has undergone modification by Goff (1940), Battle and Hisaoka (1951), and Jones (1957).

The Zebra fish used in this project were kept in a battery of five-gallon aerated aquaria placed in a cabinet which could be sealed off from the light. About 30 fish were in each tank with a ratio of two males to one female. The tanks were maintained at a temperature of 26±2° C (79°F). The laboratory was air-conditioned and kept at a year around temperature of 70-80°F. Water in the tanks was kept at a constant depth of six inches by the addition of either aerated tap water, distilled water, or both.

Due to the carnivorous habits of the adults some means had to be taken to prevent them from eating the eggs as they were laid. Nylon nets of a fine mesh were placed in the tanks which allowed the eggs to drop to the bottom where they were safe from the adults. Some of the aquaria had glass marbles covering the floor instead of nets. The eggs were small enough to drop between the interstices of the marbles and be out of reach of the adults.

The adult fish were fed two types of commercial fish food, plankton, daphnia, dwarf white worms of the family Enchytraeidae, and a prepared baby food mixture. The baby food consisted of a mixture of one can strained spinach with enough pablum to thicken to a paste. This misture was cooked one hour and stored at a temperature below 0^{o} Centigrade.

B. rerio are known to ovulate a short time after being exposed to strong light following complete darkness (Jones, 1957). A timer was set to switch on the lights over the tanks a few minutes before the cover was removed. The eggs were then siphoned from the tanks with rubber tubing and collected in a tea strainer. They were washed in a decanting bowl where the excess debris which was lighter than the eggs was removed. The eggs were then removed with the aid of a pipette to culture dishes where they were rinsed and sorted according to the test stage desired.

Brachydanio rerio eggs are about 0.5 mm. in diameter, and can be seen with the unaided eye. The first clevage usually occurs 30-40 minutes after fertilization and subsequent cleavages occur approximately every 18-20 minutes thereafter at 26^{o}C (Roosen-Runge, 1938).

The eggs were placed in culture dishes containing 50 ml. each of concentrations of 1.0, 0.56, 0.32, 0.18, and 0.10 parts per million of 17-methylestradiol. These particular concentrations were used because the resulting figures lend themselves to ease in graphing on semilogarithm paper (Doudoroff, 1951). The results obtained are included at the end of this paper.

The chemical was diluted with aerated tap water to obtain the proper concentration. A control dish containing 50 ml. of aerated water and another dish containing 1% propylene glycol dilluted in 50 ml. of water was also set up. The stock solution was 1.0 mg. of 17-methylestradiol

dissolved in 1 ml. of propylene glycol. The dish of propylene glycol served to show if there was any abnormal effect brought about solely by its presence.

Each culture dish contained ten embryos, or less if there was a scarcity of eggs on a particular day, and every concentration was replicated twice in each test. Observations were made at 6, 12, and 24 hours after exposure and every 24 hours thereafter up to 96 hours.

Three separate tests were run in this manner. Eggs in early cleavage (2-16 cell stage), eggs in late cleavage (30-blastula stage), and 24 hour embryos were tested separately to determine the differences brought about by the age of the embryos alone. A test on the recovery ability of the embryos after 24 hours in the chemical and subsequent transfer to fresh water was also run.

The median tolerance limit (TL_m) was run on eggs exposed to the lower concentrations of 17-methylestradiol in both early and late cleavage. The TL_m values were found in order to determine the most accurate concentration of the chemical in ppm. that killed half of the embryos tested. In testing the stronger concentrations of 17-methylestradiol the same general pattern was followed with some revisions. The concentrations of the chemical were 10.0, 5.6, 3.2, and 1.8 parts per million. Continuous observations from the time of exposure using a phase microscope and a Spencer AO triocular microscope were made as long as definite changes in the cellular makeup were taking place rapidly. Two or three embryos from each concentration were placed in small dishes or shallow-well depression slides for aid in ease of observations.

Exposure to these concentrations of 24-hour embryos was also undertaken as in the case of the lower concentrations. Recovery ability of

the embryos after two hours in the chemical and subsequent transfer to fresh water was also tested.

A wide-field binocular stereomicroscope with eye piece of 10X magnification and lens objectives of 1.0 to 7.5X was used in the daily readings on the lower concentration tests and 24 hour tests. A phase microscope equipped with Kohler illumination and a triocular Spencer AO microscope with built in illumination and objectives of 10 to 93X were used for cytological and cellular observation in the tests on higher concentrations.

Photographs of treated and untreated embryos exhibiting various characteristic effects produced by the chemical were taken. A 35 mm. Leica camera attached to the phase microscope fitted with a Micro-Ibso attachment was used when specific high-contrast pictures especially of nuclear structure were needed. A 35 mm. Kodak Pony IV camera attached to the Spencer triocular microscope was also used. Panatomic X and High Contrast Copy film were used depending on the type of result desired.

Photographs of the characteristic abnormalities produced by the chemical, as well as control specimens at all of the test stages, are included in plates at the end of this paper.

The chorions were removed from some of the 2-8 cell stage eggs with microdissecting needles, the yolk was punctured and allowed to run to one side of the slide. The free cells were then examined under the phase microscope and the nuclear structure observed. Some of the cells were removed from the chorions, treated with higher concentrations of 17-methylestradiol and then observed under the phase microscope to determine what effect the chemical had on the nuclear structure.

Several of the embryos at various stages of development were preserved for future study. They were fixed in Bouin's fluid, the chorions

removed, and the embryos transferred to pieces of glass tubing which were plugged at both ends with absorbent cotton. A small strip of paper with date of fixation, age of embryo, and type of treatment was inserted in the tube. The tubes were placed in small vials which were immersed in large jars containing 60-70 percent isopropyl alcohol.

Some of the fixed material was dehydrated in isopropyl alcohol, cleared in xylene and embedded in paraffin. Serial sections were cut with a rotary microtome and the sections mounted on a slide. They were then stained with Mallory's triple stain and eosin. Because of the large amount of yolk present on embryos of this age these sections did not help much in establishing causes for the abnormalities.

A tape recorder was used to aid in observation when continuous changes were taking place in the cellular makeup of the eggs tested in the higher concentrations of the chemical. Recording changes was aided in this manner since both hands remained free to take photographs and manipulate the specimen. The recording was then played back and the information transcribed and correlated with the photographs. A special experiment sheet was used for each separate test and provided space for the following needed information: the date of the experiment, developmental stage of the eggs at the time of 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 at designated time periods and the information that was recorded after each observation period consisted of the following: the number dead, the number dying, the number abnormal and in what way they were affected, the number retarded, the amount of pigmentation present. There was also space in which to record any unusual observations which might indicate

the effect of the treatment on the normal embryonic differentiation. A sample date sheet is given in Table VI.

EXPERIMENTAL DATA AND OBSERVATIONS

The embryos used in this experiment demonstrated abnormalities in direct proportion to the concentration of the chemical used and the age of the embryo. Retardation was exhibited in solutions as low as 0.18 ppm. Due to the fact that the effects produced by the higher concentrations of 17-methylestradiol differ so from those exhibited by the lower concentrations no attempt will be made to speak of total effects of the chemical. The two series will be disucssed in separate sections and the characteristic effects resulting from the chemical will be related.

EXPOSURE TO LOWER CONCENTRATIONS

Two sets of tests were run on embryos at concentrations of 1.0, 0.56, 0.32, 0.18, and 0.10 ppm. Eggs were tested in early cleavage (2-16 cells), and also in late cleavage (32-blastula). Characteristic abnormalities were produced in both series, the major difference being the time at which they appeared. These abnormalities will now be discussed in detail.

There was a general retardation of both the size of the embryo and the time at which the organs appeared and became functional. Pigmentation was slowed in development not only in the amount of pigment present but also in the quantity of melanophores present. This retarding of pigment may be partly due to propylene glycol in which the chemical is in solution. However, a dish of embryos in 1% propylene glycol was always run simultaneously with the test chemicals and the effect brought about by propylene glycol was not the same as that brought about by

17-methylestradiol.

The cranial region was affected as to size, as was the development of the eye. The head, instead of appearing expanded, was not much larger in diameter than the rest of the trunk. The concentration of pigment in the eye was not as great as in the control, although this retardation of pigment was usually overcome after 48 hours.

The tail and gut were very susceptible to damage by the chemical. The tail, instead of curving above the head within the chorion was bent or twisted out of line, projecting out at an angle from the trunk. In many cases dead cells appeared and there was a sloughing of cells at the tip of the tail. The gut which in normal embryos appeared as an elongated area posterior to the yolk sac and immediately under the anterior portion of the tail was noticeably shortened. In shortening it took on a swollen appearance and also had an accumulation of dead cells over the surface.

The pericardial region was also characteristically affected. The heart normally appears as a twisted tube in this region at about 28-30 hours and blood can be seen coursing throughout the embryo shortly thereafter. In affected specimens not only does the pericardial sac swell but the beating of the heart was very sporadic and the blood was seen to be congested in various areas of the body.

Exposure during early cleavage

The series of tests run on eggs in early cleavage gave results which appear in Table III. Concentrations of 1.0 and 0.56 ppm. were too lethal to allow much if any development at all. They both showed a total kill at the 12-hour reading. Concentrations of 0.32 ppm. showed 50 percent of the embryos abnormal at the 6 hour reading, with this concentration showing

a progressively steady rise of abnormal and dead embryos as time progressed. At the end of the readings at 72 hours, 0.32 ppm. exhibited 75 percent abnormal development with about half of the total, dead. Concentrations of 0.18 ppm. exhibited no toxicity until 12 hours and then only very slight. By the end of the test it produced less than half abnormal or dead specimens.

The data for the median tolerance limit (TL_m) run on these embryos are as given in Table I.

TABLE I

TL_m DATA FOR EMBRYOS IN EARLY CLEAVAGE

Concentration in ppm.	Number of embryos	Number of test animals surviving after			
		6 hrs.	12 hrs.	24 hrs.	48 hrs.
.56	112	78	0	0	0
.32	196	186	136	105	92
.18	100	-	-	96	73

The median tolerance limits derived from these figures give the following hourly values. The TL_m at 12 hours (TL_m^{12}.37). The TL_m at 24 hours (TL_m^{24}.325). The TL_m at 48 hours (TL_m^{48}.29).

Exposure during late cleavage

The data compiled on this series of tests are shown in Table IV. Eggs in late cleavage were not as noticeably affected. Concentrations of 10.0 and 0.56 ppm. showed a total arrest but no dead embryos, while 0.32 ppm. showed no effect at all. The 12-hour reading showed a complete kill in 1.0 ppm. and a 50 percent kill at 0.56 ppm. At this reading 0.32 ppm. had only a slight effect and 0.18 ppm. none. At 24 hours there was a complete kill in the 0.56 ppm. concentration, a slightly more significant

effect at 0.32 ppm. and the first indication of abnormalities appearing at 0.18 ppm. The readings thereafter showed only a slight increase in abnormalities in the solutions tested. At the end of the reading 0.32 ppm. had about a 50 percent effect and a slight kill. Concentrations at 0.10 ppm. were discontinued in both the early and late exposures as being too weak for practical use.

The date for the median tolerance limit (TL_m) run on these embryos are as given in Table II.

TABLE II

TL_m DATA FOR EMBRYOS IN LATE CLEAVAGE

Concentration in ppm.	Number of embryos	Number of test animals surviving after 6 hrs.	12 hrs.	24 hrs.	48 hrs.
.56	100	94	40	0	0
.32	180	-	-	154	152

The median tolerance limits derived from the above table give the following hourly values. The value at 12 hours ($TL_m{}^{12}.51$). The value at 24 hours ($TL_m{}^{24}.40$).

Recovery ability of the embryos

A series of tests was run to determine the rate of recovery of embryos subjected to the lower concentrations for 24 hours. They were removed from the chemical by means of a pipette and transferred to culture dishes containing aerated water. Those embryos that were already dead at this time were not included. The results of this series seemed to show that the chemical must be present for any future morphological change to take place. It also appeared that the damage which had already been inflicted

could not be overcome after removal from the chemical and transfer to fresh water. Abnormalities present at the time of transfer remained the same and no further retardation was expressed. Pigmentation was the only characteristic which could be recovered. If a specimen exhibited a bent tail, swollen gut, or abnormal heart this remained even after 24 or more hours in fresh water.

Exposure of 24 hour embryos

A few tests were run on 24-hour embryos to see what effect would be produced on embryos which had already undergone most of their organ differentiation. In this series of tests the embryos were transferred from aerated water to culture dishes containing concentrations of 17-methylestradiol from 0.56 to 0.18 ppm. Results of this test showed that the chemical had no affect on these embryos at all. Those specimens in the test dishes appeared similar in structure to those in the control dishes and hatched at the same time.

This would appear to mean that after the embryo has developed this far the chemical at these low concentrations is not strong enough to bring about any noticeable affect. This corresponds with the finding of Stockard (1928) who stated that the earlier the arrest the more numerous the types of defect and the later the arrest the more limited the variety of deformities, since there are fewer organs to be affected during their rapidly-proliferating primary stages.

EXPOSURE TO HIGHER CONCENTRATIONS

When concentrations of 17-methylestradiol of 10.0, 5.6, 3.2, and 1.8 ppm. were used they immediately impared cell division so much that no further cleavage took place. Higher concentrations of the chemical were tested in the same manner as those of lower concentrations, with

both eggs in early and late cleavage used. The finding from these series of tests will be discussed separately.

Exposure during early cleavage

After the first division B. rerio eggs undergo cleavage approximately every 18-20 minutes (Roosen-Runge, 1939). However, when eggs were placed in solutions of 17-methylestradiol at higher concentrations these subsequent cleavages failed to occur. Not only did the egg fail to undergo normal cleavage but those cleavage lines present at the time of exposure began to fade as well. The fading of cleavage lines begins at the germ ring of the egg and progresses toward the animal pole. The speed with which it takes place depends on the stage of division as well as the concentrations of the chemical used.

Thirty minutes after exposure the cells in solutions of 10.0 ppm. appear to have a lessened surface tension. Instead of the individual cells being distinct and separated from one another the cell mass appears smooth with only faint cleavage furrows at the pole. The loss of turgidity and subsequent flattening of the cells was exhibited to a lesser degree by the eggs in the other concentrations as well. This striking difference produced by the chemical is shown in some of the photographs at the end of the paper.

After the chorion and yolk were removed from 2-8 cell eggs the free cells were observed at a magnificantion of 430X. At this time some chromosomal movement could be observed. It appeared that the action of the chemical produced a stoppage of mitosis in whatever stage it was, when the chemical was applied, with a preference to metaphase.

In normal mitosis the chromosomes are visible in prophase immediately before the breakdown of the nuclear membrane (Roosen-Runge, 1939). After

this they are not visible again until they reappear as chromosomal vesicles in telophase. When 17-methylestradiol was applied to the free cells observation through high magnification disclosed the immediate effect of the steroid on the nucleus. If the cell was in prophase the nuclear membrane brokedown and no further mitotic activity was observed. If the cell was in telophase when the chemical was applied the nucleus appear to degenerate with the subsequent loss of all inner differentiation. It appeared that the chemical was specific for a particular stage of mitosis, although more detailed study in this area might prove otherwise.

Exposure during late cleavage

Eggs in the 32-cell stage and above exposed to higher concentrations are affected in much the same manner as those in early cleavage. Due to the fact that more cells are present the coalescing of the cells can be observed easier. Many of the peripheral cells appear large after they have been exposed to the chemical for a few hours. The cell membranes appear to break down and adjacent cells merge. A complete disorganization of the cells takes place with a lessening of surface tension allowing a flattening of the cap of cells as exhibited in the lower concentrations.

That these results were brought about solely due to chemical means was tested by subjecting embryos treated with higher concentrations of the chemical to temperatures of 40° F. Embryos in the control dishes exhibited a cessation of cell division but did not show the characteristic coalescing of the cell membrane as did the embryos in the test dishes. They exhibited instead a quick-freeze type of reaction with the cells remaining in the same form for many hours, as when first subjected to the low temperature.

Exposure of 24 hour embryos

In this test normal embryos were transferred from fresh water to concentrations of 17-methylestradiol ranging from 10.0 to 1.0 ppm. Whereas the 24 hour embryos exposed at lower concentrations were not adversely affected, those at the higher concentrations showed very definite abnormalities. These abnormalities affected not only the areas designated before as those susceptible to the lower concentrations on eggs in both early and late cleavage, but additional areas as well.

In normal embryos the pericardial sac and yolk are separated by a membrane, with both areas composing a definite spatial area. In the treated embryos the pericardial region swelled to a large size and appeared entirely clear. The heart did not develop properly within the pericardial sac during this time.

The embryos, after only 6 hours in the higher concentrations of the chemical, appear to be covered by blisters. This gives the surface of the animal a bubbly, translucent appearance which lasts until death.

The pigment on the yolk was either absent or entirely obscured by this transluscence. Dead cells appeared on the surface of the yolk as well as on the gut and in the cephalic region. The general development of the embryo was so affected that after only 24 hours in the chemical most of the specimens had succumbed.

Concentrations of 10.0 ppm. had so strong an effect that embryos exhibited a swollen pericardium and gut after only six hours in the solution. Embryos in concentrations of 5.6 ppm. and 3.2 ppm. exhibited this characteristic at 12 hours. After an exposure of 24 hours all concentrations produced abnormal characteristics and in the 10.0 ppm. half were dead. After 48 hours of exposure there was complete toxicity

in concentrations of 3.2 ppm. and half of the embryos in 1.8 ppm. were also dead. At the 72 hour reading the only specimens surviving were in the 1.0 ppm. concentration. The severity of the effects produced by the higher concentrations of 17-methylestradiol on 24 hour embryos is shown in Table V.

Observations of the effects of 17-methylestradiol on other organisms

The tails of one-week-old tadpoles were snipped off and studied in aceto-carmine squash preparations. A concentration of 5.6 ppm. of 17-methylestradiol produced stoppage of mitosis, for the most part, in the metaphase or anaphase stage.

Personal correspondence with Dr. Roy W. Jones (July 5, 1961) on his work with pig kidney tissue cultures disclosed the fact that 17-methylestradiol produced a colchicine-type effect on cell division. After 48 hours a concentration of 10.0 ppm. added to the culture medium of PK_{13} cells grown in monolayer tissue cultures produced many multinucleate cells. The number of cells showing mitotic figures was sharply increased with the chromosomal organization and arrangement similar to that observed in colchicine-treated cells. Treated cells were larger then untreated cells and had an increased number of chromosomes. Many cell clusters exhibited a bubbly appearance similar to that shown by 24 hour embryos of B. rerio treated with higher concentrations of 17-methylestradiol (Plate IV, Fig. 5). It had not been determined at that time whether the multimucleate condition of the cells was due to the cells coalesing,or whether it was due to the nuclei dividing and the cytoplasm failing to divide.

DISCUSSION

17-Methylestradiol in concentrations as low as 0.32 parts per million has a significant effect on the embryological development of Brachydanio rerio. The effects produced by the chemical are characteristic of it and are in direct proportion to the concentration of the chemical used and the age of the embryo involved. The severity of the effects is demonstrated by the fact that recovery is not evident after removal of the specimens from the chemical. The chemical appears to definitely inhibit cell division in such a manner that regeneration of affected parts is not possible.

It appears that 17-methylestradiol has a disrupting effect on normal cell division by breaking down the cell membranes already formed and inhibiting the further formation of subsequent membranes. This results in a coalescing of the adjacent cells, giving rise to a smooth cap rather than one distinguished by individual cells.

17-Methylestradiol produced certain characteristic abnormalities at all concentrations tested. The tail is usually affected at all concentrations and assumes a number of abnormal positions. It is also shortened and does not function properly in the characteristic movement of the embryo within the chorion. The gut is greatly distorted in size as well as in shape. The pericardium is adversely affected and it appears that circulation is hampered in many places with the appearance of congested blood cells. A condition of edema causes a large amount of swelling in the pericardial region.

All of the above-mentioned abnormalities are produced by the lower concentrations of the chemical. These abnormalities probably result from the inhibition of mitosis in the test embryos. The concentrations of the sterioid are such that mitosis is not completely halted, yet the effect produces a disorganization of the cells which brings about abnormal development.

These and even more noticeable abnormalities occur in 24-hour embryos treated with higher concentrations of the chemical. In this instance the internal organs are already fairly well formed yet the chemical produces sudden distortion of the organs and in a short time brings about death in the test embryos.

After only six hours in the chemical, the entire animal is covered with blisters causing a granular translucent appearnace. The embryos exhibit a condition of edema which is most pronounced in the region of the pericardium. The pericardial sac is quite swollen and lined with irregular cells. It encroaches on the area at the top of the yolk and much of this area is filled with granular like blisters.

The trunk of the treated embryos does not develop in a manner corresponding to that of the untreated embryos. The growth of the myotomes is slowed, resulting in little muscular activity of the embryos within the chorion. The cephalic region also is adversely effected. At the time of exposure to 17-methylestradiol the brain of normal embryos is changing from a solid to a hollow tube. The chemical probably affects mitosis in such a way that this morphological process is disrupted, causing the expansion of the cephalic region to be greatly hindered. The eyes, already developed in normal embryos, undergo severe degeneration and are covered with dead and dying cells.

The yolk, due to the retardation of normal development in the tail and trunk, assumes a much larger spatial area in relation to the embryo as a whole. The gut is not demarcated as noticeably from the yolk and is much shortened due to the corresponding shortening of the entire animal.

The effect of the chemical on the differentiated tissue of 24-hour embryos is probably a disruption and disorganization of the cells which organize the tissues, as these abnormalities produce death in the embryos within 24 to 48 hours.

One of the most important effects of 17-methylestradiol is the stoppage of cell division in eggs. Once the higher concentrations of 17-methylestradiol are applied to the eggs no further development takes place. The steroid erases the cleavage lines present in the eggs at the time of exposure, within a matter of a few hours. This coalescing of cell membranes appears to give rise to multinucleate cells as the adjacent cells merge.

17-Methylestradiol in higher concentrations stops mitosis, in the majority of cells, during metaphase. In doing so it has an effect similar to the alkaloid, colchicine. When applied to the free cells of eggs in early cleavage the nuclear membrane is broken down and mitotic activity quickly comes to a halt. This stoppage of mitosis results in a disorganization of all further cellular differentiation. The combined phenomena of arrested mitosis and the coalescence of cell membranes gives rise to larger-than-normal cells with an apparently increased number of chromosomes per cell.

The effect of higher concentrations of 17-methylestradiol on eggs in both early and late cleavage is quite significant. The chemicals'

ability to completely stop cell division might prove profitible in treatment of cancer-type growths. Further work along this line is advised as the exact manner in which the steroid produces its effect is not known.

SUMMARY

A study of the effects of 17-methylestradiol on the embryological development of the Zebra fish, Brachydanio rerio (Hamilton), shows that these embryos are sensitive to this chemical at concentrations as low as 0.18 parts per million. Three developmental stages were used in the test. Early cleavage (2-16 cell stage), late cleavage (32-cell to blastula) and 24 hour embryos.

Concentrations as high as 10.0 ppm. were used and it was found that the effect of the chemical was directly proportional to the concentration of the chemical used and the age of the embryos involved.

Some embryos were given a recovery test after being exposed to the chemical. In no case was it found that complete recovery was possible. However, it was found that the removal of the chemical does away with any further retardation of development.

A listing of some of the specific abnormalities characteristic of the chemical follows:

1. 17-Methylestradiol on embryos in early cleavage affects tail development in concentrations as low as 0.18 parts per million.
2. Test specimens were retarded as to size and the time of formation of organs.
3. The tail and gut were especially sensitive to even the lower concentrations.
4. The pericardium of day old embryos was very sensitive to the higher concentrations, exhibiting great swelling and distortion.
5. The chemical produced a blistered effect over the entire 24 hour embryo causing a translucent appearance and obscuring pigmentation.

6. Cell membranes were broken down in the higher concentrations resulting in the complete disorganization of cell division in the eggs.

7. Mitosis was stopped for the most part during metaphase in the higher concentrations.

8. The combined action of inhibited mitosis and coalescence of the cell membranes produced large multinucleate cells.

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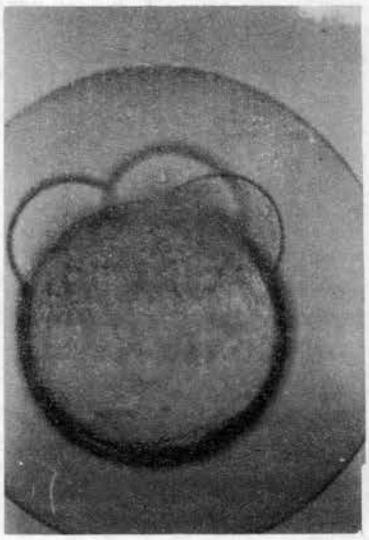
APPENDIX

Plate I. . . . . .

Figure 1. Normal four cell egg of Brachydanio rerio.
2. Four cell egg of Brachydanio rerio, five minutes after exposure to 5.6 ppm. of 17-methylestradiol.
3. Normal eight cell egg of Brachydanio rerio.
4. Eight cell egg of Brachydanio rerio, two hours after exposure to 3.2 ppm. of 17-methylestradiol.
5. Eight cell egg of Brachydanio rerio, two hours after exposure to 10.0 ppm. of 17-methylestradiol.
6. Eight cell egg of Brachydanio rerio, five hours after exposure to 5.6 ppm. of 17-methylestradiol.

Plate I



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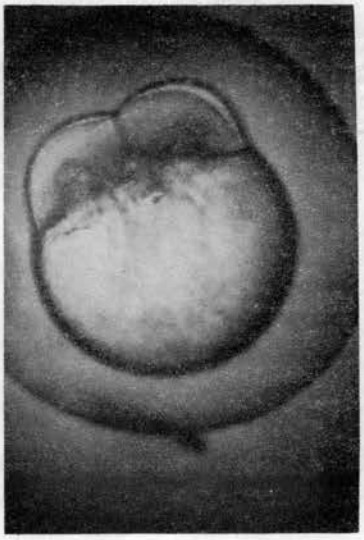

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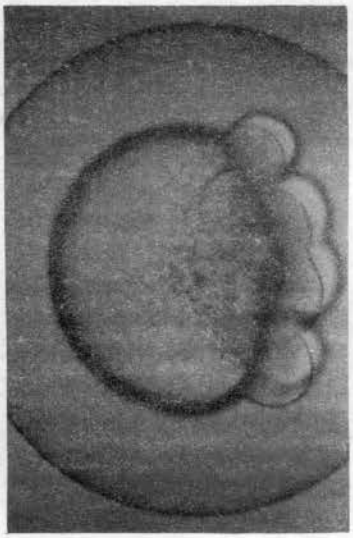

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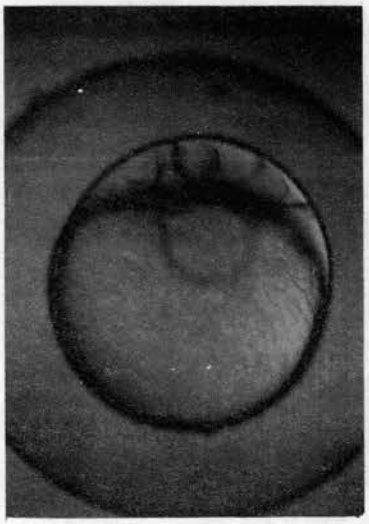

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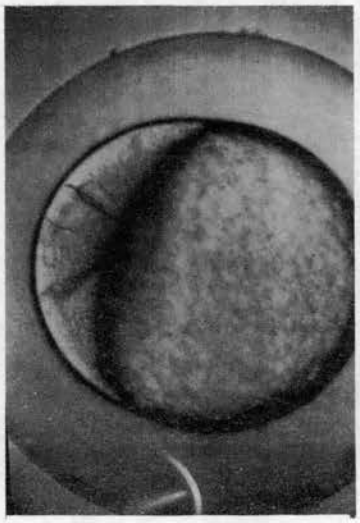

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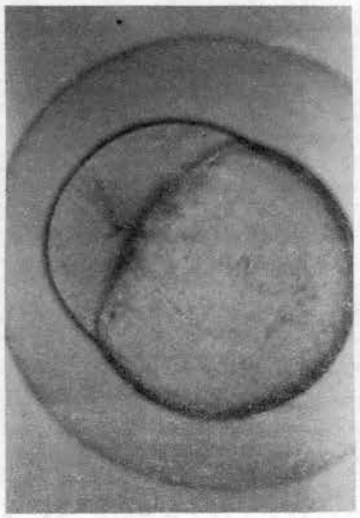
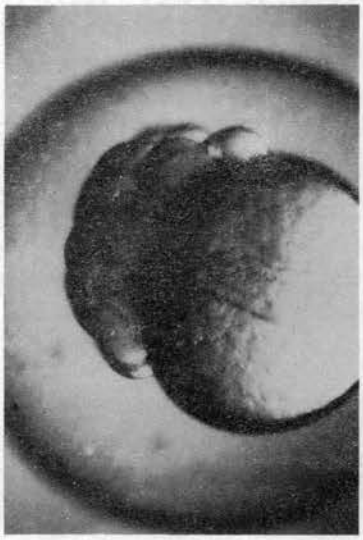


Figure 6.

Plate II. . . . . .

Figure 1. Normal 32-cell egg of Brachydanio rerio.
2. 32-cell egg of Brachydanio rerio, 15 minutes after exposure to 3.2 ppm. of 17-methylestradiol.
3. 32-cell egg of Brachydanio rerio, 45 minutes after exposure to 3.2 ppm. of 17-methylestradiol.
4. 64-cell egg of Brachydanio rerio, two hours after exposure to 5.6 ppm. of 17-methylestradiol.
5. 32-cell egg of Brachydanio rerio, five minutes after exposure to 3.2 ppm. of 17-methylestradiol.
6. 32-cell egg to Brachydanio rerio, two hours after exposure to 5.6 ppm. of 17-methylestradiol.

Plate II



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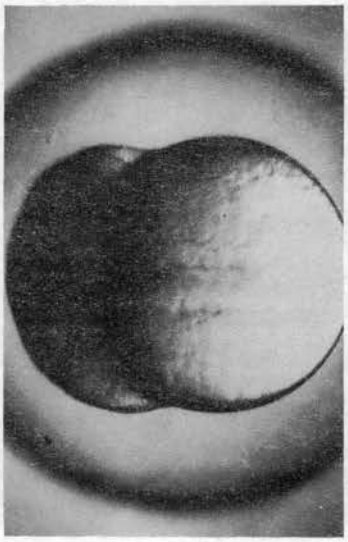

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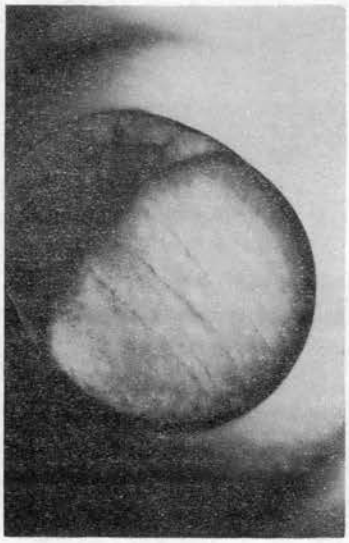

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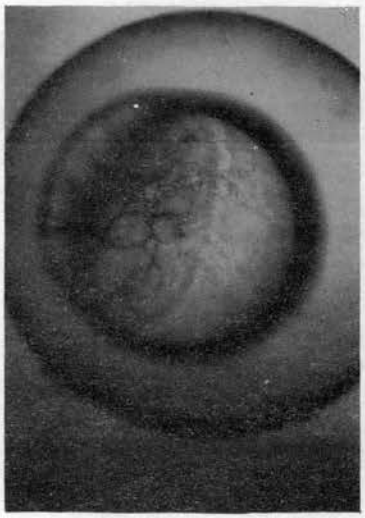

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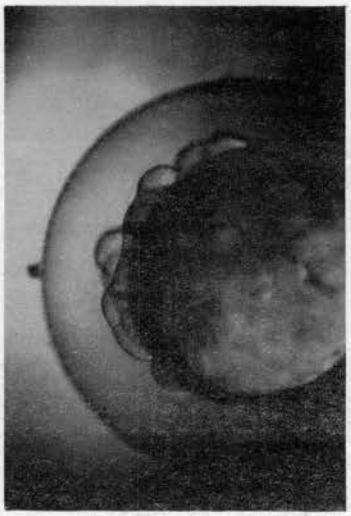

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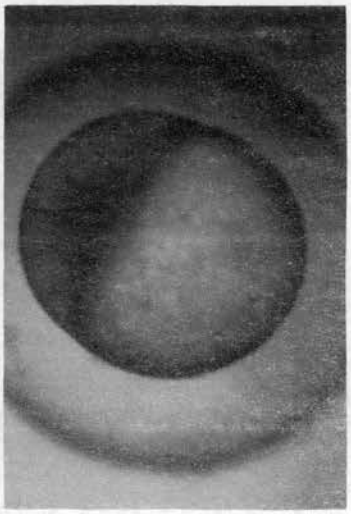


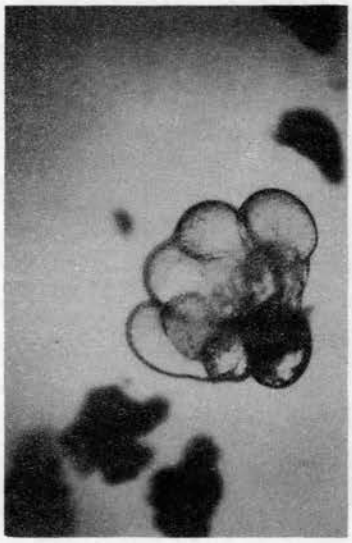
Figure 6.

Plate III. . . . . .

Figure 1. Four cell egg of Brachydanio rerio, with chorion and yolk removed, immediately after exposure to 5.6 ppm. of 17-methylestradiol. 100X magnification, same as that of eggs shown in Plate I.

2. Four cell egg of Brachydanio rerio, with chorion and yolk removed, five minutes after exposure to 3.2 ppm. of 17-methylestradiol.
3. 32-cell egg of Brachydanio rerio, with chorion and yolk removed, five minutes after exposure to 5.6 ppm. of 17-methylestradiol.
4. 64-cell egg of Brachydanio rerio, treated with podophyllotoxin.
5. 32-cell egg of Brachydanio rerio, treated with podophyllotoxin.
6. 64-cell egg of Brachydanio rerio, two hours after exposure to 5.6 ppm. of 17-methylestradiol.

Plate III



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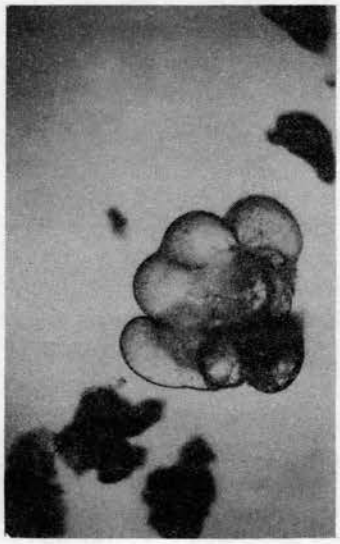

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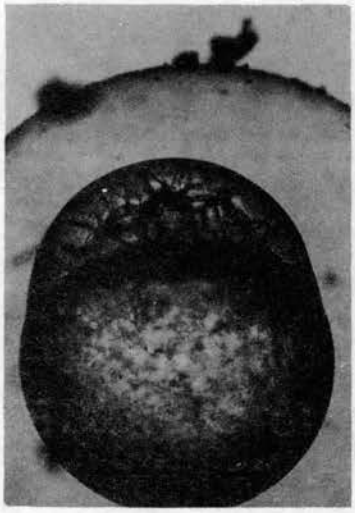

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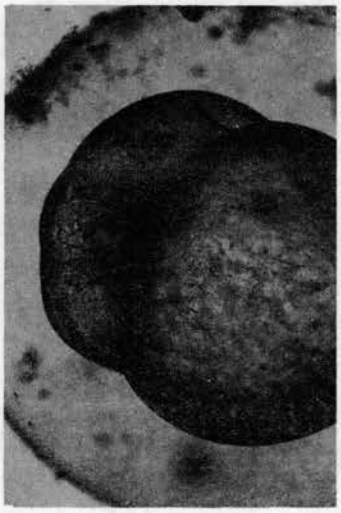

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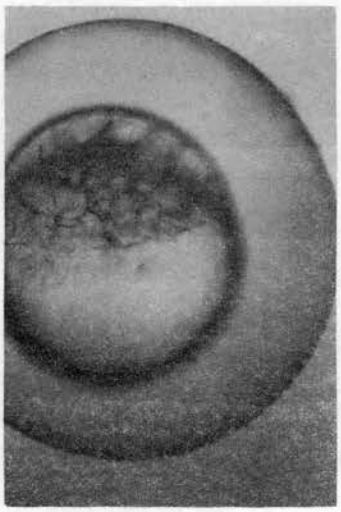


Figure 6.

Plate IV. . . . . .

Figure 1. Normal Brachydanio rerio embryo, 24 hours after fertilization.

2. Normal Brachydanio rerio embryo, 24 hours after fertilization.

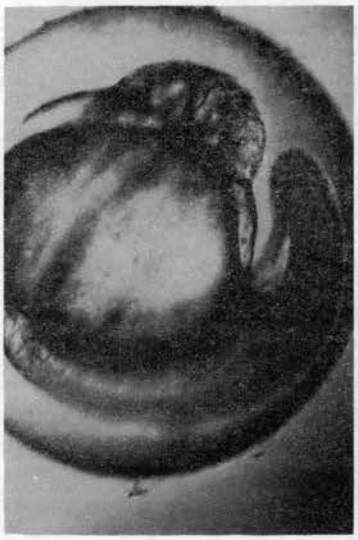
3. Brachydanio rerio embryo, 24 hours after fertilization. Exposed during late cleavage to 0.32 ppm. of 17-methylestradiol. Note abnormal development of gut.

4. Brachydanio rerio embryo, 24 hours after fertilization. Exposed during late cleavage to 0.32 ppm. of 17-methylestradiol. Note general retardation of growth especially in head and tail regions.

5. Brachydanio rerio embryo, 30 hours after fertilization. Exposed at 24 hours to 10.0 ppm. of 17-methylestradiol. Note appearance of blisters over entire surface of embryo.

6. Brachydanio rerio embryo, 36 hours after fertilization. Exposed at 24 hours to 5.6 ppm. of 17-methylestradiol. Note abnormal development of peridardial region.

Plate IV



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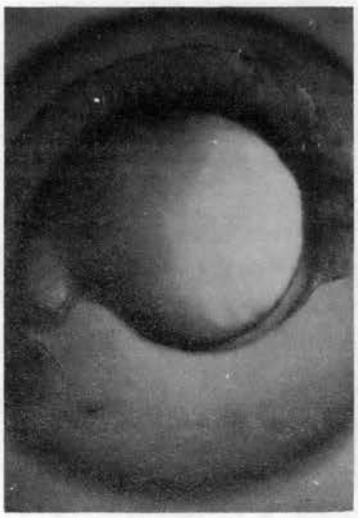

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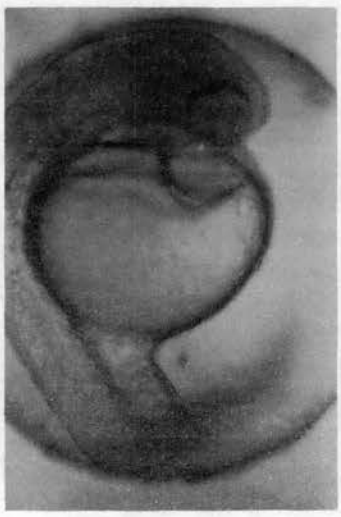

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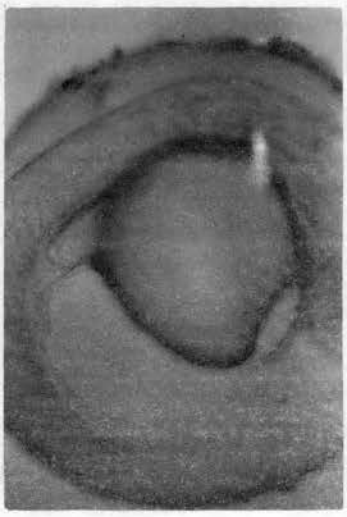


Figure 6.

Plate V. . . . . .

Figure 1. Normal Brachydanio rerio embryo, 48 hours after fertilization.

2. Normal Brachydanio rerio embryo, 48 hours after fertilization.

3. Normal Brachydanio rerio embryo, 72 hours after fertilization.

4. Brachydanio rerio embryo, 48 hours after fertilization. Exposed at 24 hours to 5.6 ppm. of 17-methylestradiol. Note abnormal development of pericadial region and presence of blisters.

5. Brachydanio rerio embryo, 48 hours after fertilization. Exposed at 24 hours to 5.6 ppm. of 17-methylestradiol. Note abnormal development of gut and retarded growth of trunk.

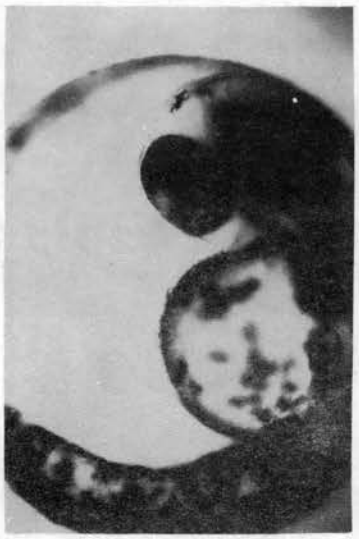
6. Brachydanio rerio embryo, 48 hours after fertilization. Exposed at 24 hours to 3.2 ppm. of 17-methylestradiol. Note granular appearance of pericardial region.

7. Brachydanio rerio embryo, 48 hours after fertilization. Exposed at 24 hours to 3.2 ppm. of 17-methylestradiol. Note twisted tail.

8. Brachydanio rerio embryo, 36 hours after fertilization. Exposed at 24 hours to 3.2 ppm. of 17-methylestradiol. Note abnormal development of pericardial region and gut.

9. Brachydanio rerio embryo, 36 hours after fertilization. Exposed at 24 hours to 3.2 ppm. of 17-methylestradiol. Note retardation of growth in cephalic region and trunk.

Plate V



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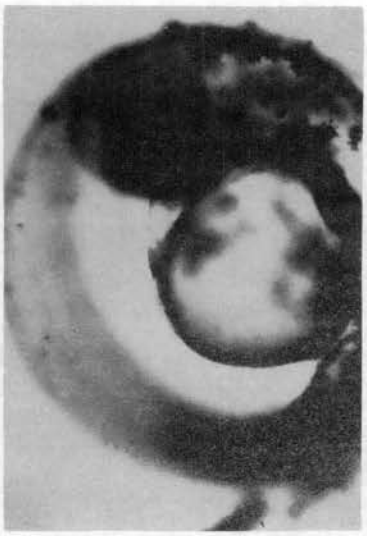

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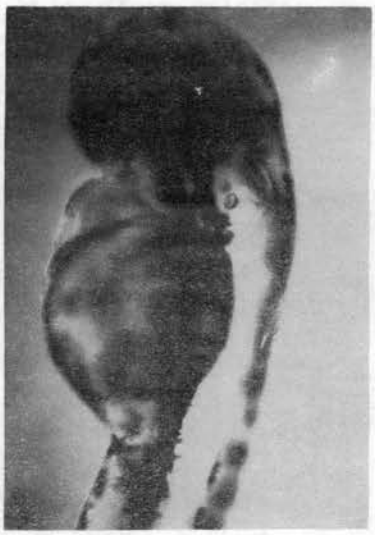

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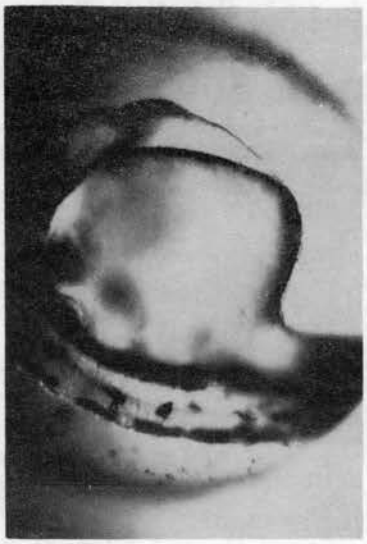

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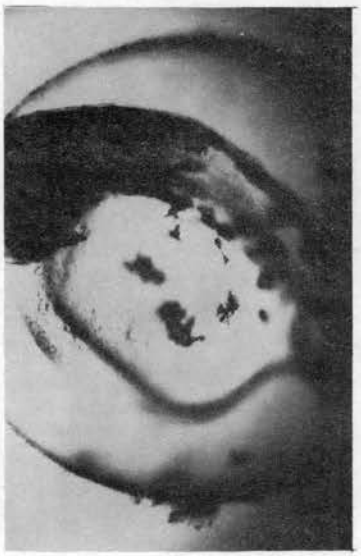

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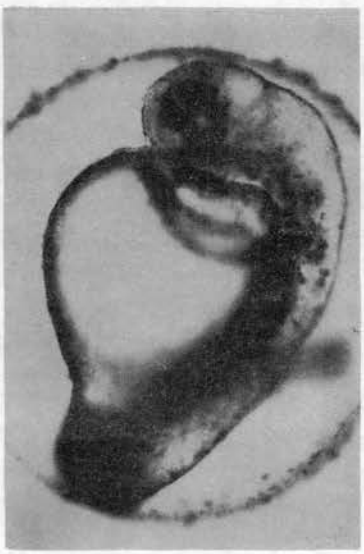

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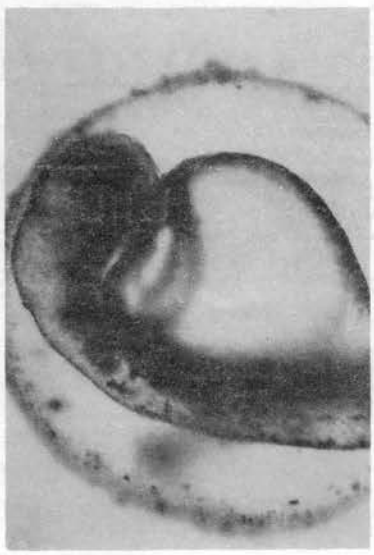
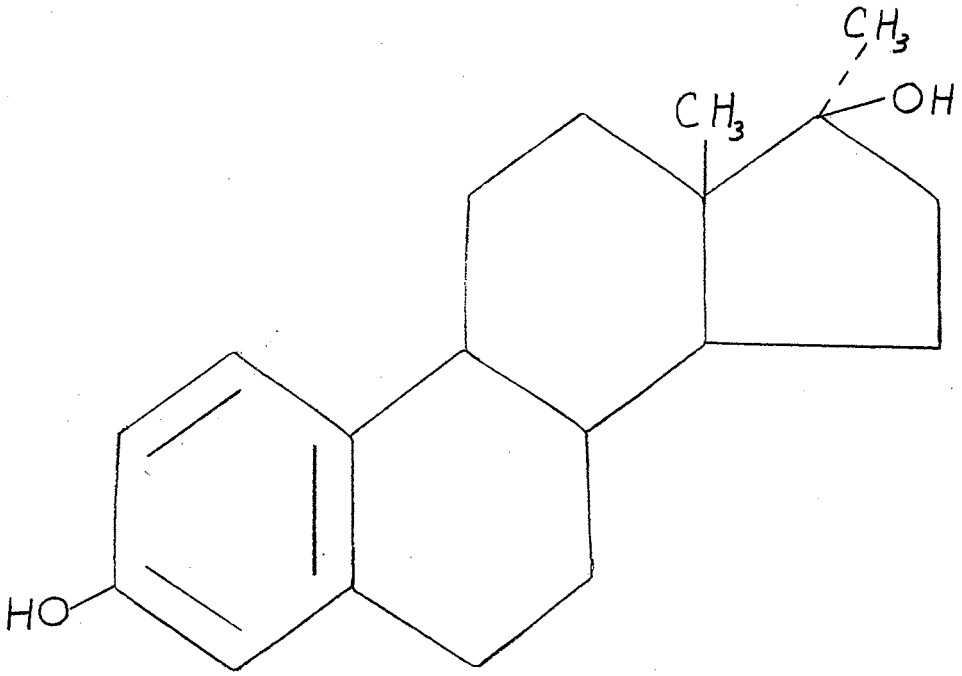


Figure 9.

Plate VI



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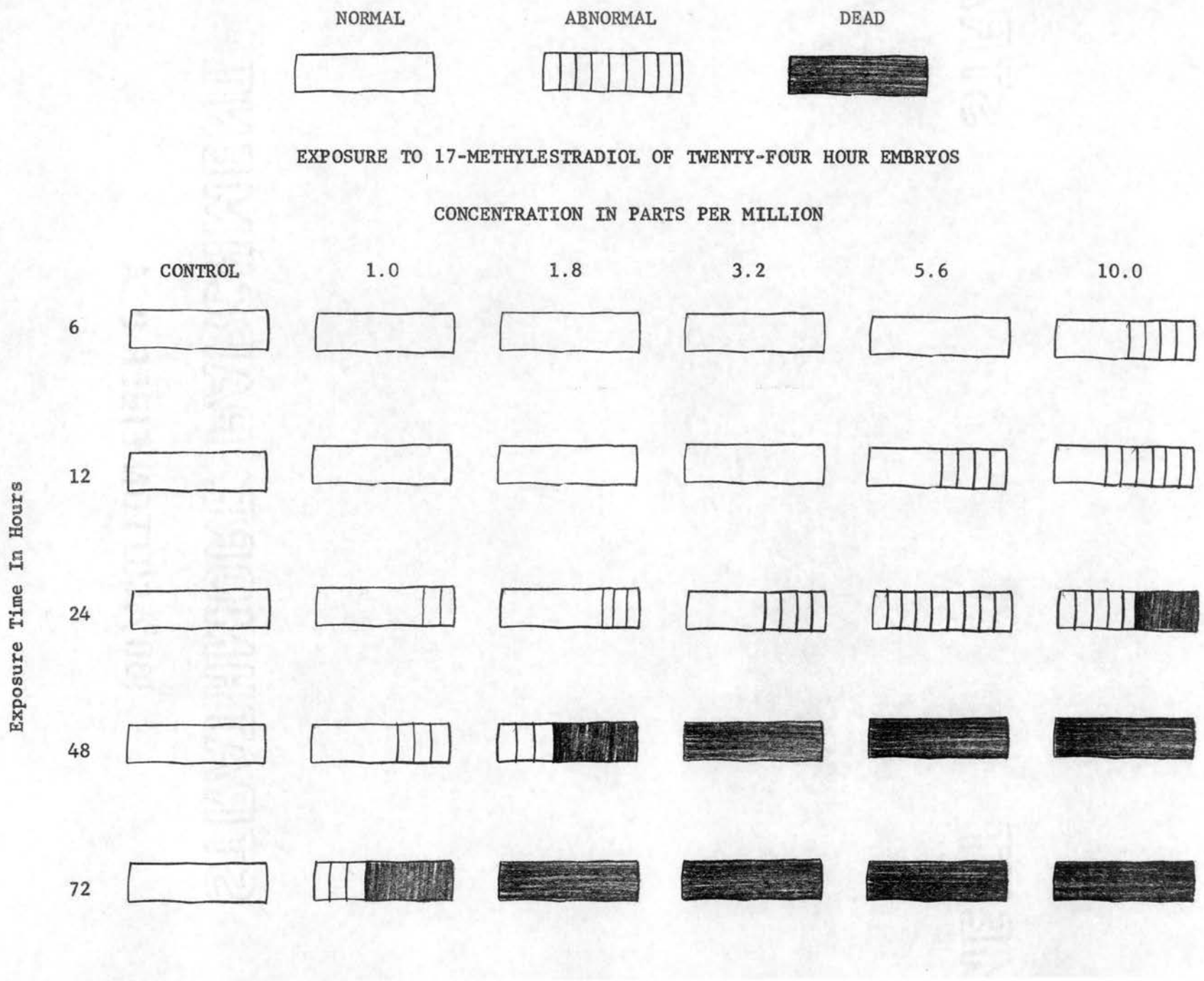
NORMAL
ABNORMAL
DEAD
EXPOSURE TO 17-METHYLESTRADIOL OF TWENTY-FOUR HOUR EMBRYOS
CONCENTRATION IN PARTS PER MILLION
CONTROL
1.0
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6
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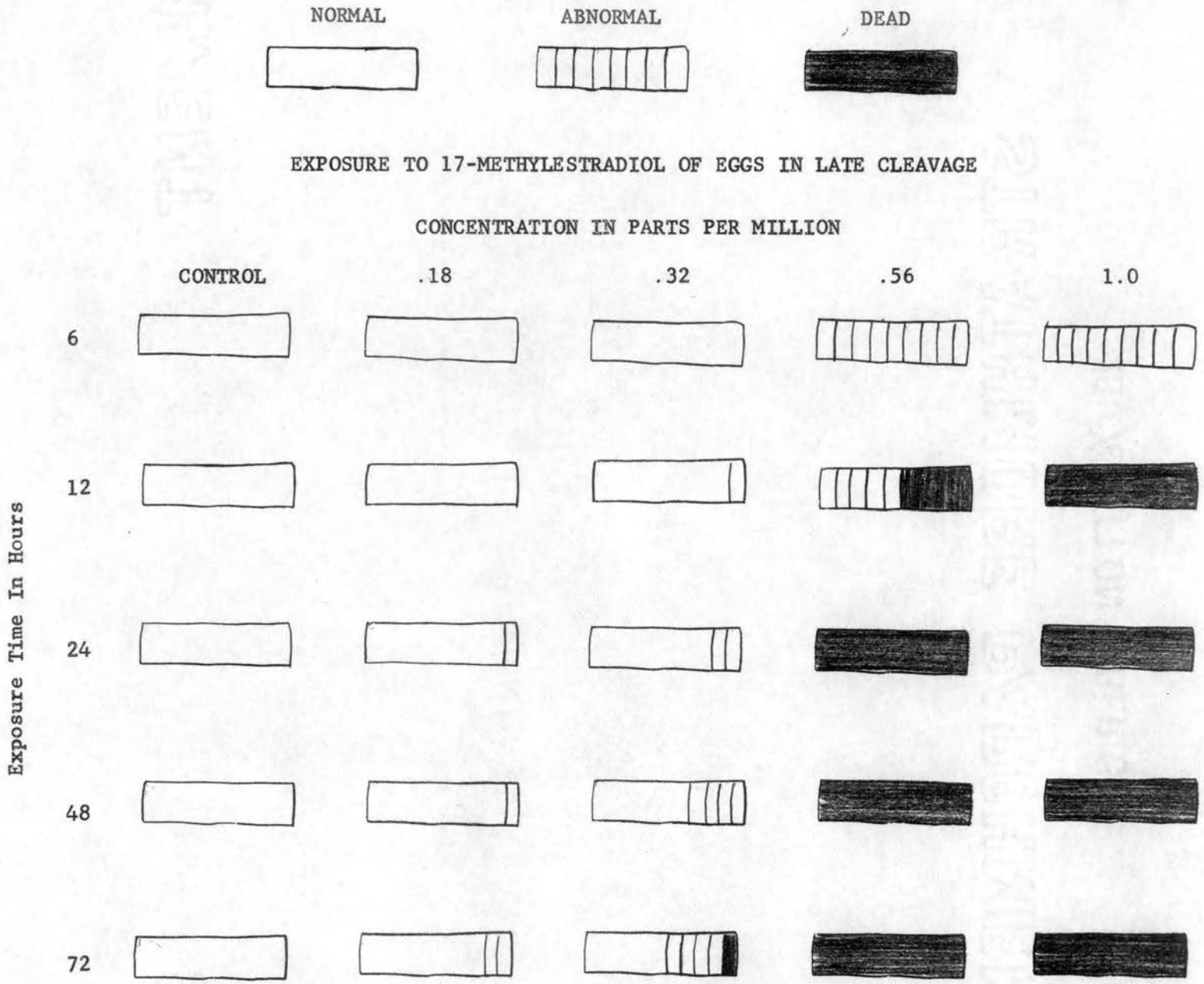
NORMAL
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CONTROL
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.32
.56
1.0
Exposure Time In Hours
6
12
24
48
72

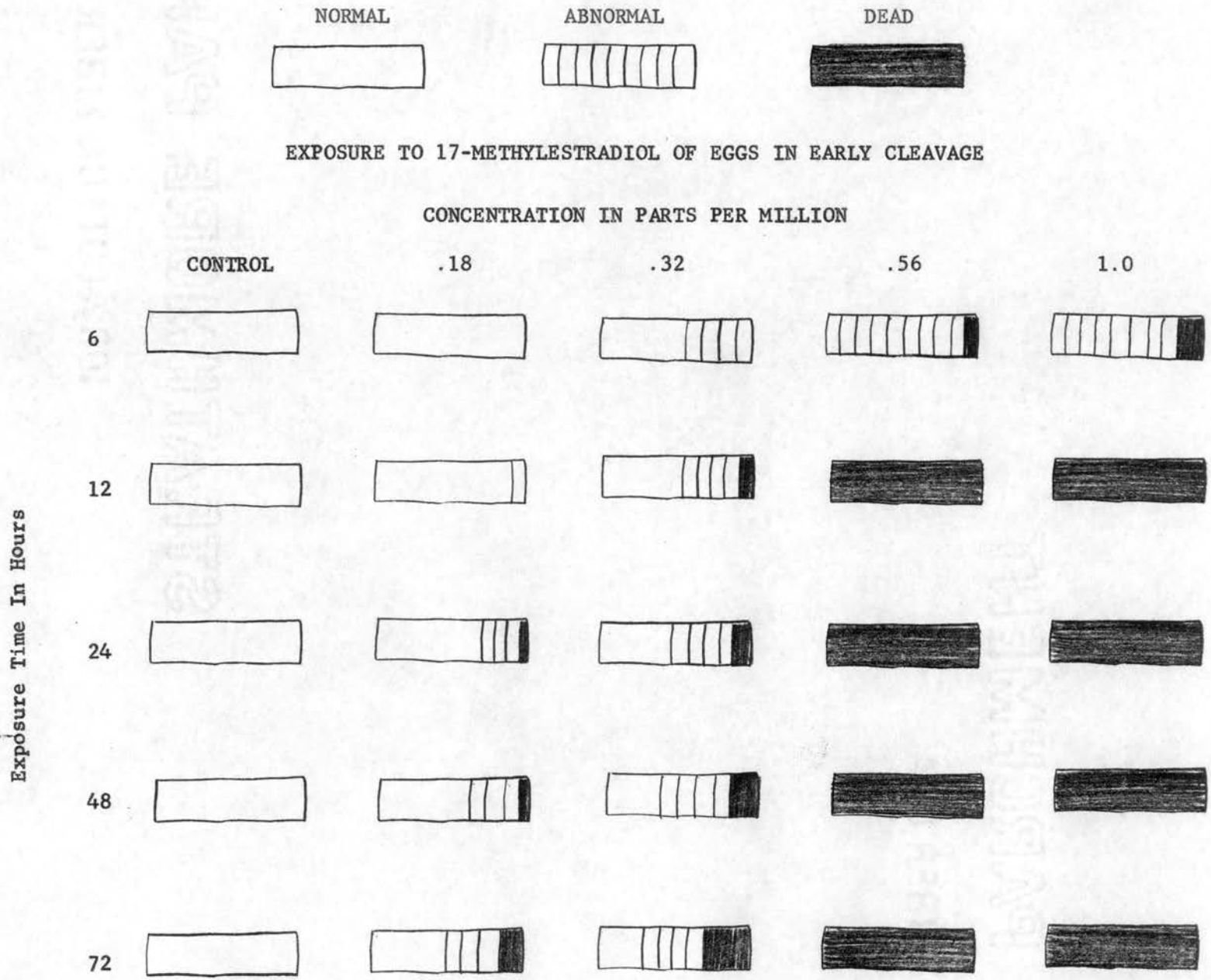
NORMAL
ABNORMAL
DEAD
EXPOSURE TO 17-METHYLESTRADIOL OF EGGS IN EARLY CLEAVAGE
CONCENTRATION IN PARTS PER MILLION
CONTROL
.18
.32
.56
1.0
Exposure Time In Hours
6
12
24
48
72

TABLE VI

TYPICAL LABORATORY EXPERIMENT

Date started 4/29/60 Time 0920 Date closed 5/2/60 Time 0920

Experiment No. 837

Developmental stage of eggs at time of exposure 32-cap		Control No. 1	1.0 ppm	0.56 ppm	0.32 ppm	0.18 ppm	PG 102 1%	Control No. 2	1.0 ppm	0.56 ppm	0.32 ppm	0.18 ppm	PG 102 1%
No. of eggs		10	10	10	10	10	10	10	10	10	10	10	10
6 hours exposure	No. dead & dying	0	4	2	0	0	1	0	2	1	0	0	0
	No. abnormal												
	No. affected by treatment	0	6	8	0	0		0	6	4	0	0	0
	Stage of development	10 ok			10 ok	10 ok	9 ok	10 ok			10 ok	10 ok	10 ok
	Comments	Chemical toxic at 1.0 ppm. Some slowing at development at 0.32.											
12 hours exposure	No. dead & dying	2	10	8	2	0	1		10	8	0	1	0
	No. abnormal	0		2	1	1	0			2	0	0	0
	Comments	0.56 shows dead cells and abnormal cell division; retardation of development. 0.32 may prove active.											
24 hours exposure	No. dead & dying	2	10	10	3	0	1	0	10	10	0	1	0
	No. abnormal	0			1	0	1	1			2	0	0
	Comments	0.32 development of total embryo slowed 0.18 developing normally											
48 hours exposure	No. dead & dying	2	10	10	3	0	2	0	10	10	0	1	0
	No. abnormal	0			1	0	0	2			2	0	0
	Comments	0.32 tails bent or twisted out of line; large number of dead cells in caphalic region and gut. Reduced growth and pigmentation. PG very retarded in pigment.											
72 hours exposure	No. dead & dying	2	10	10	3	0	2	0	10	10	0	1	0
	No. abnormal	0			2	3	0	2			5	0	1
	Comments	Pericardium swollen, tails bent, retarded embryos slowed in development of organs and general size. Not much change from 48 hours. 0.18 active at this time.											

VITA

Virginia Ann Garner

Candidate for the Degree of

Master of Science

Thesis: STUDIES ON THE EFFECTS OF 17-METHYLESTRADIOL ON THE EMBRYOLOGICAL DEVELOPMENT OF THE ZEBRA FISH, Brachydanio rerio (Hamilton).

Major Field: Zoology

Biographical:

Personal data: Bron in Oklahoma City, Oklahoma, January 15, 1938, the daughter of James R. and Jewel S. Garner.

Education: Attended grade school in Bradley, Illinois, and Bethany, Oklahoma; graduated from Bethany High School in 1955; received the Bachelor of Arts degree from Bethany Nazarene College, with a major in Biology, in May, 1959; completed the requirements for the Master of Science degree July, 1961.

Professional Experience: Three semesters as Laboratory Assistant in General Zoology labs, January 1960 to May 1961; Graduate Research Assistant September 1959 to August 1961.