MORPHOGENESIS AND METABOLISM OF AMPHIBIAN LARVAE AFTER EXCISION OF HEART

I. MORPHOGENESIS OF HEARTLESS TADPOLES OF RANA PIPIENS [1]

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SEVENTEEN FIGURES

That isolated parts of amphibian embryos will continue to differentiate even without circulating blood has been realized ever since Born's (1897) pioneer experiments involving fusion of pieces of embryos at tailbud stages. Nevertheless, the author was surprised to discover in the course of studies on the development of the digestive tract (Kemp, '51a) that entire tadpoles of Rana pipiens would live up to 6 days after excision of the heart at Shumway stage 22 or 23 (approximately two days after establishment of circulation). Since it was known that circulating blood is necessary for normal differentiation of the digestive viscera and various external anomalies were apparent in heartless animals, a detailed study of the differences between morphogenesis and metabolism in normal and circulationless larvae was initiated. From such a study one should glean valuable information about the manifold functions of the blood stream during larval development. The present paper, embodying results reported previously only in preliminary fashion (Kemp, '51b), deals with the major morphological changes resulting from loss of circulation.

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Credit for the earliest work on this subject must go to Knower ('07). His descriptions indicate that he removed the heart prior to the initiation of circulation, but the results closely parallel those reported here. He noted that the digestive tube developed only a single loop; liver and pancreas were arrested; the dorsal mesentery developed into a large bag with the dorsal aorta opening directly into it; the brain was smaller than normal and the walls of the optic cup clung closely about the lens, leaving little cavity. The sinus of the postcardinal vein around the pronephric tubules was greatly enlarged, also the lumen of the pronephric tubules. A few blood corpuscles were noted, mostly in the mesenteric bag but also in the aorta, postcardinal vein and postcardinal sinus. The development of these and other anomalies is traced in the present paper, followed by a discussion of their meaning and their relation to results of other workers who have undoubtedly produced impairment of circulation through treatment of embryos with a variety of chemical and physical agents.

MATERIALS AND METHODS

Excisions of heart were performed on approximately 350 larvae of Rana pipiens at Shumway stage 22 or 23, about two days after establishment of circulation. The digestive tract at this time is indented slightly in two places — anteriorly on the left side and posteriorly on the right. After anaesthetizing animals by electric shock, the technique of removal was to make a single mid-ventral, longitudinal incision with a needle-knife into the pericardial cavity. The knife was then inserted into the cavity and maneuvered into a position dorsal to the bulbus arteriosus. With the aid of a tamp, the heart was then transected through the bulbus. The knife was next inserted dorsal to the thin-walled sino-atrial region and brought upward (ventrally). By a combination of cutting and tearing, it was easy to sever the heart again through the sino-atrial region. The excised portion, consisting of the posterior part of the bulbus, the entire ventricle and the atrial

region, was then discarded. Less than a minute is required for the entire operation. Considerable blood was lost at the time of operation, but bleeding soon stopped and the incision in the body wall healed over within a day. Likewise the two stumps of the heart healed over and in some specimens continued to beat separately. Although no continuous flow of blood was possible, some ebb and flow of fluid did occur in the blood vessels near the heart. There was also some pumping of body fluids through the action of the dorsal lymph hearts. Animals were cultured in Holtfreter's solution and transferred to 50% Holtfreter's solution or, in some cases, to water after one day. Specimens for sectioning were fixed in Bouin's fluid at daily intervals after operation. Serial sections were cut at 10 μ and stained in Harris' haematoxylin and eosin. Dissected whole specimens were photographed on 35 mm film with a Kine-Exacta camera. Photomicrographs of sections were also taken on 35 mm film, utilizing the Spencer photomicrographic camera.

I wish to acknowledge my indebtedness to Miss Barbara L. Quinn for technical assistance.

OBSERVATIONS

A. Gross changes

The most obvious grossly discernible effects of excision of heart had to do with the development of the head, particularly of the forebrain and eyes; with changes in the external body form resulting from accumulation or loss of fluid in body cavities, lymph spaces or blood vessels; with a change in color due to increased separation of melanophores; with the development of the digestive system; and with diminished muscular activity. As early as one day after the operation it was easy to distinguish experimental animals from controls by their altered shape. Because of accumulation of fluid in lymph spaces of the head, the anterior end of the tadpole had

already started to broaden out. This process continued during subsequent days so that the larva came to resemble a pear in outline, with a broad anterior end and the sides markedly divergent (fig. 1), whereas the control (fig. 2) developed in a nearly oval shape. Swelling in experimentals became obvious around the pronephroi and in the dorsal mesentery during the second post-operative day. One of the most striking anomalies in the heartless animal was the failure of the eyes to enlarge and become movable. Instead, the eyes remained small, immovable, sunken beneath the surface and in close contact with the brain. Since the brain at this level was also smaller than normal, the two eyes looked too close together.

For the first two days after operation there was little difference in color of experimental and control animals; both were dark brown with melanophores well expanded over the body. By the third day, however, heartless animals came to look lighter than normal over the swollen sides of the head and body. Directly over the brain and spinal cord, there was no appreciable difference from controls. It was noted that controls sometimes had practically all the melanophores contracted to a round dot while at other times they were expanded and in close contact. The pigment cells of experimentals, on the other hand, were always expanded. Since individual melanophores remained expanded, the lighter color clearly resulted from greater separation of the melanophores in those areas where internal swelling caused the skin to stretch. Expansion of melanophores suggests that intermedin was being produced by these animals. If so, it was distributed chiefly by diffusion, although the pumping of the anterior lymph hearts may have speeded its distribution. It was observed also in circulationless larvae that the digestive tract failed to elongate beyond an advanced S-shape, although it was clear that differentiation proceeded slowly. This was the expected result in view of the finding reported earlier (Kemp, '51a) that circulation is necessary for the completion of intestinal coiling. Swelling of the dorsal mesentery through uptake of water was another major anomaly in these circu-

lationless animals. This balloon-like enlargement of the dorsal mesentery undoubtedly offered mechanical resistance to intestinal elongation.

During the first day after excision of heart, larvae were approximately as active as control specimens. Thereafter they became less responsive to tactile stimuli. When prodded or when the culture dish was shaken, they would start to swim but stop after a short excursion. Controls, on the other hand, would react much more vigorously and for a longer period when similarly stimulated.

The majority of animals in these experiments survived up to 4 days after operation; some lived 5 days and one specimen was still healthy when fixed on the 6th day. For one group of 50 animals, the following data were recorded: growth in length in 5 days from 7 mm to 10 mm (compared to 11 mm in control); survival — 50 (100%) through second day, 48 (96%) on third day, 38 (76%) on 4th day, 13 (26%) on 5th day. Death frequently appeared to be preceded by bursting following inordinate swelling.

B. Microscopic changes

1. Stage of operation. Larvae at the time of operation (stage 22 or 23) were approximately 7 mm long with circulation well established. Figures 3, 4, and 5 illustrate the degree of differentiation through the levels of the eye, gastroduodenal and pronephric region, and the yolk-packed midgut. In the eye at this stage (fig. 3) one can recognize all the layers of the fully differentiated larval eye. Sensory projections of the rod cells have started to grow into the narrow space between tapetum and sensory layer of the retina. Inner and outer nuclear layers are just beginning to separate in the central part of the retina and the ganglionic layer is clearly delimited. The body of the lens is surrounded by lens capsule save on the inner side.

At the level of the pronephric tubules (fig. 4) one sees the stomach, already possessing a prominent lumen. Be-

tween stomach and duodenum is the anlage of the pancreas. Erythroblasts, containing both yolk platelets and pigment granules, are abundant in dorsal aorta, glomi and postcardinal sinus; some were undergoing mitosis. In the midgut (fig. 5), the endodermal cells are stuffed with yolk platelets. The section illustrated shows a large vitelline vessel in the short dorsal mesentery. Close examination reveals that yolk platelets and pigment are found not only in the gut but also, in lesser quantity, in other structures — neural tube and spinal ganglia, notochord, mesenchyme, somites and epidermis.

2. Changes during first two days after excision of heart. Comparison of figures 6 and 7 will reveal that as early as one day after operation the eye in a heartless larva is abnormal. It is conspicuously smaller than that of the control and the vitreous chamber has collapsed so that retina and lens are in direct contact. It is also seen that the abnormal eye is in close contact with the brain, whereas in the control the two organs are well separated. It is inferred that the hydrostatic pressure maintained by circulation in the normal animal is necessary for the maintenance of the fluid within the vitreous chamber of the eye. This same force is probably necessary also to maintain intercellular fluid within the mesenchyme surrounding the eye.

Changes in the region of the pronephros and midgut were not strikingly different in experimentals and controls during the first post-operative day. Pronephric tubules looked approximately normal in the heartless animals, but only a few blood cells could be found in the postcardinal sinuses. Some of these were in various stages of mitosis. Many pycnotic nuclei, presumably of presumptive blood cells, could be seen in the liver. Digestion of yolk had proceeded further in the stomach, liver, pancreas and erythroblasts of control specimens. By two days there was considerable swelling of the pronephric tubules, the dorsal aorta, and the postcardinal sinuses (fig. 8). Swelling was also occurring in the dorsal mesentery (fig. 9). The mesentery in the section depicted appears to be separated into two wings connecting with the

stomach and duodenum and enclosing a large space containing undifferentiated pancreas surrounded by unorganized endodermal cells with pycnotic nuclei.

3. Changes from three to 6 days after excision of heart. Continued uptake of water occurred in both heartless and control specimens during the third day, resulting in further expansion of the body cavities. In the control, intestinal coiling reached completion, although some yolk platelets remained in the intestinal epithelium. Although most heartless animals survived up to 4 or 5 days, degeneration of certain tissues had started as early as one day after operation. Figure 10 illustrates the beginning degeneration which has taken place in the central part of the retina and in the mantle layer of the brain of a 4-day experimental. Evidently these tissues are most susceptible to the harmful results of failure of circulation, chief among which probably is insufficient oxygenation. In the damaged areas, cells are disrupted and the nuclei pycnotic, very much as in the eyes of Amblystoma larvae exposed to nitrogen mustard, described by Bodenstein ('48). In the eye of a control of 4 days (fig. 11) one sees the normal process of sclerosis of the central lens fibers. Figure 12, photographed at the level of the pronephros in a 4-day experimental, shows a greatly expanded postcardinal sinus, an enlarged lymph space in the body wall and the swollen dorsal aorta. Splitting of the dorsal mesentery has resulted from accumulation of fluid. Undifferentiated endodermal cells separate liver from stomach epithelium. Farther posteriorly (fig. 13) the intestine in this animal looks very abnormal. The dorsal mesentery is ballooned and the intestinal epithelium, while partly differentiated, appears disorganized instead of forming a continuous layer around the gut. Undifferentiated endoderm and yolk are still abundant, whereas only a little yolk still remains in the well-differentiated intestinal epithelium of the control.

Several specimens which lived 5 days and one which lived 6 days were available for examination. A search for blood cells in one 5-day specimen revealed that some were still

present in the dorsal mesentery and undergoing mitosis; some were also present in persisting blood vessels in the branchial arches. By this time yolk was entirely used up in the control but was abundant in the digestive tract of the experimental. The 6-day specimen showed no signs of degeneration in any of its tissues. The blank space in the lens (fig. 14) was caused presumably by loss of sclerotic central lens fibers during sectioning. Recall that sclerosis was shown to occur normally in control animals two days younger (fig. 1). Note the continued absence of vitreous chamber, the small size and sunken position of the eye in experimental as compared with control (fig. 15). Swelling of the pronephros (fig. 16) is apparent but is not so pronounced in this specimen as in the 4-day specimen described above. The dorsal aorta is collapsed and opens into the dorsal mesentery. Undifferentiated endoderm occupies most of the dorsal part of the digestive tract, but stomach and intestinal epithelia are present ventrally. Possibly a more successful osmoregulation accounted for the longer life and healthier appearance of the tissues in the 6-day animal. Good evidence that differentiation and utilization of yolk have continued up to 6 days after shutting off the circulation is presented in figure 17. Here the greatly hypertrophied intestine is completely surrounded by epithelium still containing yolk pletelets. Its lumen is filled with digested and partly digested endoderm cells, indicating that functional differentiation (e.g., development of digestive enzymes) has kept pace with structural differentiation.

DISCUSSION

Lack of circulation in a developing embryo results in an internal environment which differs from that in an embryo with normal circulation both physically and chemically. Knower ('07) recognized the significance of circulation for morphogenesis in stating that "on the whole it would be difficult to find a better example, to illustrate the influence of a complete and efficiently functioning vascular system on the later elaboration of structure and form." Circulation of blood is vitally

concerned with the maintenance of hydrostatic pressure in the developing intestine (Kemp, '51a) and other tubular organs (Holtfreter, '45). Indeed Holtfreter ('45) believes that hydrostatic pressure "deserves to be listed as a fundamental morphogenetic principle, equivalent to the principles of cell division, morphogenetic movements and cytological differentiation." Circulatory deficiency is probably the immediate cause of the edema so often seen in haploid amphibian embryos (Fankhauser, '45; also personal observations). To say precisely what environmental alterations produce each of the anomalies found in a circulationless larva is impossible; yet for purposes of discussion we may distinguish between those (1) resulting chiefly from altered hydrostatic pressure and those (2) resulting from the lowered metabolism attending reduced oxygen supply. To the former category belong such anomalies as accumulation of fluid (ballooning) in the dorsal mesentery, swelling of lymph spaces, pleuroperitoneal cavities and postcardinal sinuses, and collapse of spaces such as the vitreous chamber of the eye. Reduced metabolism is probably the primary cause of reduced hemopoiesis, slow utilization of yolk, retarded growth in intestinal length, and microcephaly. Undoubtedly some anomalies arise through a combination of mechanical and metabolic changes.

One of the most interesting observations made by Knower ('07) was that his heartless embryos developed much like embryos exposed to x rays or to certain poisons. The obvious question which comes to mind is: To what extent are the harmful effects of x rays and certain drugs explainable as a result of impairment of circulation? It is well known (Rugh, '49a) that x rays cause damage to regions of actively proliferating cells, such as the vascular, glandular and germinal tissues of the adult or to rapidly growing parts of embryos. X rays tend to prevent cells from undergoing mitosis. Rugh ('49b) also showed, however, that irradiated embryos suffered an initial rise in heart beat followed by a steady decline to a low of 20 beats per one-half-minute as compared with a rate of 30.6–40.8 beats per one-half-minute in controls.

Blood frequently became congested in the heart and adjacent blood vessels and peripheral capillaries were damaged. Damage to the circulatory system cannot help but affect other tissues indirectly.

A similar picture is seen in the action of nitrogen mustards on developing embryos. Gillette and Bodenstein ('46) initiated a series of valuable studies in an investigation of the effect of methyl-bis (betachloroethyl) amine hydrochloride (MBA) on embryos of Triturus torosus. Half of the 35 animals exposed were dead by the 4th day. After 10 days the surviving exposed animals responded to tactile stimuli but showed little spontaneous activity. Their last unfixed specimen died on the 15th day. Although they mention that circulation was visible in balancers and the poorly developed external gills, it is likely from the history that circulation was impaired and eventually ceased. The time of survival of their last exposed animal (15 days) is approximately the same as for larvae of Amblystoma punctatum rendered circulationless by excision of the heart (Kemp and Quinn, '51). Gillette and Bodenstein felt that the primary effect of the mustard was to inhibit mitosis in cells. They demonstrated that growth and differentiation of cells might continue even though mitosis was completely blocked. They concluded, however (pp. 30–31), that "many systemic symptoms caused by exposure to the agent, such as the disturbance of the water-electrolyte balance, for example, do not fit into such a unitary hypothesis and it would be an improper forcing of the facts to classify these symptoms as secondary effects." In a later investigation on Amblystoma punctatum, Bodenstein ('47) states that most of his experimental animals developed a "bloated appearance." It is highly probable that this condition resulted from the altered water balance which follows reduced circulation. In another paper Bodenstein ('48) compared the effects of nitrogen mustard on the eyes of Amblystoma punctatum embryos exposed at stages 28, 32, 37, 38, 39 and 43. From his photographs, it appears that circulation was blocked in embryos exposed prior to stage 39, because they resemble

closely the eyes of heartless animals (figs. 5, 6, 10, 14) seen in the present study. Embryos exposed at stages 39 or 43 in Bodenstein's experiments, however, had persisting eye cavities and showed less retinal damage than those exposed earlier. It is reasonable to believe that circulation persisted in these older animals. All but one of 9 different vesicants tested by Bodenstein and Goldin ('48) resulted in collapsed eyes and severe retinal damage.

Nieuwkoop and Lehmann ('52) report that exposure of embryos of Triton to chloroethylamine during gastrulation and neurulation may cause, among other things, destruction of the blood island and rudimentary development of the heart. At eye cup or tailbud stages, embryos became more resistant to the poison but impaired circulation and abnormalities of the eyes and skin continued to be common.

What has been said concerning the harmful effects of x rays and nitrogen mustards applies also to various drugs. The effects of 2,4-dinitrophenol have been studied by Dawson ('38) on embryos of Rana pipiens and Waterman ('39) on embryos of Oryzias latipes. Dawson relates that "almost innumerable evidences of defective development were found." Among these were: rudimentary hearts, intestines which formed but a single loop, heads which often were small and undifferentiated, gills often lacking, eyes frequently abnormal. It is significant that blocked circulation may cause all of these symptoms. Dawson realized that many instances of atypical eye development "appear to be secondarily induced by the general disturbance of development and differentiation rather than specifically induced by the drug." Waterman discovered that the heart and extra-embryonic blood vessels of Oryzias were "particularly sensitive" to the drug. Similar to the effects of 2,4-dinitrophenol are those caused by indolebutyric acid, tested by Copenhaver and Detwiler ('41). They mention that "cases with rudimentary hearts and delayed circulation were particularly numerous." The same workers in 1948 reported the results of exposure of Amblystoma embryos to sulfanilamide and sulfadiazine. A 1% solution of the former drug

frequently caused a reduction in erythrocytes and in size of the heart. Ryanodine, tested by Bodenstein ('50), causes the heart of Amblystoma to slow down and eyes may show collapsed vitreous cavities.

X rays, nitrogen mustards and certain other drugs must have profound effects on the metabolism of individual cells, and it is important to understand these specific effects. In evaluating the damage to a particular organ or tissue, however, investigators using these agents on whole animals should realize that impaired circulation of blood may also account for considerable alteration in tissues. Deciding to what extent particular anomalies are caused by a specific agent acting directly on sensitive cells or indirectly through damage to the circulatory system may be very difficult when animals with normal circulation are employed as test objects. It is suggested that decisions in experiments of this sort on amphibian larvae might be reached most directly by subjecting both normal and circulationless larvae to the same experimental procedure.

SUMMARY

1. Larvae of Rana pipiens usually live 4 or 5 days at room temperature after excision of heart at Shumway stage 22 or 23. One specimen lived 6 days.

2. Gross changes in heartless animals include: broadening of the anterior end due to swelling of lymph spaces of the head; microcephaly; immobility and reduced size of eyes; development of light areas along the sides of the body where individual dermal melanophores are forced apart by the hydrostatic pressure of swollen lymph spaces in the body wall; swelling around the pronephroi and ballooning of the dorsal mesentery; failure of the digestive tract to elongate beyond an S-shape; reduced response to tactile stimuli.

3. In sections one sees that in the eye the posterior chamber collapses within the first day after operation. The eye remains small and in close contact with the brain, although differentiation of both retina and lens continues.

4. By two days after excision of heart, considerable swelling is manifest in the pronephric tubules, postcardinal sinuses, dorsal aorta and dorsal mesentery.

5. Erythroblasts, some of which are undergoing mitosis, may still be present in the dorsal mesentery and gill arches 5 days after excision of heart.

6. Intestinal epithelium differentiates and considerable yolk is digested during the period of survival, but these processes are decidedly slower than in control specimens and did not reach completion in any of the experimental animals.

7. Some of the anomalies reported by other workers in studies on the effects of x rays, nitrogen mustards and certain other drugs on developing amphibian or teleost larvae are interpreted as resulting from impaired circulation.

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PLATE 1

EXPLANATION OF FIGURES

1 Dorsal view of heartless animal 4 days after excision of heart. Note broadening of head due to accumulation of fluid in lymph spaces. × 5.5.

2 Control of same age as animal in figure 1. The body is oval in outline, the tail longer and wider. × 5.5.

3 Eye of tadpole at stage of operation. All cellular layers of retina (inner and outer nuclear layers and ganglionic layer) are recognizable. × 75.

4 Section through region of pronephros of tadpole at stage of operation. Stomach, already showing lumen, half surrounds the pancreas. × 75.

5 Section through yolk-packed midgut at stage of operation. × 75.


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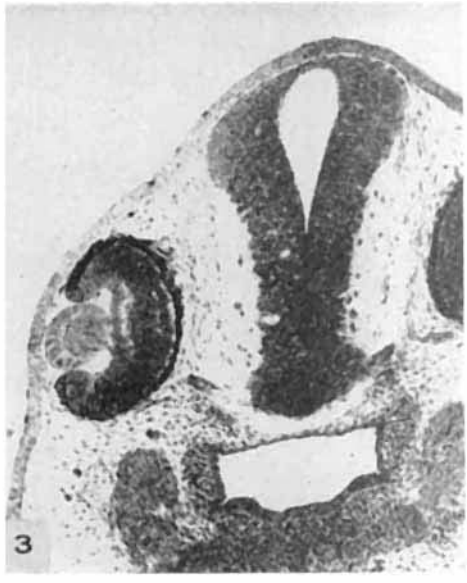
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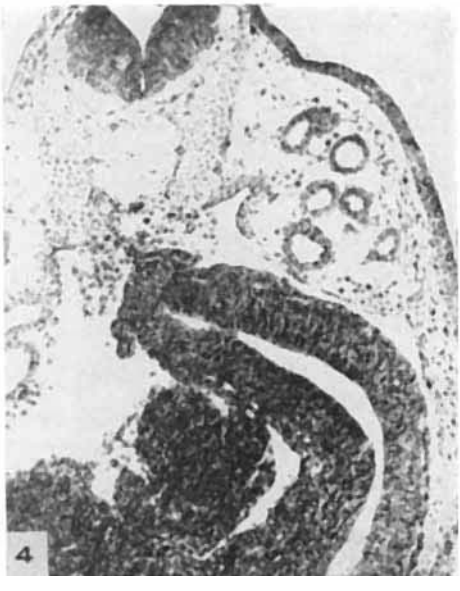
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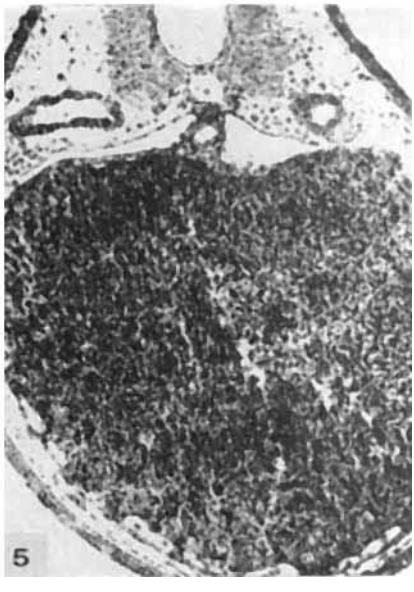
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PLATE 2

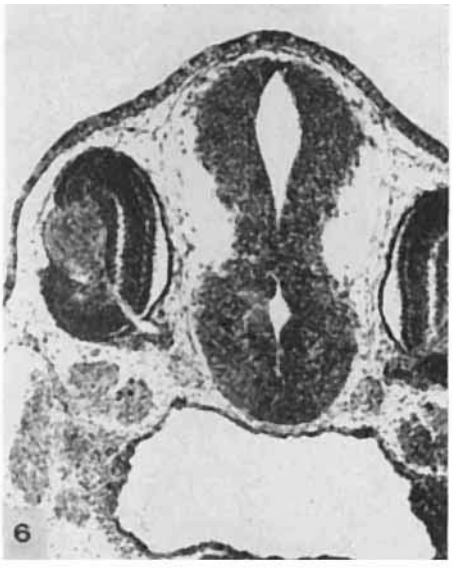
EXPLANATION OF FIGURES

6 Eye and brain of experimental animal one day after excision of heart. Lens and retina more differentiated than in figure 3, but vitreous chamber has collapsed, bringing lens and retina into close contact. Also eye is in close contact with brain. × 75.

7 Eye and brain of control one day after operation. Eye conspicuously larger than that in figure 6. It is clearly separated from the brain and causes the overlying skin to bulge outward. Vitreous chamber separates inner surface of lens from retina. × 75.

8 Section through pronephros of experimental animal two days after operation. Note that pronephric tubules are enlarged, as are the postcardinal sinus and the dorsal aorta. × 75.

9 Section through level of stomach and duodenum of animal in figure 8. Dorsal mesentery is split into two leaves joining stomach (to the right) and duodenum. Pancreas and undifferentiated endodermal cells enclosed within the mesentery. × 75.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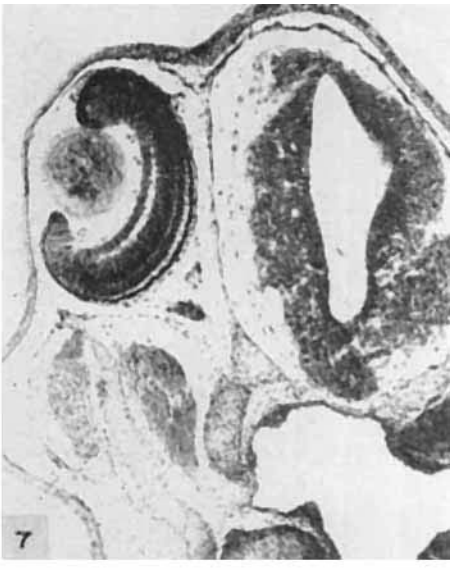
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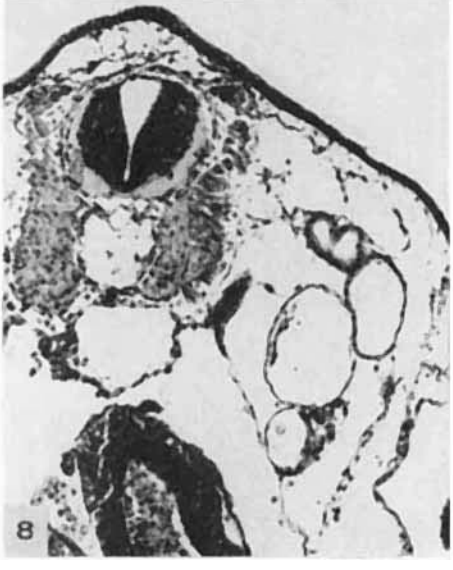
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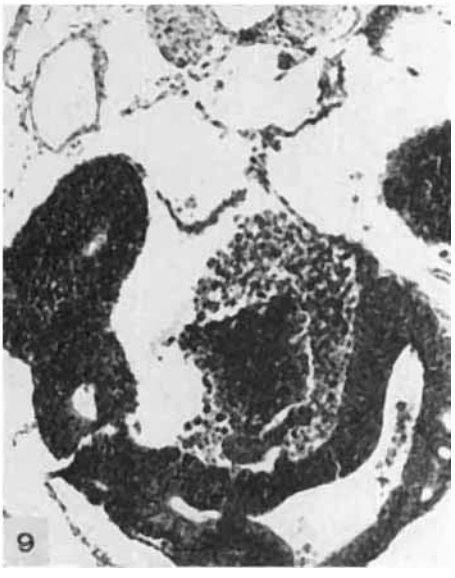
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PLATE 3

EXPLANATION OF FIGURES

10 Eyes and brain of 4-day experimental. Note pyenotic nuclei in central part of retina and in mantle layer of brain. × 75.

11 Eye and brain of 4-day control. Sclerosis of central lens fibers has commenced. Empty space in lens due to loss of lens fibers in sectioning. × 75.

12 Section through level of pronephros of 4-day experimental. Dorsal aorta is greatly distended, as are pronephric tubules and postcardinal sinus. Enlarged lymph space (lower left), below sinus. Paired lung buds below aorta. Dorsal mesentery split into two leaves connecting (on the left) with liver epithelium and (on the right) with stomach epithelium. Undifferentiated endodermal cells nearly fill space within mesentery. × 75.

13 Section through level of intestine in 4-day experimental. Intestinal epithelium has differentiated but is abnormally organized. Ballooning in dorsal mesentery and large amount of undigested yolk are evident. × 75.


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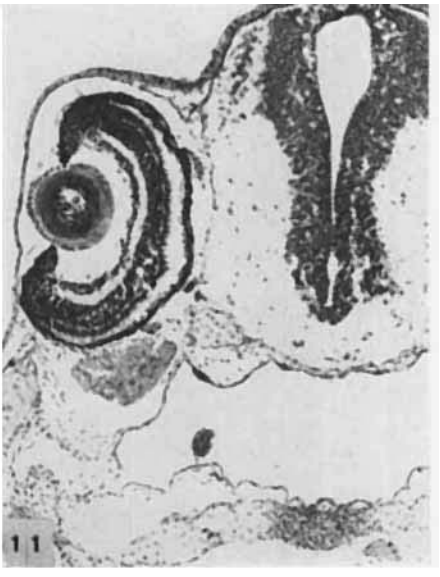
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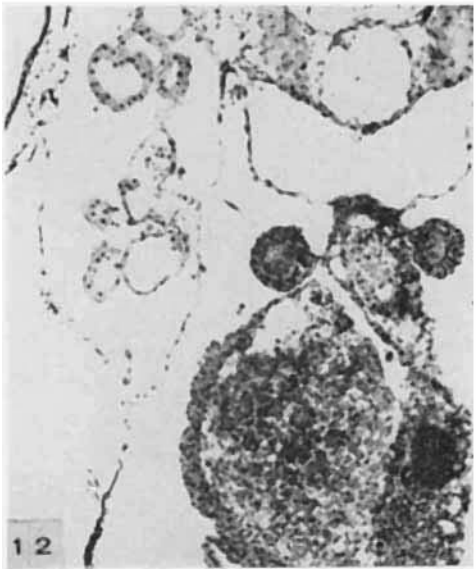
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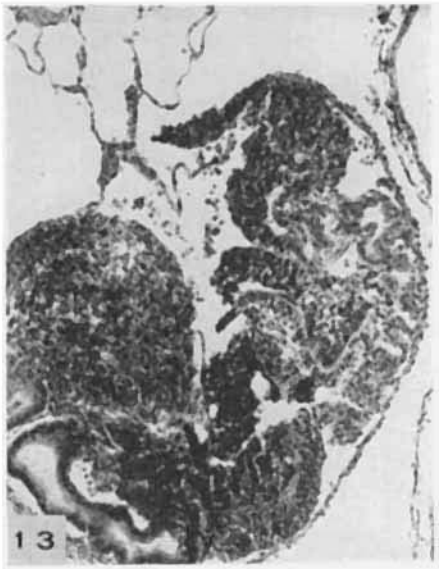
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PLATE 4

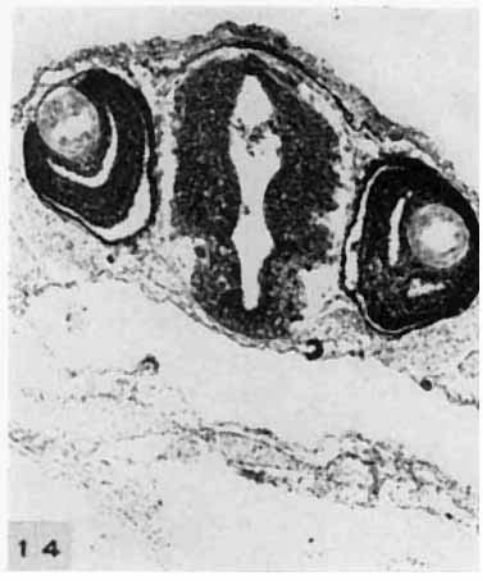
EXPLANATION OF FIGURES

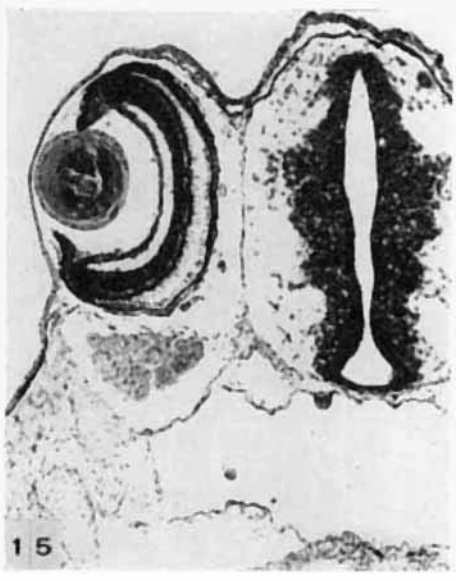
14 Eyes and brain of 6-day experimental. Small size of eye, collapsed vitreous chamber, sunken position of eye in contact with brain are obvious features. Central lens fibers lost in sectioning. × 75.

15 Eye and brain of 6-day control. Compare large size and protruded position with figure 14. × 75.

16 Section through level of pronephros of 6-day experimental. An enlarged pronephric tubule is present, but swelling is not so pronounced in postcardinal sinus or in dorsal aorta as in two-day or 4-day experimentals (figs. 8 and 12). Stomach and intestinal epithelium partially surround mass of undifferentiated endodermal cells. × 75.

17 Section through intestine of 6-day experimental. Epithelial lining of gut is complete. Partially digested endodermal cells and yolk in lumen. Considerable yolk still present in epithelial cells. × 75.


14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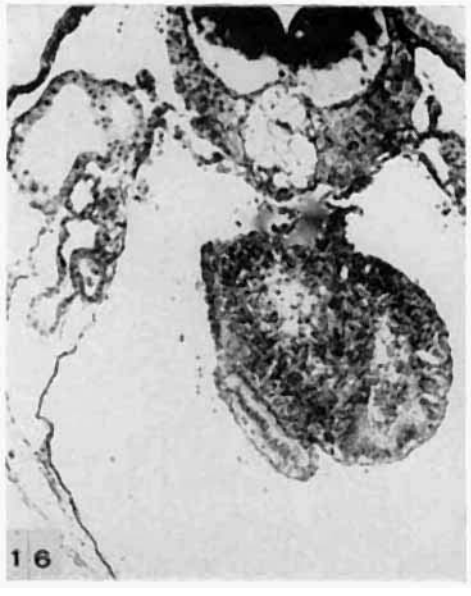
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