THE CHRONOLOGICAL DEVELOPMENT OF THE EMBRYO OF THE ZEBRA FISH *Branchydanio rerio* (Hamilton) AT A CONSTANT TEMPERATURE

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I. INTRODUCTION

The purpose of this study was to determine the time and sequence of morphogenesis in the Zebra fish, Brachydanio rerio (Hamilton). The study began with the 16-cell stage and considered the development of various organs up to 72 hours after fertilization. Hatching usually had occurred and the major organ systems had been established by the 72nd. hour after fertilization. "Critical periods," or periods during which certain basic organ structures were established or altered, were identified.

The Zebra fish is an excellent animal for studying the actions of hormones and other organic compounds upon cell division and embryonic development, Gibson (9), Jones (17 and 18), Jones et. al. (16), and Battle and Hisaoka (3). Anderson and Jones (1), Goff (10), and Whittington (41) have utilized these embryos in studying the effects of physical forces upon embryonic development. The description and identification of the developmental stages of this fish should be of value in future studies.

The embryos of Brachydanio rerio offer many advantages as material for embryonic studies. The fertilized eggs are easily obtained daily in large numbers. The embryos undergo rapid cleavages and morphogenesis, so that within 72 hours they undergo the morphological changes that often take much

longer in other laboratory animals. Most important, the animal is completely transparent during the formation of the major organs. After the development of pigment it is translucent in the non-colored areas. The researcher is thus afforded the opportunity of observing the morphogenetic changes as they occur in the living animal. Culture of the embryos at a constant temperature is comparatively easy. A hundred or more embryos can be kept in water in a fingerbowl stored in an incubator. Experimental chemicals whose effects are to be studied can easily be added to the culture water in the concentrations desired.

II. REVIEW OF THE LITERATURE

One of the papers which is considered a classic in the field of fish embryology is, "The Embryology of the Sea Bass," by Wilson (40). This work contains complete gross and histological descriptions of the derivations and fates of the primary germ layers. Wilson not only summarized the previous work in fish embryology, but also compared teleost development with the studies of embryos of the higher vertebrates. Studies on fish embryology which have followed his work generally confirm his observations.

Some authors believe that the chronological age of a teleost, stated in hours or days, is not a satisfactory way of expressing the stage of embryonic development, because the rate of growth varies with the supply of oxygen and environmental temperature. Oppenheimer (24) used a stage-naming system, in which development was expressed in terms of the degree of organ and tissue differentiation.

Other authors, Solberg (34), Price (26, 27, and 28), Watling and Brown (39), and Ingersol (14), observed that when oxygen supply and temperature were kept constant, embryological differentiation occurred as a function of time. By maintaining a constant temperature, each gave a chronological account of embryonic development of their respective species of fish. The hatching of fish from the chorion was accepted in each

case as the arbitrary point at which embryogeny ceased.

Studies involving the development of viviparous fish were compiled by Hopper (13), Tavolga (38), and Tavolga and Rugh (37). The latter article included a comparison of oviparous to viviparous fish embryology.

Teleosts appear to vary in the sequence of the appearance of certain structures. Bolin (4) found that in the development of the fish Oxyjulis californicus, the embryonic axis becomes visible before the ring of the blastodisc reaches the equator of the yolk, and that Kuffer's vesicle appears before the closure of the blastopore.

Orton (25), discussed the development and migration of pigment cells in teleosts, and Oppenheimer (23), described the organization of the teleost blastoderm.

Many authors have reported observations on fish embryology and larval development in connection with life history and/or ecological studies, e. g. Battle (2), Budd (5), Carr (6), Fry (8), Harrington (12), Moore (21), and Morris (22). Many of these papers give chronological times of organ development utilizing structures already established as a reference point. In only a few cases were the embryos kept at a constant temperature and the sequence of morphogenic events noted.

Jones (19) and Self (33), studying the killifish and the Gambusia respectively, used the mitotic index to determine relationships between cell division and differentiation.

Stockard (36), investigating the formation of monsters and other anomalous formations in vertebrate embryos, contri-

buted much to the field of experimental and descriptive teleost embryology. He discussed various abnormalities possible in fish embryos and attempted to explain their origin.

Lewis and Rossen-Runge (20), utilizing time lapse photography, produced a motion picture of the early development of Brachydanio rerio. Cytological and protoplasmic events which occurred during early cleavage and gastrulation and the formation of certain organ systems during later embryonic development were the main themes.

Roosen-Runge (30), described the cytological and protoplasmic events occurring in the living egg of the danio before the 32-cell stage. Photographs of various stages of the early embryos of the fish are available.

Goff (10), while describing the effects of increased atmospheric pressure upon the development of Brachydanio rerio, recorded some observations on the chronological sequence in normal embryonic development. A constant temperature similar to the one used in the present study was maintained.

Effects of various physical and chemical changes in the external enviroment of the danio have been studied by many workers. Sensitivity to drugs and ultrasonic vibrations were recorded by Gibson (9), Battle and Hisaoka (3), Anderson and Jones (1), and Jones et. al. (16, 17, and 18).

An anomalous early development of Brachydanio rerio without cleavage was observed by Rossen-Runge (30).

A technique for collecting and handling eggs of the danio was reported by Creaser (7).

Goodrich and Nichols (11), working with regeneration of portions of the fin of the adult fish, Brachydanio rerio studied the actions of chromatophores in the newly formed appendages. They found that there is the appearance of self coloring followed by a localized destruction of chromatophores, thus leaving an altered color pattern.

Whittington (41) found that chromatophores in the young adults of the Zebra fish are definitely affected by variations in light and background and migrate in response to changes in these stimuli.

Observations were made by Schmidt (32) concerning the hybridization of Brachydanio rerio and Brachydanio albolineatus.

III. MATERIALS AND METHODS

Care of adults:

Adult Zebra fish, were kept in 5 gallon aerated aquaria with water six inches deep and at a temperature of about 26° C. The number of fish per tank varied from 25 to 35. The males were more abundant than females. A layer of marbles was placed on the bottom of each tank. The water was filtered through nylon or glass wool, or replaced with freshly aerated water each time the tanks were cleaned.

Two types of commercial fish food, plankton, daphnia, dwarf white worms of the family Enchytraeidae, dried shredded shrimp, brine shrimp of the genus Artemia, and a baby food mixture were used to feed the fish. The baby food mixture was made by mixing one can of strained liver, one can of cooked eggyolk and two cans of strained spinach with pablum to thicken to a paste. This mixture was cooked one hour and refrigerated below 0° Centigrade.

Conditions for egglaying:

The initiation of spawning was helped by a diet of live foods. The tanks were kept darkened from early evening to morning by either turning off all of the lights, or by masking the tanks. When the tanks were lighted again, a pinch of dried shrimp was dropped into each tank to initiate excitement. Spawning usually occurred soon thereafter. The eggs dropped

to the bottom of the tank into the interstices between the marbles. There they were comparatively safe from the carnivorous adult fish.

Collection of fertilized eggs:

The eggs were collected by siphoning them from among the marbles. This process also collected much debris. The eggs were separated from the debris by repeated rinsing and decanting. The debris, being lighter than the eggs, was removed during the decanting process. The eggs were removed from the heavier detritus with the aid of a rubber-bulbed pipette. The eggs were sorted for developmental stages under a dissecting microscope over a dark background.

Culture of the embryos:

Twenty-five eggs were incubated in 100 ml. of water in standard, covered culture dishes. The incubation temperature was maintained at 26 ± 1° C. All dead (white) embryos were removed from the culture dishes as soon as observed to prevent contamination.

Methods of obtaining data concerning the embryonic development:

A. Utilization of direct observation.

A wide-field binocular microscope with magnifications of 10x and 30x was used for observation of gross anatomical features. This lent a three-dimensional aspect to the observations of development.

The chorions of the embryos were removed after the 24th. hour of development, to aid in the observation of the embryos. At this stage the embryo begins to curl upon itself,

and the chorion becomes adhesive, picking up dust particles which obstruct the view. The chorion was punctured with micro-dissecting needles and then torn open, releasing the embryo.

B. Utilization of photography.

Photographic records of the embryo were made with a 35 mm. Leica camera attached to a standard Spencer microscope fitted with a Micro-Ipso. Kohler illumination with a ribbon-filament lamp was used throughout. Photomicrographs were made at 50, 60, and 100 diameters. Phase contrast was used in studying the more advanced stages.

Early in the study, pictures were made every hour. As the embryo got older, observable morphologic changes became more frequent, and exposures were made every half hour. Towards the end of the study, the observable changes became less frequent and photographs were made once every forty-five minutes.

C. Utilization of a wire recorder.

Verbal descriptions of the development observed under the microscope were recorded on magnetic wire. This method was especially important in studying rapid morphological changes. The observations were later transcribed or summarized on paper. There are several advantages to this method of recording. The use of the recorder leaves the observer free to take photographs, remove chorions, and watch the embryo develop without moving his eyes, hands and attention to a note pad. Photographs should be so labeled that identification with the recorded description is possible later.

D. Utilization of a written record.

Data, such as number of pictures taken at one observation period, illumination intensity, use of phase contrast, frame magnification, and chronological age of the embryo at the observation period were recorded. Sketches were made of particular structures which the author feared would not show in the photographs.

Methods of preserving embryos for future study:

Embryos of various ages were killed, fixed in Bouin's solution, and stored in 60% isopropyl alcohol. All chorions were removed before killing. After fixation, 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 was included in the tubes. The tubes were immersed in a jar containing 60% isopoply alcohol and stored. Fixed material was thus available for reference and confirmation of in vivo observations.

IV. OBSERVATIONS

General description of the embryonic development:

According to Roosen-Runge (31), the first cleavage takes place 30 ± minutes after fertilization, and each successive group of cleavages, to the tenth, occurs at 17-20 minute intervals at 28-29° C. After the tenth cleavage, the rate slows as the blastodisc begins its movement downward over the surface of the yolk. Many studies in our laboratory indicate that these observations are correct. At the incubation temperature of 26 ± 1° C., the first cleavage took place in 35 ± minutes, and subsequent cleavages occurred at 18-20 minute intervals.

The edge of the blastodisc, once it begins to envelop the yolk mass, forms the germ ring. It reaches the equator of the yolk mass at about the fourth hour of development, at which time the embryonic shield begins to appear. By the middle of the seventh hour the blastopore closes, and the embryonic axis is well defined. The notochord appears below the primitive nerve chord by the middle of the ninth hour. This is followed by the formation of the first somite during the twelfth hour.

The solid nerve chord delaminates to form a tube by the 15th. hour. The tail begins to constrict from the yolk by the middle of the 20th. hour, and is entirely free by the

25th. hour. The hind gut, being an integral part of the tail is also freed. Motion in the embryo was first observed during the 21st. hour, suggesting the beginning of function of the nervous and muscular systems.

The heart beat begins about the 28th. hour of development, followed 30 minutes later by circulation of the blood. At about the same time, pigment begins to appear in the anterior dorsal portion of the eye cup. About the 28th. hour, pigment cells can be observed on the ectoderm of the sides of the embryonic axis, in the region covering the anterior somites. Some of the pigment cells appear to migrate caudoventrally and eventually to form two lateral rows of melanophores. Other pigment cells migrate ventrally to the area of the yolk sac.

The anterior limb buds appear during the 30th. hour of development as a cluster of cells in the area lateral to the midgut, just above the stalk of the yolk sac. By the time of hatching, the pectoral fins usually have differentiated. Hatching time varies with individuals in this animal. The majority of specimens hatch between the 60th. and the 77th. hour of development. There are various reasons for such wide latitudes of time in which hatching takes place. Hatching depends upon two factors, the lysis of the chorionic material and the activity of the embryo inside the chorion. Since neither of these factors is constant for any group of embryos, the time of hatching varies.

Observations concerning the embryo in general:

The yolk and the body cells of these fish remain transparent from the time of fertilization of the ovum to the time of hatching. The cells containing the melanin pigment become opaque. Dead cells and embryos turn white and become opaque. Dying cells become translucent.

After the onset of muscular activity in the embryo, it was observed that movement of the embryo increases in the presence of strong light. Upon the removal of this light, the embryonic movements became less intense. Since muscular contraction is closely allied with enervation of the muscles, it is possible that the increase in embryonic movement is associated with nervous stimulation by light.

In order to observe more accurately and to photograph better, the embryos were removed from their chorions. The embryo usually dies if removed from the chorion during the first 14 hours of development. If the chorion is removed after the first day, the chances of the embryo remaining viable is greater and the development is usually normal. Possibly, the integument of an embryo of less than 24 hours of age is not sufficiently developed to be protective without the aid of the chorion. After 24 hours, the outermost layer of the embryo is much better adapted to protect the embryo and chorionic removal can be effected.

Gross early morphology and organogenesis in chronological sequence:

Developmental times in the following discussions are placed in parenthesis. Development here refers to change in

form of an organ or structure, or its appearance chronologically. All times appearing in parentheses refer to the number of hours elapsed after fertilization had occurred.

1. Development from the sixteen cell stage to the closure of the blastopore:

The 16 cell stage (1½ hours) exists as four rows of four cells each, all lying above the yolk mass. The blastodisc cells continue to cleave and form a clump of cells hemispherical in shape, transparent, and continuous with the yolk mass. The embryo now resembles a perfect sphere. The top third of this sphere is covered by the blastodisc (2½ hours), and the uncovered remainder is the yolk mass. This stage is termed by the writer, the "Early Cap Period." (Fig. 1A).

The cell mass continues to expand in the dorso-ventral plane until it is somewhat paraboloid in shape. When considered with the yolk mass the entire structure is shaped like a chicken egg with the small end up. The top or cell mass is parabolic in shape and the bottom or yolk mass is hemispherical. Such a stage is termed the "Middle Cap Period" (3½ hours). (Fig. 1B).

Further growth and multiplication of the cells in the cell mass results in a return of the blastodisc to the hemispherical appearance. This is accomplished by an apparent shrinkage of the cellular cap. The early cell cap consists of a loosely packed mass of large cells extending dorsally from the yolk. The cells first become

more closely joined, and through subsequent divisions become smaller in size. The cells being more tightly packed, give the "cap" an appearance of having shrunk. Later, the cells lose their definition as individual cells at 10x magnification, and the cap takes on a granulated appearance. The germ ring and periblast are quite evident. The blastula stage (4 hours) is termed the "Late Cap Period." (Fig. 1C).

After a period of further division, the cells begin a downward migration over the yolk mass, adhering to, and covering the yolk. When the mass of decending cells (the germ ring) reaches the equator of the yolk, a thin, short, wide, anterior projection is observed (5 hours). This projection extends from one side of the germ ring, over the pole, and one third of the way down on the other side. The projection is the embryonic shield. The writer terms the stage the "Equatorial Period."

The germ ring continues to move downward over the yolk mass, but the diameter of the germ ring does not increase to accommodate that of the yolk. As a result, an elongation of the entire system occurs. (Fig. 4). A graphic analogy that illustrates such a phenomenon would be the forcing of an orange (yolk) into a rigid spherical container (the germ ring and blastula). The orange would necessarily have to elongate, as a result of the pressure in order to fit into the container.

The germ ring constricts and continues to move toward the vegetal pole (uncovered yolk end) of the system, thus keeping the diameter of the germ ring smaller than that of the yolk. A constant pressure is kept on the yolk mass, and the system becomes more elipsoidal in form. As the germ ring advances toward the vegetal pole of the system, the embryonic shield continues to elongate. The shield extends from the germ ring, across the animal pole to a region just dorsal to the equator of the ellipsoid on the other side of the germ ring. (Fig. 2). The body axis, a narrow band of cells, lies in the median aspect of the broad flat embryonic shield ($6\frac{1}{2}$ hours). The axis elongates posteriorly as the germ ring advances over the yolk. (Fig. 5).

The diameter of the germ ring becomes progressively smaller until the yolk is completely covered by cells except for a small area. An analogy is the forcing of an orange into a balloon. Once the orange is inside of the balloon, it is still not completely covered by the balloon at the open end. The still-uncovered yolk forms the yolk plug. The ring continues to shorten its diameter and suddenly disappears. This is the closure of the blastopore ($7\frac{1}{2}$ hours). A thickening of tissue appears a few seconds after the closure of the blastopore, directly below the former yolk plug. A depression appears in the center of the thickening, thus giving an appearance of a ring of tissue. The center of the ring enlarges leaving

a thin cellular layer covering the place that was over the yolk plug. (Fig. 5). The action is analogous to dropping a pebble in a pond. Where the pebble strikes the water, a depression in the water surface is momentarily seen. The depression enlarges and the water on all sides ripples away from the place in which the pebble was dropped. This is called by the author "The Closure of Blastopore Period."

2. Development and differentiation of the notochord and the somites:

The notochord first appears as a homogeneous rod (9 hours). It is a delaminated portion of cellular material from the cells composing the body axis. The rod is granular in appearance and extends from the tail region to the head. (Fig. 7). The first somite appears (12 hours) as a condensation and constriction of cells in the mesoderm on each side of the notochord. The somite is slightly anterior to the middle of the body axis. (Fig. 12). Further development of the somites continue in a caudal direction. As the undifferentiated tissue in the tail condenses into somite blocks, the anterior somites become larger and more sharply defined. The typical "V" shape of the myomeric segments begins to appear as a result of the oblique angularization of the dorsal and ventral borders of the muscle blocks at 18½ hours. (Fig. 13). The notochord continues as described for some time, becoming more vacuolized in appearance on or about the

time of hatching.

3. Development of the brain and the spinal cord:

The neural cord first appears as a single elognated structure (8 hours), extending from the anterior end of the body axis to the posterior. The cord is somewhat enlarged anteriorly and tapers at its posterior end. At the time of the appearance of the notochord (9 hours), the portion of the neural cord located anterior to this rod is approximately 10 times greater in diameter, than the portion that is located above the rod. The anterior portion of the neural cord is the brain; the portion of the neural cord located above the notochord is the primitive spinal cord. The brain at this stage is composed of three parts. The fore and hind parts are at angles to the mid part, so that the entire brain resembles a trapezoid whose base (the yolk) is a convex arc. (Fig. 6). An outward growth of neural tissue occurs just anterior to the mid point of the fore portion of the brain on each side of the body axis. These swellings are the primitive optic vesicles. The vesicles are confluent with the rest of the forebrain. They converge anteriorly to form a rounded apex. The neural cord, as seen from the dorsal view, thus might appear analogous to a blunt arrow. The anterior portion or head of the arrow is formed by the evagination of the optic vesicles.

The brain becomes a five-parted structure (13 hours), each part being demarcated by a definite constriction.

(Fig. 3A). The second, third, and fourth portions of the brain (the diencephalon, mesencephalon, and metencephalon respectively) are approximately equal in length. The first portion of the brain (the telencephalon), is almost twice as long as any one of these. The fifth portion of the brain (the myelencephalon) is approximately equal in length to the telencephalon but somewhat smaller in diameter. (Fig. 9).

A cavity is seen to appear within the solid nerve cord (14½ hours) in the telencephalon area of the brain. It appears dorsoventrally flattened, and anteroposteriorly elongate. (Fig. 10). Within a time period of 8 to 17 minutes, the cavity elongates and extends through the entire brain structure. (Fig. 11). The cavity then begins to enlarge dorsoventrally and the brain becomes completely tubular, consisting of equally thick walls surrounding an enclosed chamber. All this occurs within one hour after the first appearance of a cavity (14½-15½ hours). (Fig. 12).

The otic placodes appear in the posterior half of the myelencephalon of the brain (16½ hours).

Muscular activity in the trunk was observed 18½ hours after fertilization.

By the end of the first day (24 hours), a diamond-shaped invagination appears in the roof of the mesencephalon. The long axis of the diamond concides with the long axis of the brain. The short axis of the diamond is perpendicular to the long axis. The invagination known

as the mesocoele is covered by a thin layer of nervous tissue. The mesocoele is the first enlargement of the neural canal. (Fig. 16).

A constriction just posterior to the mesocoele is seen to occur about the entire perimeter of the neural tube, the constriction being perpendicular to the long axis of the tube.

Immediately following the constriction (24½-25 hours), two more median cavities appear in the brain. One cavity lies posterior to the mesocoele in the region of the myelencephalon, and is triangular-shaped. The base of the triangle lies close to the mesocoele, while the vertex extends posteriorly. The invagination, being roofed by a thin velum of nervous tissue becomes the myelocoele. (Fig. 2B).

The tissue between the chambers of the mesocoele and myelocoele which is pushed dorsally by the afore-mentioned constriction becomes the primitive cerebellum. The tissue housing the myelocoele becomes the medulla oblongata.

Another invagination appears anterior to the mesocoele. Its shape is complex, being composed of two somewhat triangular figures attached at their bases by an hourglass-shaped figure (Fig. 2B). After the formation of the cavity the canal may be considered a telo-diocoele, the anterior triangle composing the telecoele and the hourglass figure and posterior triangle forming the diocoele. The walls of the telocoele become the cerebral

lobes of the brain. The tissue around the posterior triangular portion of the diocoele becomes the optic lobes of the diencephalon.

The tissues on the sides of the mesocoele become the midbrain.

4. Development of the eye:

The optic vesicles appear as outpocketings from the posterior part of the prosencephalon (9 hours). An inpocketing at the center of each vesicle is seen to be formed (13 hours) as a longitudinal slit which deepens in the spherical surface of the oval vessicles. The ectoderm above the optic cup thickens and sinks down into the cup. The thickened ectoderm (the lens placode) separtes from the rest of the ectoderm and becomes the primitive chrystalline lens. Optic stalk formation takes place by the constriction of the basal portion of the optic cup. All the foregoing events take place generally at the same time, no one action preceeding the other. The entire mechanical process is completed in one half-hour and is followed by organ growth and cell proliferation.

As the optic cup deepens, as a result of the continued growth of the lip of the cup, the choroid fissure becomes more easily visible (24 hours). It appears as an invagination of the anteroventral portion of the optic cup. (Fig. 23).

Pigmentation of the eye is first noted to occur 24½-25 hours after fertilization.

The iris of the eye is first noted to appear (24½-25 hours) as a ring shaped organ limited on its outer border by the optic cup and the inner circular area, the pupil. (Fig. 17).

An increase of diffuse black pigment, and the further addition of diffuse gold pigment in the eye, gradually occurs up until the time of hatching. The exact time of appearance of the gold pigment was not determined. This was probably due to its diffuse nature and its lack of refractivity of the light coming thru the microscope.

5. The development of the internal ear:

The auditory placodes first appear as thickenings of ectodermal tissue which lie in the region of the ventral portion of the posterior half of the myelencephalon (16½ hours). (Fig. 13). The placodes elongate in the antero-posterior direction, sink below the surface, and separate, from the ectoderm (26½ hours). Within one half-hour two black round bodies, the otoliths, appear in each otic chamber. These are pigmented granules, and are presumed to have a function in equilibrium. (Fig. 21).

6. Development of the olfactory apparatus:

A pair of shallow inpocketings of ectoderm appear in the region exterior to the ventrolateral aspects of the telencephalon. These develop a slit extending dorsoventrally (34-36 hours). (Fig. 28). The dorsal ectoderm of each olfactory sac thickens as the invagination deepens. The invagination appears to close at the surface soon

afterwards. Further development was not observed.

7. Formation of the gut and the tail:

At the closure of the blastopore, the archenteron appears as a flattened chamber floored by the yolk mass. The brain is observed to be curved about the anterior ends of the archenteron and notochord. The notochord is ventral to the nerve cord and dorsal to the archenteron, and extends posteriorly from the most anterior end of the archenteron (9 hours).

Just anterior to the point of blastoporal closure is an undifferentiated mass of cells. This cell mass, composing the tail bud, is continuous with the posterior portions of the nerve cord, the neural tube, and the roof of the archenteron (9 hours). (Fig. 11).

The first major change to take place is a constriction of the tail, away from the yolk mass (18½ hours). It resembles the action of an invisible pair of shears, each of its two blades being placed on one of the two sides of the body axis. As the blades of the imaginary shears come together, the tail bud is separated from the yolk mass. (Fig. 15).

During the process of the separation of the tail bud from the yolk mass, the tail bud gives the illusion of turning in on itself. This is not so. An inpocketing at the ventral portion of the tail bud occurs, which when seen from the lateral surface of the embryo, gives the afore-mentioned illusion. This inpocketing, roofed by a

thin strip of tissue, is the Kuffer's vesicle.

As the shearing of the tail bud from the yolk continues, the yolk sac which up to this time has maintained an ovoid shape, begins to constrict in the region ventral and anterior to the tail bud. (Fig. 14). The continued constriction forms the hind gut (20-21 hours), which is located ventral to the anterior portion of the tail. The tail is formed (20-21 hours) concurrently with the hind gut by the posterior elongation and differentiation of the tail bud. (Fig. 15).

As the hind gut continues to lengthen by the continued constriction from the yolk mass, the Kuffer's vesicle becomes shallower. It finally disappears (24 hours).

As the tail continues to lengthen, it becomes free from the yolk sac, and contractions of the musculature can be seen. The contractions begin as weak motions becoming stronger so that the entire tail eventually moves in whip-like strokes. This motion causes the entire embryo to rotate within the chorion ($20\frac{1}{2}$ hours). The stage is named by the author, "The Tail-Free Period."

The tail bud gradually differentiates into notochordal tissue and myotomal substance. A vertically compressed flap of tissue is seen to extend from the anteriormost ventral and dorsal borders of the tail posteriorly, where it is continuous around the tip of the tail ($26\frac{1}{2}$ hours). After hatching this tissue containing cartilaginous rods, becomes the ventral and dorsal caudal fins.

The anus appears as an invagination of the ectodermal (epidermal) tissue which connects the hind gut to the exterior (27 hours). (Fig. 24).

As the tail and hind gut undergoes final constriction to attain full length, the head of the embryo begins to emerge as a definite structure from the surface of the yolk. The same shearing motion observed in the formation of the tail occurs here (24 hours).

As the heart is formed, the ventral portion of the head from the region of the mesencephalon anteriorly is completely separated from the yolk. The subcephalic pocket, an invagination of epidermal tissue between the head and the yolk, becomes apparent at about this time (35 hours). (Fig. 29). The development of this pocket further accentuates the separation of the head and foregut from the heart and yolk mass.

8. The heart, pericardium, heart beat, and circulation:

The pericardial sac first appears as a thin, flat sac lying upon the yolk (27 hours). It makes it's appearance within a period of 30 minutes. The pericardium is discoidal in shape and somewhat concave on its ventral side adhering closely to the yolk sac. Its concave portion fits the convexity of the yolk sac. The pericardium extends in a perpendicular plane from beneath the eyes to the ventral side of the yolk sac (Fig. 20).

The heart appears as a simple tube within the pericardium (28-29 hours). (Fig. 25). Later the post-

erior bifurcation in the region of the sinus venosus of the tubular heart becomes apparent (35-36 hours). (Fig. 27). As a constriction in the center of the tubular heart separates the atrial chamber from the ventricle, the ventricular portion is seen to bend to the right. The atrial portion is also seen to bend so that the entire tube assumes an "S"-shape disposed in two planes with the upper portion of the "S" perpendicular to the bottom portion.

Soon after the formation of the heart, it begins to beat (28-29 hours). The beat, although at first erratic, soon became regular.

The beginning of circulation follows the onset of the heart beat within one-half hour. Large irregularly-shaped colorless cells are seen to move posteriorly along the dorsal border of the notochord in the region of the tail. These cells move through regular channels, suggesting the formation of blood vessels (the Dorsal Aortae). An anterior motion of these cells towards the anterior of the body axis in regular channels, suggest the formation of other blood vessels. Circulation of blood cells over the ventral and lateral surfaces of the yolk was also observed.

The cells are few in number at the onset of circulation, and the rate of motion is slow. Within a short time, the number of moving cells increases. The cells appear to become smaller in size and the rate of movement accelerates. Gibson (9), pointed out that as the embryo

becomes older, the rate of heart beat increases. These observations indicate that as the rate of heart beat increases, so does the speed of motion of the blood. The increase in number of blood cells is attributed to increased formation of these within the developing system.

The pericardium grows larger and finally covers one-third of the anterior portion of the yolk sac (45-46 hours). (Fig. 31). At the time of hatching, the pericardium covers the anterior half of the yolk.

9. Pigmentation and pigment distribution:

The first sign of pigmentation appears in the eye in the anterocranial portion of the optic cup (25 hours). It was observed as a definite diffuse graying of a previously colorless locus. The area becomes darker and wider, covering more and more of the optic cup (27-28 hours). (Fig. 23).

Somatic pigmentation appears first as two lines of chromatophores on each side of the tail (28-29 hours). The chromatophores are small and widely spaced. They lie in two rows parallel to the top and bottom of the notochord. Other chromatophores appear upon the yolk sac in close proximity to the medullary portion of the brain.

A third row of chromatophores similar appearance to the other two appears along the dorsal portion of the embryonic tail bud (30 hours). The size of the chrom-

atophores and intensity of pigmentation gradually increases in the tail.

A fourth row of chromatophores then appears along the ventral border of the hind gut (31 hours). These chromatophores continue to multiply in number and extend onto the yolk sac where they appear on successively lower planes. In the same manner chromatophores appear on the brain capsule laterally at first and then proceeding dorsally.

By the end of the 36th hour of development two more rows of chromatophores make their appearance. One borders the dorsum of the hind gut, the other forms a superficial border separating the pericardium and the yolk sac (Fig. 32). The chromatophores that have appeared on the brain case line up in two main lines which are continuous with the two lateral lines of chromatophores on the dorsal aspects of the tail.

10. Development of the pectoral appendages:

The appearance of a bud like growth on each side of the median body axis near the junction of the myelencephalon and the neural cord denotes the arrival of the pectoral fin anlagen (30 hours). The fin bud as seen from the lateral view is elliptical, with its major axis parallel to the main body axis. The fin bud becomes larger and extends farther and farther from the body axis (Fig. 30). From a dorsal view, the bud resembles a parabolic figure. The apex represents the distal portion of the fin, and the

base represents the proximal portion.

As further development takes place the base of each bud begins to constrict, giving the structure an oval outline when viewed from above. The distal edge of the bud becomes irregularly serrated. The bud continues to grow distally, and may or may not develop into a functional fin before hatching.

Critical development periods of the fish Brachydanio rerio (Hamilton):

The following sections are discussions of the various states of development of organs and tissues at particular "critical" periods. The periods, refer to the age in hours of the embryo incubated at a constant temperature of 26 ± 1° C. at which time certain cell movements occur. The word "critical" is herein used to mean, a point in the development where certain organs and other structures arise as a result of a rearrangement of cell masses. At this time the form of the adult animal is determined. These periods should aid in the comparison of any species of embryo to that of Brachydanio rerio (Hamilton).

1. The early cap period (2½ hours).
2. The middle cap period (3½ hours).
3. The late cap period (4 hours).
4. The equatorial period (5 hours).
5. The closure of the blastopore period (7½ hours).

The above named periods have been described and named

in the section entitled "Development from the sixteen-cell stage to the closure of the blastopore."

6. The hollow brain formation period (14½-15 hours):

The body axis, prior to this time, has been divided anteroposteriorly into two parts: the brain and the nerve cord. The posterior portion has been divided dorsoventrally into the nerve cord, the notochord, and the gut. The brain has been subdivided into five embryonic parts. The entire nerve cord having been originally solid, now develops a fissure the walls of which separate to form a cavity. Thus the brain becomes a hollow tube. The optic vesicle had previously developed into the optic cup and the lens placode had developed. The chrystalline lens now rests in the invaginated portion of the cup.

The notochord is located beneath the nerve cord. It extends from the myencephalic portion of the brain into the tail bud. The somites, numbering 12-14, lie upon the notochord and extend posteriorly from its most anterior portion. The tail bud is an elongated structure, located posterior to the last somite. (Fig 11).

7. The tail-free period (19½-20½ hours):

The posterior portion of the yolk sac constricts, forming the hind gut. Kuffer's vesicle is prominent in the tail bud. The tail bud shows some detachment from the yolk as a result of the lateral constriction of the ectoderm.

The otic placodes have previously formed and appear

on the posteroventral aspects on both sides of the myelencephalon.

The diencephalon enlarges in the lateral plane, preparatory to the formation of the optic lobes.

The somites number 15-18. The anterior somites appear to be fiberous and become obliquely angular forming mature myomeres. (Fig. 14).

8. The brain differentiation period (24-25 hours):

The five-part embryonic brain develops ventricles. The roofs of these ventricles are composed of thin layers of nervous tissue. The cerebrum, cerebellum, midbrain, optic lobes, and medulla oblongata become apparent as definite structures as a result of the foldings of the nervous tissue.

The eye developes an iris. The choroid fisure becomes quite apparent.

The otic placode is quite prominent.

Kuffer's vesicle has disappeared from the tail bud. The hind gut is completely formed. The head and foregut are separated from the substance of the yolk as a result of the constriction of the ectoderm at the sides of the body axis. (Figs. 18, 21, and 22).

9. The heart formation period (27-28 hours):

The pericardial sac appears. Heartbeat is initiated after the formation of a tubular heart. Circulation follows, and the heartbeat adjusts from an erratic to a constant rate. The blood cells become uniform in size

and the rate of blood flow increases. The number of circulating blood cells increases. The development of vessels can be inferred from the seeming appearance of channels in which the blood flows.

Eye pigmentation develops on the anterodorsal border of the optic cup.

The cloacal aperture becomes prominent.

The otic capsules are covered over by a thin membrane. Within each capsule the otoliths (two refractile granules) develop. (Figs. 19, 23, 24, and 26).

10. The body pigmentation period (35-36 hours):

The tail exhibits five rows of melanogenic chromatophores on each side. The epidermis covering the brain exhibits two rows of melanistic pigment cells on its dorsal aspect. The pericardium is demarcated from the yolk by a chain of melanogenic chromatophores, extending from one side of the body axis, around the yolk sac-pericardial sac sphere, to the other side of the body axis.

The subcephalic pocket is observed as an inpocketing of the surface ectoderm between the fore gut and the yolk sac. The head is raised from the surface of the ectoderm.

The olfactory apparatus appears as a vesicle which develops a vertical slit and then sinks below the surface of the ectoderm and disappears.

The pectoral limb bud makes its appearance in most

embryos as a disc-shaped outgrowth from the body axis. (Figs. 28, 29, 30, and 32).

V. DISCUSSION

Since Brachydanio rerio is an egg laying fish, the collection and study of both fertilized and unfertilized eggs is an easy matter. The ability to simultaneously fertilize a large number of eggs aided in the study of their chronological development. Eggs that have been fertilized at the same time generally develop at the same rate provided their environmental conditions remain the same. Should an abberation in development occur within some eggs, it could be easily detected by comparison to the other normally developing embryos, and then discarded. On the other hand, Hopper (13), Tavolga and Rugh (37), and Tavolga (38), working with Platypoecilus maculatus, a live bearing fish, were at a disadvantage because the newly born larvae of any one female parent varied from one to four hours in development. The most probable cause of this is that simultaneous fertilization of a clutch of eggs within the body of the female fish is rather unlikely. Thus a variation in chronological age of the embryos developing in a clutch of such eggs is expected.

Oppenheimer (24) believed, "The chronological age of a teleostean embryo, expressed in hours or days, does not represent its actual age, which varies according to conditions of temperature, oxygen supply, etc. The precise state of development, of older embryos cannot be expressed in somite

numbers, since this varies when compared with differentiation of other organ systems, in embryos maintained under different conditions, and probably differing in genetic constitution." The statement disregards that the standardization of the environment allows a chronological description to be presented.

Most embryonic development in all vertebrates is affected by the factor of a time-temperature relationship. In the study of chick embryology, variations in development are due to the difference in length of time that the zygote remains in the oviduct. Yet, when the freshly-laid chicken egg is incubated at a constant temperature, humidity, and atmospheric pressure, the developmental conditions can be predicted fairly accurately from the time incubated.

The same situation holds true in fish embryology. In the embryonic development of Brachydanio rerio, since it is fertilized externally, variation due to length of time that the ovum is in the oviduct of the animal does not exist. The temperature, pressure, and oxygen gradient of the environment of the embryo from the time of fertilization to the termination of the experiment can be controlled and/or recorded. Thus, an accurate and pertinent account of embryonic development can be attained, and stages at which experimental studies are made can be designated by age in hours.

Stockard (36), in his paper, concluded that: "All embryonic stages are passed at a specific rate of development, dependent upon the rate of oxidation in the protoplasm of the species." The rate varies within certain limits, beyond which develop-

ment results in modification or distortion. Although in any given group of danio embryos, anomalies were sometimes found to be present, there was no evidence that there was a change in protoplasmic oxidation as a result of the incubation medium. The cause of the anomaly may have been due to a difference in the protoplasm itself.

Bolin (4) found in a study of the Labrid fish, Oxyjulis californicus, that Kuffer's vesicle appears before the closure of the blastopore. He also found that the embryo begins to differentiate extremely early. Both the optic cups and Kuffer's vesicle appear before the germ ring reaches the equator of the yolk. This is quite different from the development of Brachydanio rerio. When the germ ring reaches the equator of the yolk, the embryonic shield can barely be discerned. The embryonic axis does not come into full prominence until the closure of the blastopore.

An interesting axiom can be derived from the comparison. The development of an organ or organs does not necessarily have to take place in the same sequence among animals of different species.

Blood cells of the Brachydanio were observed to change from an irregular to a more rounded shape, and from a larger to a smaller diameter as the rate of body circulation increased. Stockard (35), in his paper describing the origin of blood in Fundulus heteroclitus, found that primitive blood cells (erythroblasts) are present in beginning circulation. The erythroblasts degenerate at a later time or through continued contact with

the yolk are transformed into normal ichthioid erythrocytes.

Since erythroblasts are found in the early circulation of fish, and, since they have an ameboid shape, they might well be the large irregularly shaped cells observed in the danio. Erythrocytes are smaller than erythroblasts and spherical in shape. These might also correspond to the smaller rounded bodies seen later.

A possible explanation for the apparent change of the shape and size of the blood cells in circulation is the transformation of erythroblast to erythrocyte and/or the degeneration of the erythroblasts with a replacement of erythrocytes in the circulating blood.

It was found that the chronological time of hatching of the embryos of Brachydanio rerio varied from individual to individual. It is also known that the hatching times of individuals of other species vary. The reason is a rather interesting one. Embryonic development depends upon the physiochemical reactions present within the embryo. If the temperature, pressure and other outer environmental conditions are kept constant, early embryonic development is fairly constant. As the embryo gets older, individual variation, supposedly genetic, involving rate of growth and differentiation takes place. Thus, the older a clutch of simultaneously fertilized embryos becomes the more the variation in growth and size. Although this variation is not great, it is nevertheless encountered. Hatching on the other hand is not a developmental action, but rather a physical action on the

part of the embryo. It involves embryonic activity such as motion within the chorion. The chorion which encloses the embryo is not a living portion of the specimen, but rather an inanimate body which is part of the embryo's environment. In order for the embryo to hatch, it is necessary for it to break out of the chorion. This is accomplished by activity such as motion within the chorion. Utilizing this motion, the embryo actually tears its way out. The amount, strength, and speed of motion that the embryo exerts seems to be a matter of individual variation. Usually retarded embryos show less motion, and advanced embryos more activity than do normal embryos. Another factor affecting the hatching time is a softening of the chorion as the embryo gets older. The softening is brought about by a lytic action upon the chorion. Rate of lysis varies from one individual chorion to another, depending upon enviromental conditions surrounding the chorion. The force needed to tear the chorionic material also varies. Thus, an exact chronological time cannot be assigned for hatching.

Wilson (40), in the description of the gastrulation of the Sea Bass, maintained that the posterior pole, or tail end of the embryo remained a comparatively fixed point, the anterior traveling rapidly around the yolk. In observing Brachydanio rerio, the process was somewhat different. The germ ring as a whole moves ventrally over the yolk, no fixed point being apparent. Further movement downward of the entire germ ring continues with a thin layer of tissue connecting the

posterior portion of the embryonic shield to the germ ring. The posterior portion of the embryonic shield continues to differentiate as the germ ring moves down. This differentiation continues to the closure of the blastopore. At the three-quarter mark of the yolk, the embryonic axis barely begins to appear. The embryonic axis continues to differentiate along with the embryonic shield to the closure of the blastopore. Upon the closure of the blastopore, a wave of movement of the protoplasm is observed. The posterior portion of the embryo is marked approximately by the place where the blastopore closes and the anterior portion is located somewhat above the equator. Thus, the comparatively fixed point or anterior pole of this fish is located about that point in the embryo that will give rise to the posterior end of the brain and the anterior end of the notochord.

Goff (10), presented a list of the chronological times of development of various tissues and organ structures of the fish Brachydanio rerio. A constant temperature was utilized, and this temperature, as cited, corresponded somewhat to the one used by this author. Variations of 30 minutes at most between the observations of Goff and this author were noted. An explanation for this discrepancy is a possible spread of three degrees centigrade in a fifteen hour period. This might be accomplished by one author observing development at one temperature, while the other author's observations were made at the other temperature. On the whole, the embryonic developments as observed by both authors complement one another.

Jones (19) and Self (33), working with the mitotic index upon various fish came to these interesting indications:

(1) Cell division produces cells which go into the process of cell differentiation, but does not cause the major action in differentiation.

(2) Growth and differentiation do not occur together, and differentiation of an organ is antagonistic to mitotic activity. Increase in cellular proliferation alternates with cellular reorganization and rearrangement.

(3) Changes in rates of cell division precede or occur simultaneously with growth of an organ.

These statements might lend insight concerning the development of a hollow nerve cord from a solid one. The undifferentiated five-part brain of Brachydanio rerio, which is a solid structure, delaminates in its central portion, and a hollowing of the solid structure is accomplished within one half-hour. Wilson (40), explained this as a function of cell multiplication at the center of the cord. These cells, not having any room to grow, force the delamination to occur, and thus form a hollow structure. This, on the evidence presented by Jones (19) and Self (33), is impossible, since growth and differentiation do not occur together. Another hypothesis must therefore be put forth. The answer may lie in the studies of variation of surface tension of the cells that make up the brain at that time. Another argument in favor of discarding the "Internal cell-multiplication theory" is that the brain

becomes a hollow structure in less than 30 minutes. It has been shown that after the 12th. cleavage the rate of cell division of the cells of Brachydanio rerio slows down considerably, (Roosen-Runge, (30)), and that the initial rate of cleavage is 20-30 minutes per cleavage (Roosen-Runge, (31)). The amount of hollowing therefore, cannot be reconciled with the time allowed for cellular multiplication.

It has been noted that after enervation of the muscles of the trunk, and for several hours afterward, the embryo of Brachydanio rerio will move about more violently when stimulated by light than when kept in the dark. This action suggests either a phototactic property of the muscles of the embryo, or possibly a direct discharge of nervous energy due to direct stimulation of these nerves by light, which, in itself, is a form of energy. Further investigation into the mechanism concerned with this phenomenon would be interesting.

Studies involving the distribution, development and regeneration of the pigment in Branchydanio rerio adults have been done by Goodrich (11) and Wittington (41). Orton (25) investigated the development and migration of pigment cells in several teleost fishes. Conclusions drawn from the latter investigation were that pigment cells are derived from neural crest cells, and that pigmentation primarily centers along the dorsal and dorsolateral surfaces of the body and tail. It was further stated that these cells migrate downward to secondary positions.

In the present study, Brachydanio rerio embryos first exhibited chromatophores upon the dorsolateral surfaces of the

body axis in the region of the vitilline veins. Pigmentation then appeared on other dorsolateral surfaces as well as the epidermal covering of the brain case. Later, chromatophores were seen to appear more ventrally. Comment concerning possible migration of pigment cells is not possible because constant observation of developing embryos for short periods of time showed no apparent change of position.

VI SUMMARY

1. A chronological description of the development of Branchydanio rerio is presented.

2. Eggs from the adult fish were collected and incubated at 26 ± 1° C. Atmospheric and oxygen pressures were maintained at uniform levels. The continuous development from the sixteen-cell stage to hatching was carefully studied. A wire recorder was used to aid in describing the embryos. Photographs were made to illustrate the observations. Specimens were fixed and preserved at intervals to provide materials for reference. From these studies a description of the chronological sequence of organ formation was formulated. Certain "critical periods" which can be used for identifying the state of development were defined.

3. It was demonstrated that the formation of the neural cavities by delamination was probably due to surface tension phenomena rather than to cellular multiplication and growth as previously believed.

4. No chronological time was assigned to the hatching period because it involves the action of physical forces applied to an inanimate object (the chorion) rather than of developmental forces applied to a living membrane. Since the physical force applied varies from one individual to another, and the strength of the chorion varies individually, (as a

result of thicknesses of chorion and lytic action applied to them) embryos of the same age were found to hatch at different chronological times.

5. It was found that chronological study of oviparous fish development is valid if a constant temperature is always used during their incubation. Other factors remaining constant, developmental rate in such embryos is relative to time and temperature.

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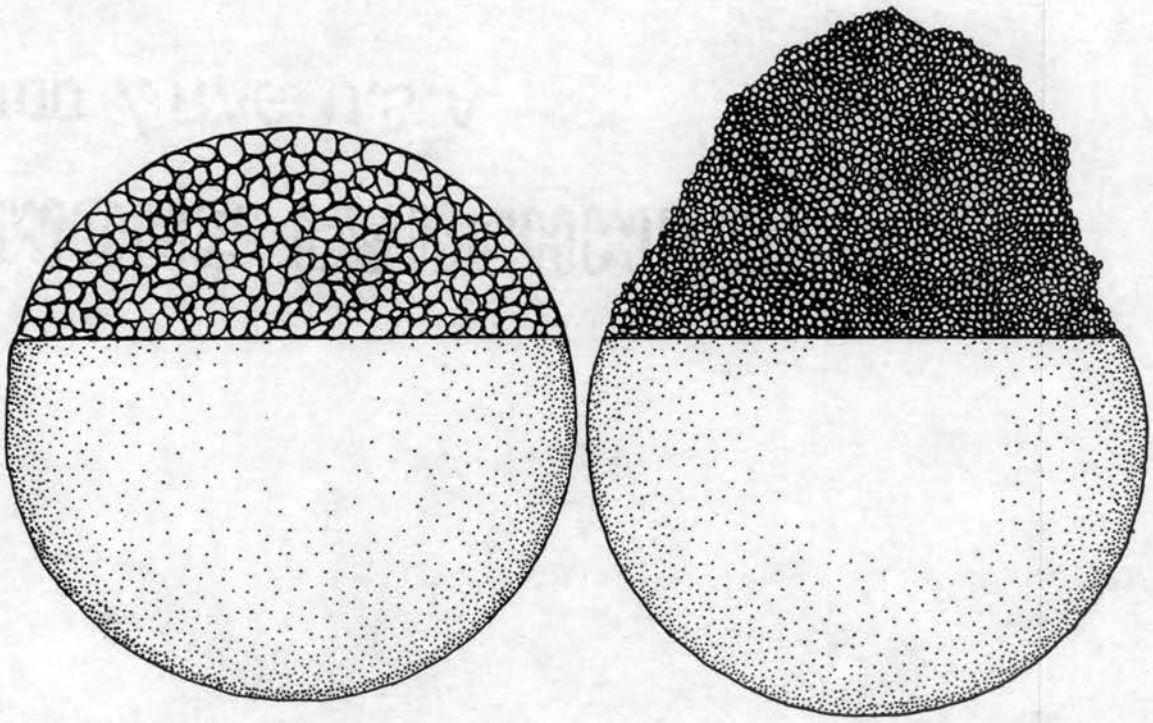
APPENDIX

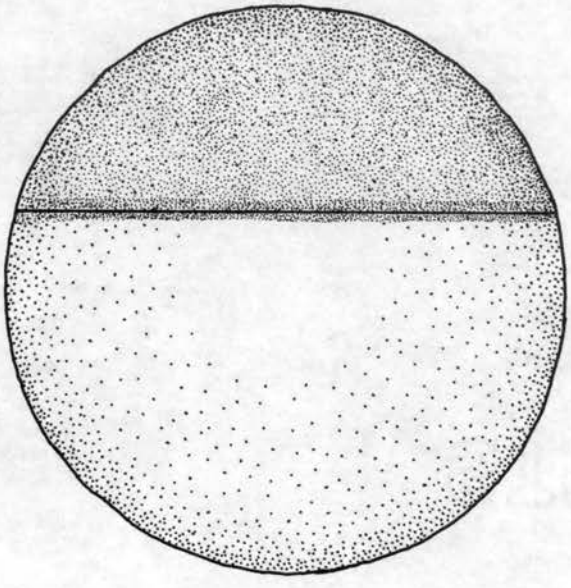
Figure 1. Stages of blastula formation of *Brachydanio rerio*.

PLATE I



A. EARLY CAP PERIOD

B. MIDDLE CAP PERIOD



C. LATE CAP PERIOD

FIGURE I.

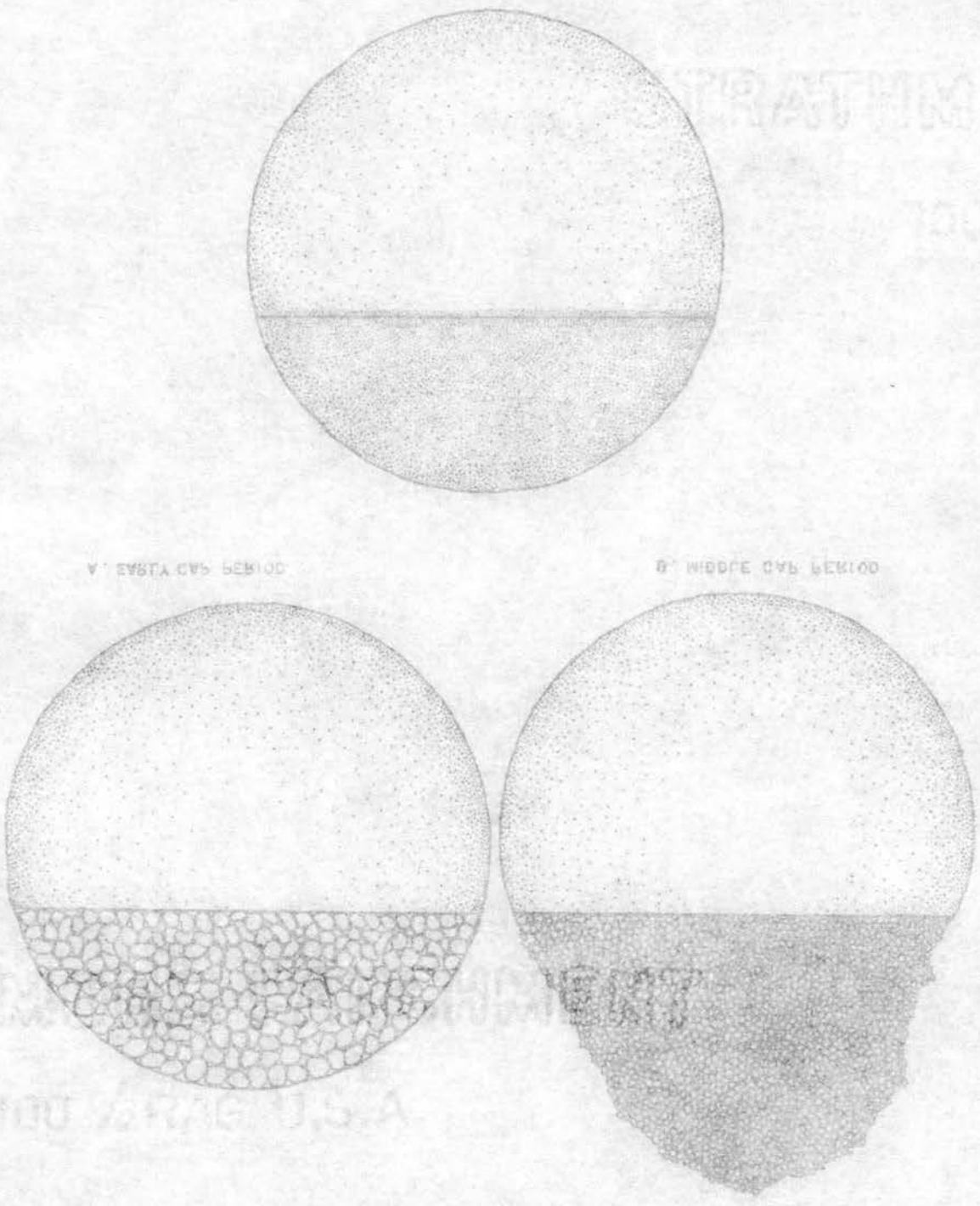
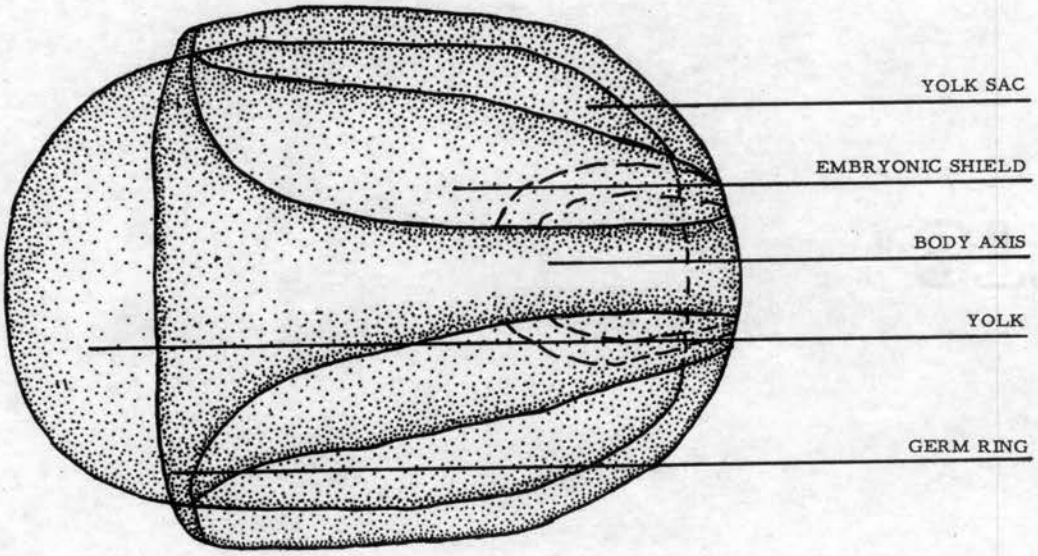
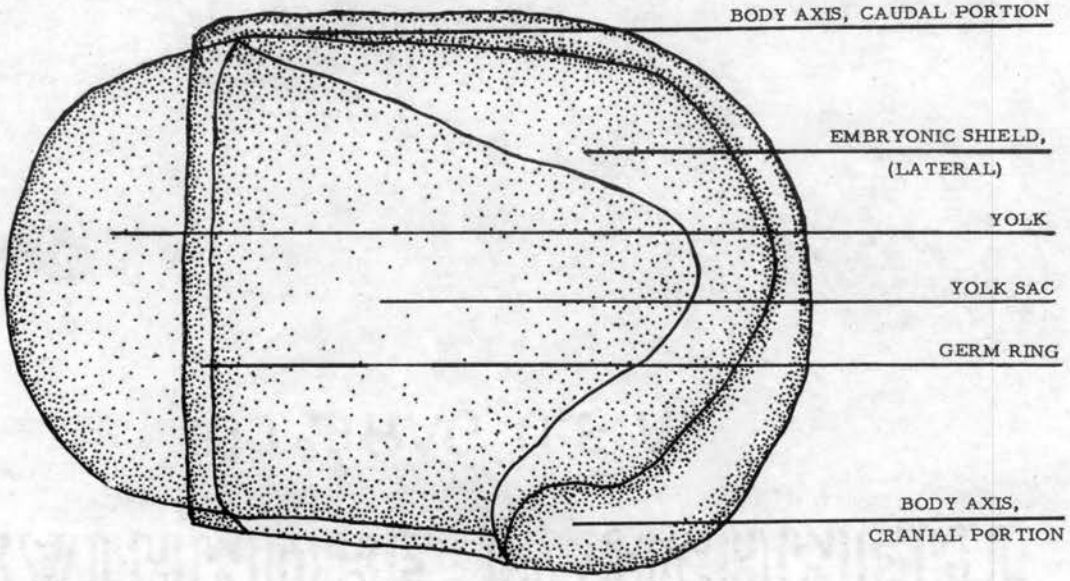


Figure 2. Brachydanio rerio embryo, 6½ hours after fertilization.

PLATE II



A. CAUDAL VIEW



B. LATERAL VIEW

FIGURE 2.

Figure 3. Dorsal views of brain organogenesis, 13 hours and 35 hours after fertilization of eggs of *Brachydanio rerio*.

PLATE III

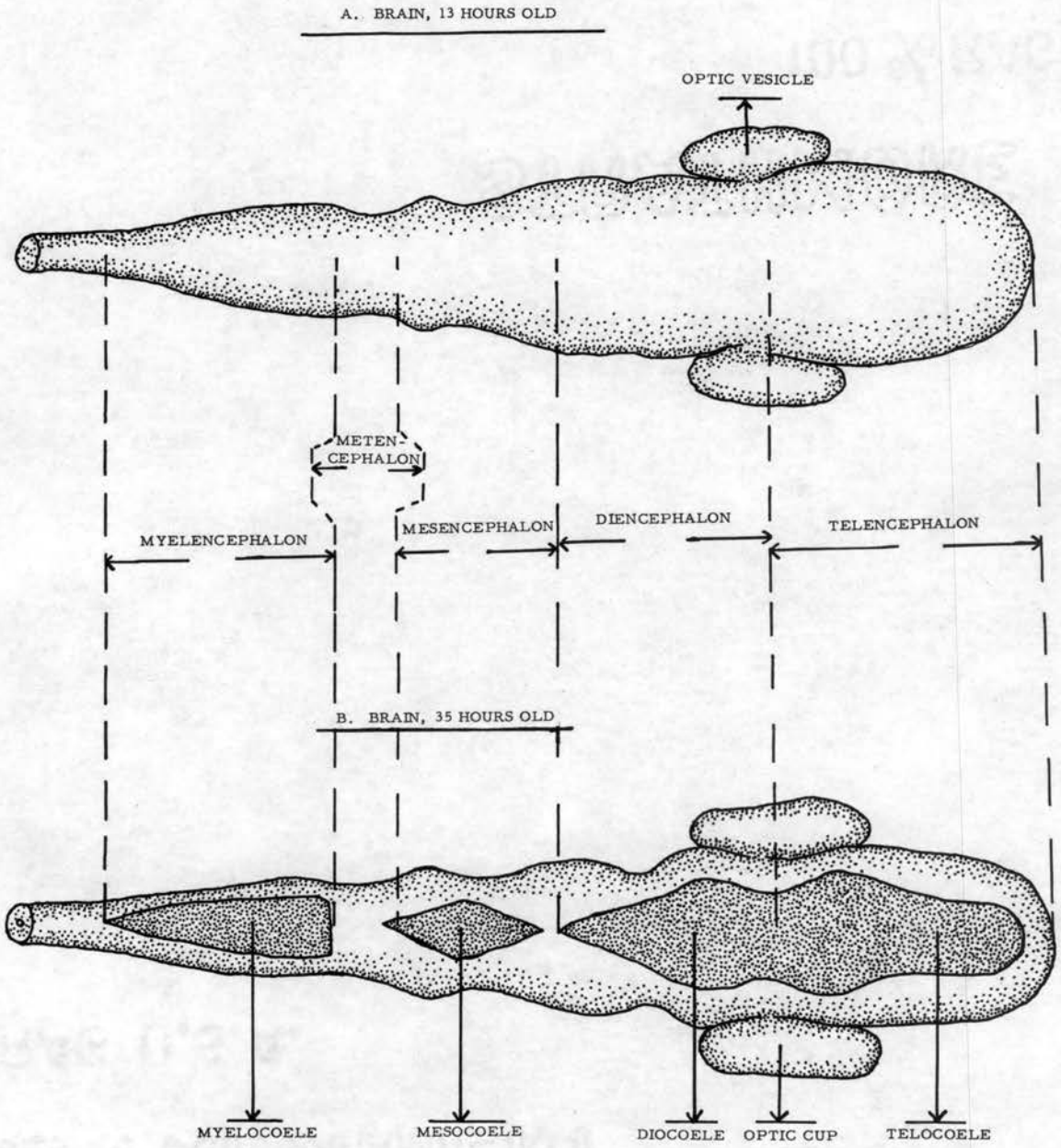


FIGURE 3.

PLATE IV

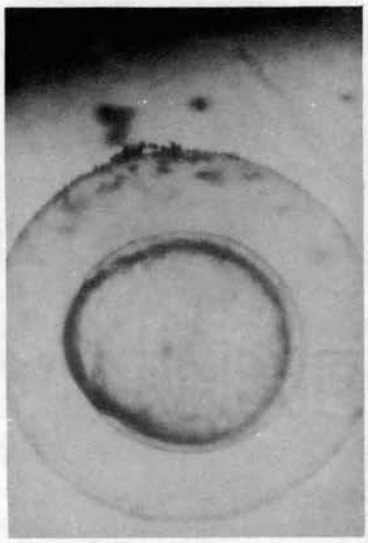


Figure 4. 6½ Hours

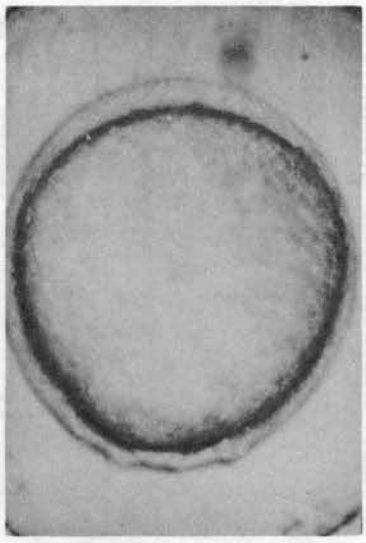


Figure 5. 7½ Hours

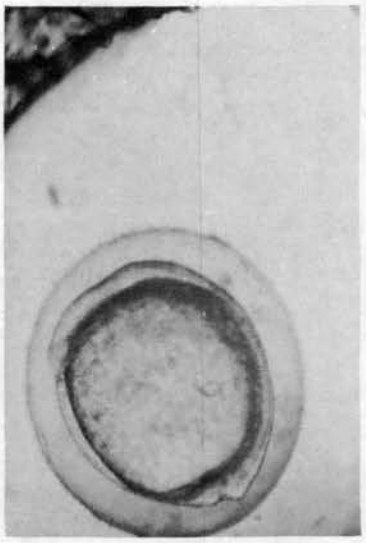


Figure 6. 9½ Hours

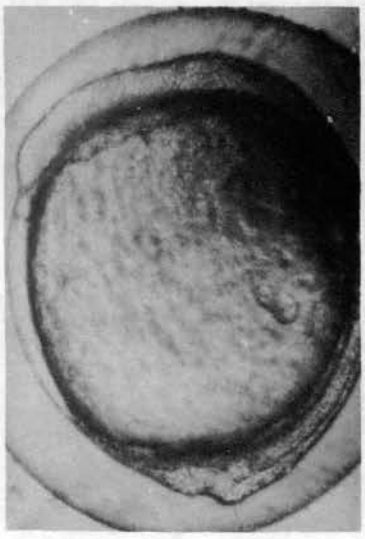


Figure 7. 9½ Hours

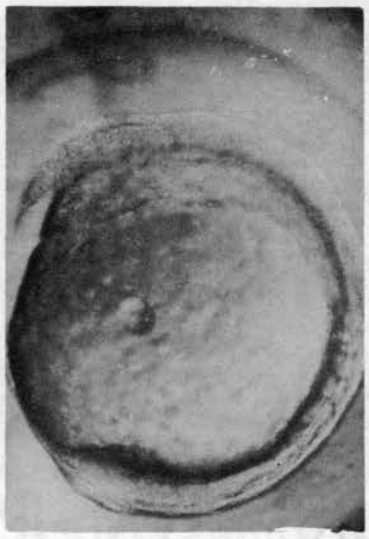


Figure 8. 12 Hours

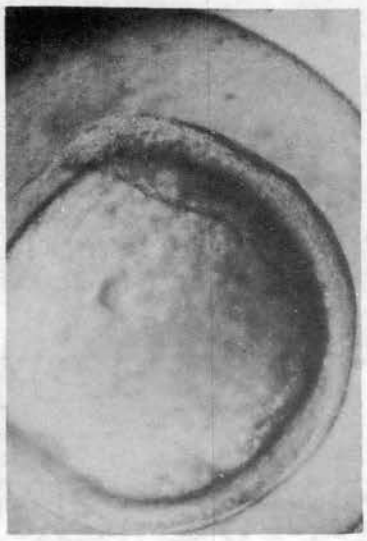


Figure 9. 13 Hours

PLATE V

Figure 10. 14½ Hours

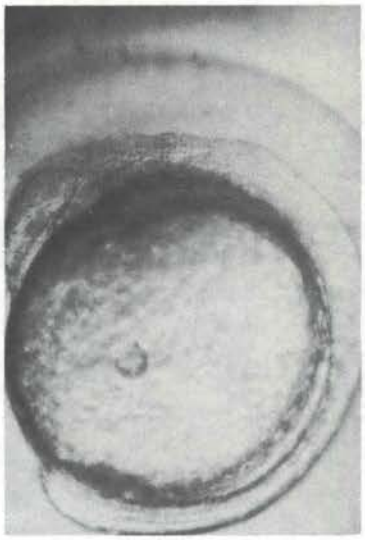


Figure 11. 15 Hours

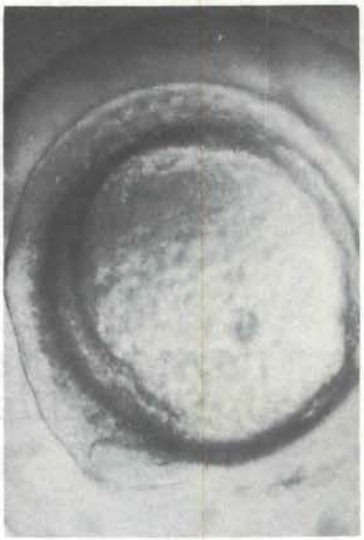


Figure 12. 15½ Hours

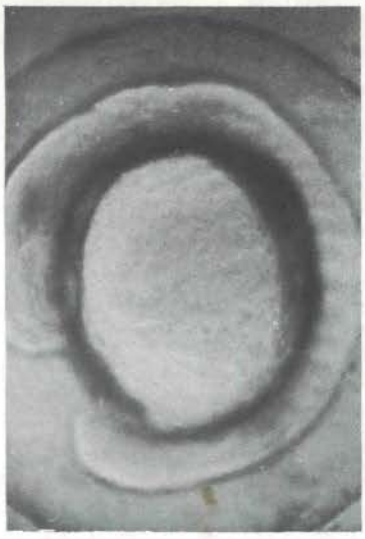


Figure 13. 18½ Hours

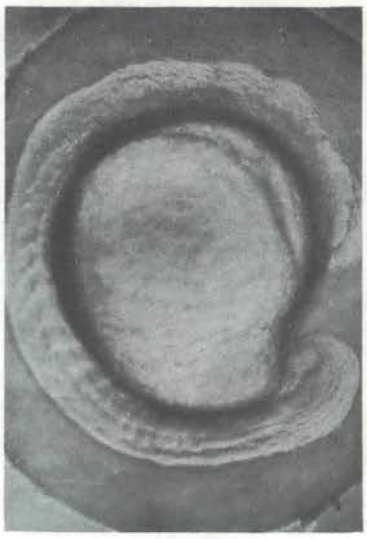


Figure 14. 19½ Hours

Figure 15. 20½ Hours

PLATE VI

Figure 16. 24 Hours

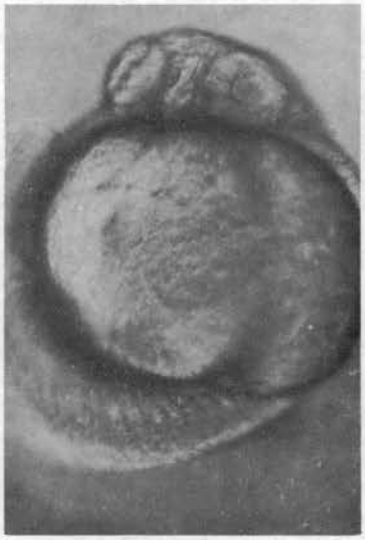


Figure 17. 24½ Hours

Figure 18. 26½ Hours

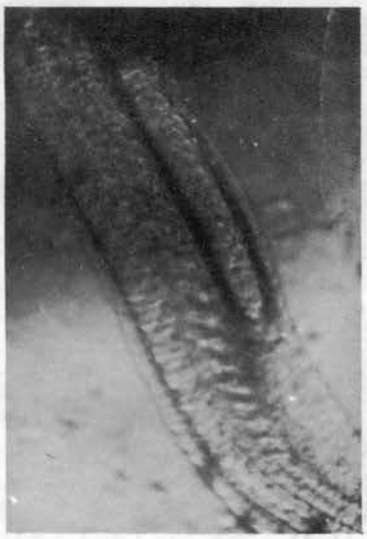


Figure 19. 26½ Hours

Figure 20. 27 Hours

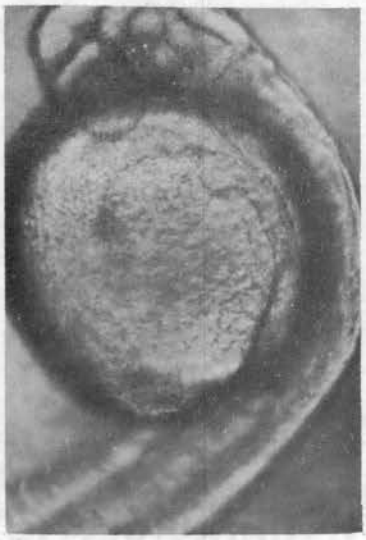


Figure 21. 27 Hours

PLATE VII

Figure 22. 27½ Hours

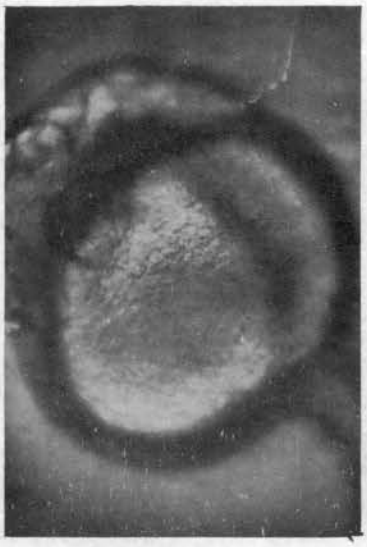


Figure 23. 27½ Hours

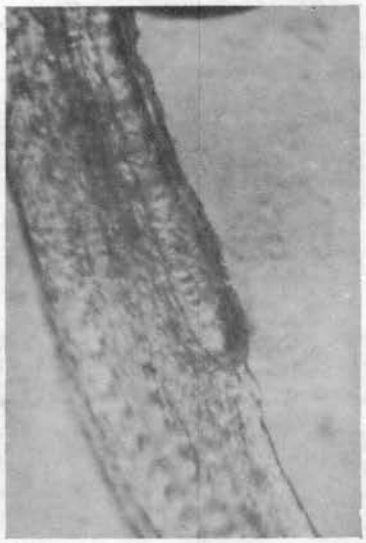


Figure 24. 28 Hours

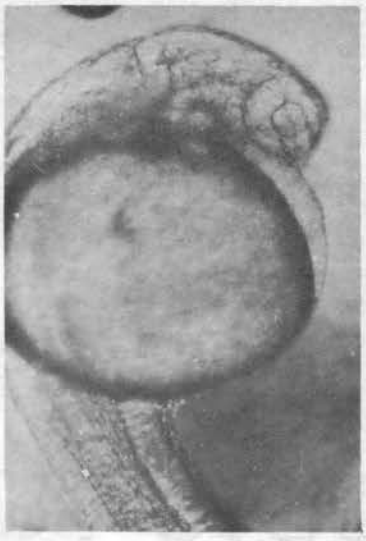


Figure 25. 29 Hours

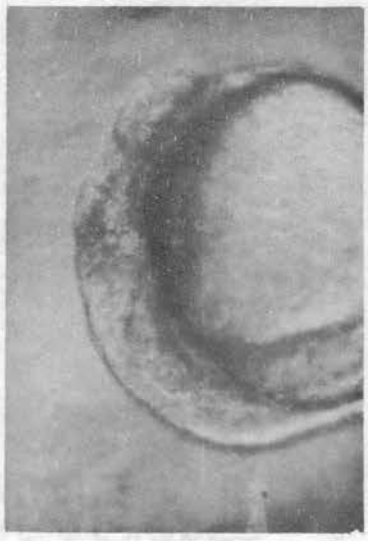


Figure 26. 29½ Hours

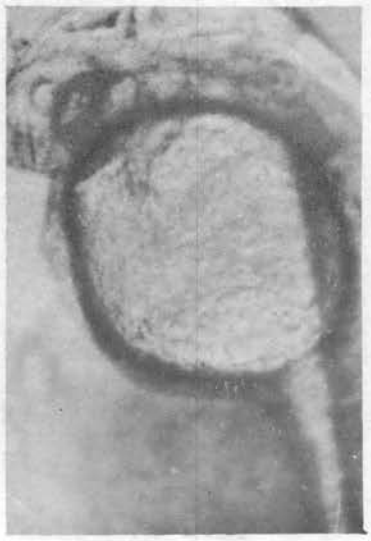


Figure 27. 35½ Hours

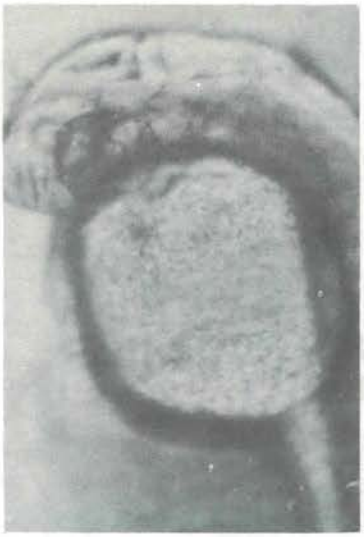


Figure 28. 35½ Hours

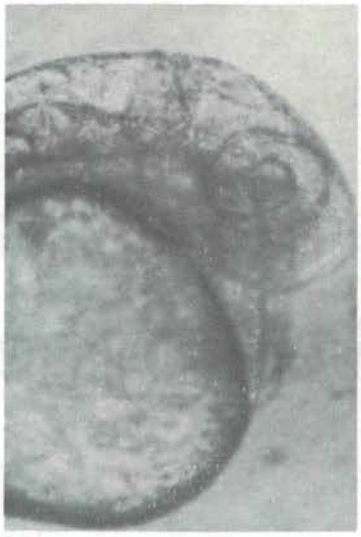


Figure 29. 36½ Hours

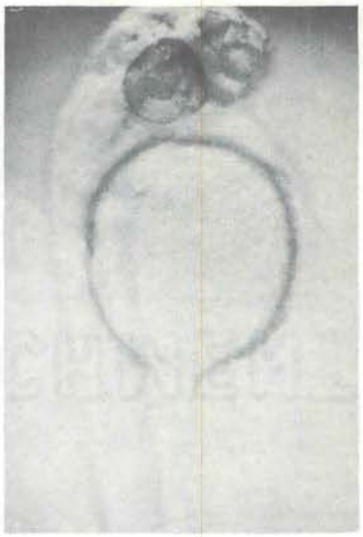


Figure 30. 37½ Hours

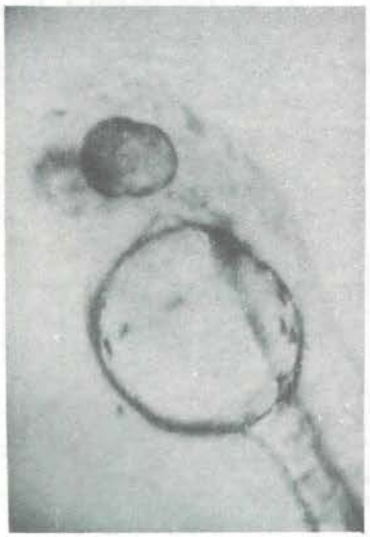


Figure 31. 45 Hours

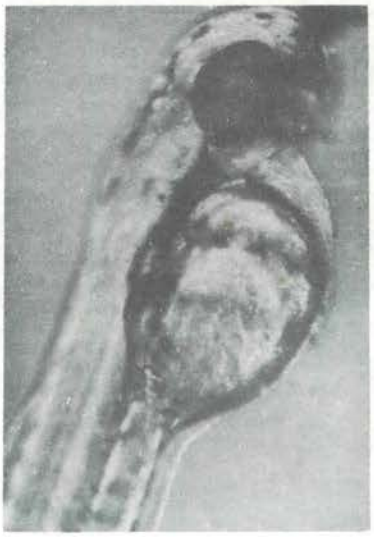


Figure 32. 68 Hours

VITA

Bertram Lawrence Blumenkrantz

Candidate for the Degree of

Master of Science

Thesis: THE CHRONOLOGICAL DEVELOPMENT OF THE EMBRYO OF THE ZEBRA FISH Brachydanio rerio (Hamilton) AT A CONSTANT TEMPERATURE

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Biographical:

Personal data: Born in Brooklyn, New York, June 6, 1932, the son of Philip and Bertha Cooper Blumenkrantz.

Education: Attended grade school in Brooklyn New York; graduated from Franklin K. Lane High School in 1950; received the Bachelor of Science degree from Brooklyn College, with a major in Biology, in January 1954; completed requirements for the Master of Science Degree August 4, 1956.

Professional Experience: Graduate Teaching Assistant September 1954 to January 1956, Graduate Research Assistant June 1955 to May 1956, Oklahoma A. and M. College. Student Teaching Assistant February 1953 to June 1953, Brooklyn College.

Professional Organizations: The Oklahoma Academy of Science.

Awards: The Edward G. Horton Certificate for Science, awarded January 1947.