MORPHOGENESIS AND METABOLISM OF AMPHIBIAN LARVAE AFTER EXCISION OF HEART

II. MORPHOGENESIS OF HEARTLESS LARVAE OF AMBLYSTOMA PUNCTATUM [1]

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ELEVEN FIGURES

INTRODUCTION

In the previous paper of this series (Kemp, '53) it was reported that tadpoles of Rana pipiens would live up to 6 days at room temperature after excision of heart at Shumway stage 22 or 23. Because of the obvious differences between anuran and urodele larvae in form and rate of development, the experiment was repeated on Amblystoma punctatum at Harrison stage 40, 41 or 42 (Kemp and Quinn, '51). It was interesting to find that heartless larvae of Amblystoma survived at room temperature up to 15 days — more than twice as long as tadpoles of Rana. Although there are many similarities in differentiation of circulationless larvae of Rana and Amblystoma, e.g., microcephaly, collapse of the vitreous chamber of the eye, swelling of pronephric tubules, postcardinal sinuses and dorsal mesentery, reduced motility and slow utilization of yolk, there are also important differences. Lymph spaces in the body wall, in which fluid may accumulate, are pronounced in Rana but negligible in Amblystoma. Anterior limb buds and balancers, on the other hand, are present in Amblystoma but absent in developing tadpoles.

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Heartless larvae of Amblystoma, furthermore, exhibit albinism due to concentration of melanin within the melanophores.

MATERIALS AND METHODS

At Harrison stage 40, 41 or 42 the heart of Amblystoma is closer to the ventral surface than is the heart of Rana at morphologically equivalent stages; hence it is easier to remove. Larvae were anesthetized by electric shock and the pericardial cavity opened by making a midventral incision through the body wall with the Burch ('42) type of microscalpel. The heart was excised by cutting through the bulbus arteriosus and the sinoatrial region. A total of 240 animals were cultured after cardiectomy, either in plain Holtfreter's solution or in Holtfreter's solution containing 0.5% to 1.0% sodium sulfadiazine as recommended by Detwiler and Robinson ('45). Survival beyond three days after operation was unusual in simple salt solution but was so increased with sulfadiazine present that 80% of the larvae cultured in the antibiotic solution survived up to 7 days, and a few survived longer — to a maximum of 15 days for one specimen. One or two days after operation, animals were transferred to pond water. At daily intervals after operation, specimens were fixed in Bouin's fluid for subsequent sectioning. Slides were routinely stained with hematoxylin and eosin. Photographs of whole animals were made with a Kine-Exacta camera and of sections with a Spencer photomicrographic camera.

OBSERVATIONS

A. Gross changes

Experimental animals could be distinguished from controls as early as one day after excision of heart. By this time both the main stem of the gills and their secondary filaments had elongated farther in the controls. During subsequent days the gills in normal animals grew larger, while those in experimental animals gradually became shorter because of degeneration at the tips. Development of the limbs provided

another distinctive contrast between normal and experimental animals. Limbs of the latter remained relatively short and never became flexed at the elbow joint; nor did they ever become more than slightly bifurcated into two digits distally. In normal animals, on the other hand, the limb elongated, became flexed at the elbow, underwent pronation and developed three digits during the period studied. As in Rana (Kemp, '53), the eyes remained small and immovable in circulationless larvae. Also as in Rana they developed in association with microcephaly of the anterior part of the head. Most of the epidermal melanophores around the head were maximally contracted to a small dot in heartless animals as early as one day after cardiectomy, likewise most of the dermal melanophores along the ventral borders of the pigmented flanks. In subsequent days more and more of the dermal melanophores became contracted until eventually the animals became "albino" (fig. 1). Experimental animals first exhibited swelling in the region of the pronephroi and anterior digestive organs of the coelom about two days after operation. Neither the head nor the posterior part of the coelomic cavity became appreciably swollen. Through the transparent body wall it could be seen that differentiation of the digestive tract was proceeding but at a slower rate in heartless animals than in normal ones.

B. Microscopic changes

1. Eye. At the time of operation the eye was well along in differentiation. Figure 2 illustrates a typical specimen showing the ganglionic layer already separated from the outer cells of the retina, but there is no demarcation between inner and outer nuclear layers. Rod cells are growing out into the space between retina and tapetum. Sclerosis has begun in the lens. Just as in Rana (Kemp, '53), the eye of Amblystoma suffers collapse of the vitreous chamber within the first day after excision of heart. For this reason the eye (fig. 3) appears smaller than that of a normal animal (fig. 4).

One day after operation the rods in eyes of experimental animals were no better developed than at the time of operation, whereas in control specimens they were longer. Pycnosis of some nuclei in the central part of the retina and in the mantle layer of the infundibulum was observed in one specimen fixed only two days after operation. Pycnosis was also observed in some cells of the eye and brain of several older specimens, but even in the oldest animal sectioned (14-day) most of the cells in these locations looked normal.

2. *Pronephros and gastroduodenal region.* Distention of pronephric tubules and postcardinal sinuses was apparent by the first day after operation. Fluid had obviously begun to accumulate and cause swelling of the dorsal aorta, dorsal mesentery, and the coelom of the gastroduodenal region. Continued uptake of fluid in subsequent days resulted in the development of large spaces beneath the mesodermal sheath of esophagus, stomach and duodenum or even in complete separation of the mesodermal sheath from the endodermal lining of the gastroduodenal portion of the digestive tract (figs. 6, 7). Hemopoiesis apparently continued within the spaces of the distended mesenteries of some specimens, for the blood cells in these spaces in older specimens often contained yolk platelets, whereas the red blood cells circulating at the time of operation were already yolkless.

3. *Intestine.* Yolk still filled the cells of the primitive intestine (fig. 5) at the time of operation. As might be expected, differentiation and digestion of yolk proceeded faster in normal animals than in experimentals. Neither group showed much change during the first post-operative day, but separation of the intestinal epithelium from the central *Nahrdotter* was beginning in the controls on the second day. By three days the epithelium in controls was completely formed and yolk had entirely disappeared from the intestinal lumen, although it was still abundant within the epithelial cells. Epithelium was completely defined in experimental animals by 4 days, but partially digested yolk still filled the lumen. No significant advance had occurred by the 6th day (figs. 8,

9). About the 7th day in controls, intestinal epithelial cells began to show vacuolar spaces of various sizes, possibly derived from digested yolk platelets. Holtfreter ('46) has described similar vacuoles in the larval epidermis of Rana pipiens and Hibbard ('28) has reported them in larvae of Discoglossus. Intact yolk platelets had practically all disappeared in control animals of 8 days or older but were abundant even in the oldest (14-day) experimental animal examined.

4. Spleen. The primordium of the spleen was recognizable in the splanchnic mesoderm adjacent to the stomach even at the time of operation. In heartless animals there was no further differentiation of spleen. In control animals, on the other hand, the spleen was noticeably larger one day after the stage of operation. By three days it was well developed. These observations indicate that circulating blood is an important factor in normal morphogenesis of the spleen.

5. Limb. At the time of operation the limb consisted of a core of proliferating mesodermal cells surrounded by epidermis (fig. 5). Blood vessels were present and at the base of the limb was a central group of precartilage cells, the primordium of the humerus. Yolk platelets were still present though scarce. Two days later control specimens possessed primordia of radius and ulna in addition to the humerus, whereas radius and ulna could not be distinguished in experimental animals until the third day. By this time the controls were definitely further advanced with respect to differentiation of cartilage. By 7 days the limb in heartless animals was only slightly longer and more differentiated than at three days, while in controls the limb had become flexed at the elbow joint and possessed well developed cartilages, including those in two well formed digits and a beginning third digit. In subsequent days there was virtually no further advance in development of the limb in experimental animals.

6. Skull and vertebral cartilages. Retardation in the rate of differentiation of cartilage in the limb was paralleled in the cranial, visceral and vertebral cartilages. Anlagen of

these cartilages were sufficiently differentiated at the stage of operation that they could be easily identified. After cardiectomy, however, there was very little further differentiation of cartilage. There was increase in basophilia of the matrix in some animals; but active multiplication of cells and marked growth, which characterized the cartilages of controls, did not occur in the experimental group (figs. 10, 11).

DISCUSSION

As stated previously (Kemp, '53), we may consider anomalies in circulationless larvae as resulting chiefly from reduced metabolism, from altered hydrostatic pressure, or from a combination of these factors. In the present study Amblystoma larvae lived up to 15 days after excision of heart, compared to only 6 days for the Rana tadpoles studied in the previous investigation. Measurements of oxygen consumption in animals of both species (Kemp, unpublished) reveal that Amblystoma respires about half as fast as Rana at comparable stages after the start of circulation. It can be inferred that the longer survival of heartless Amblystoma larvae is correlated with their relatively slow metabolism. Morphological evidence for reduced metabolism in these larvae is afforded by the behavior of melanophores, the retarded rate of utilization of yolk in the digestive tract, and the inhibition of both differentiation and growth in the spleen, gills, limbs and skeletal cartilages.

It is well known that a hormone, intermedin, elaborated by the intermediate lobe of the pituitary gland is responsible for the spreading out of the pigment granules in the melanophores of Amphibia. A number of workers, including Smith ('16, '20), Allen ('17), Blount ('32, '45), Atwell ('21), Eakin ('39) and Burch ('46) have shown that larvae lacking the intermediate lobe develop with the "albino" syndrome, characterized by concentration of melanin in both epidermal and dermal melanophores and maximal dispersion of pigment in the xantholeucophores. Of particular interest in the present investigation was the variability of response of individual

cells. Apparently the epidermal melanophores as a group are more sensitive to lack of a circulating factor, presumably intermedin, than are the dermal melanophores, since practically all of the former exhibited concentration of melanosomes within the first day after cessation of circulation. The response of the dermal melanophores, on the other hand, was variable. Those farthest ventral along the flanks tended to contract first, but as time went on the response spread dorsad so that eventually practically all of the dermal melanophores were contracted. Because of the variability in the time of response of these cells, it seems reasonable to hypothesize that the intermedin available became depleted at different rates. It is likely that a threshold level of the hormone must be maintained in the vicinity of the melanophores in order that maximal expansion of the pigment may occur. If this be true, the melanophores might remain in the expanded state only as long as the local supply of intermedin lasted or until it fell below the threshold level, or as long as the intermedin-induced reaction persisted after intermedin itself fell below the critical level.

Slow utilization of yolk in the digestive tract was anticipated in the present investigation because of the results of previous studies (Kemp, '51, '53) on anuran larvae. Blood circulating to the gut supplies something which increases the rate of digestion of yolk. Whether the circulating factor is merely oxygen or some other substance which might stimulate metabolism is unknown. It would indeed be surprising if only one substance were involved. The finding that circulating blood is important for development of the spleen is not surprising in view of the close association of the spleen with the circulatory system. Regression of gills and failure of the limbs to differentiate much beyond their condition at the time of excision of heart afford evidence that circulation is important for normal differentiation of these structures. Wilde ('52) has shown that presumptive gills have a high potential for growth and differentiation in tissue culture, even to the extent of suppressing differentiation of adjacent

limb primordia. The present study demonstrates, however, that continued growth and development of gills already well differentiated is dependent on circulation. This conclusion is in agreement with Moser's ('40) statement that "doubtless the lack of vascular connections partially, at least, explains the failure of continued differentiation of the gills." Wilde ('50) has also demonstrated that limb buds from embryos at stages 40 and 41 could differentiate humerus, radius and ulna, carpal mass and two digits in vitro. The primordium of a third digit developed in an explant from stage 43. Wilde's experiments showed that considerable organotypic growth and differentiation may take place in vitro, but it is significant that isochronous controls always showed much better development of the limb cartilages. Circulation of blood in the controls is the obvious key to this difference. The present study has shown that not only the limb cartilages but also all other cartilages of the larva are dependent on circulation for normal morphogenesis. Both Wilde ('50) and Fell and Robison ('29) have demonstrated that the degree of organotypic development in vitro depends on the stage of isolation of an explanted rudiment. One would expect that as an embryo ages it produces and stores more and more of the material needed for the synthetic activities of morphogenesis. Having more already in the warehouse would permit an older rudiment to proceed farther when isolated. Possible explanations for the poor development of cartilage in heartless animals of the present investigation are (1) that the primordia for this tissue had not accumulated sufficient reserves to permit long-continued differentiation and growth after cessation of circulation or (2) that the rate of metabolism in these primordia fell too low. One particular metabolite which appears to be implicated in the differentiation of both young cartilage and bone is alkaline phosphatase (Karczmar and Berg, '51).

Accumulation of fluid in body spaces — pronephric tubules, postcardinal sinuses, dorsal aorta, dorsal mesentery and beneath the mesodermal sheath of the gut — occurs in Amblystoma much as previously described for Rana (Kemp, '53).

One obvious difference between the two species is that in Rana fluid collects in lymph spaces of the body wall, causing the anterior end to broaden markedly. This does not occur in Amblystoma. Collapse of the vitreous chamber of the eye evidently results from the inability of the eye to maintain normal intraocular hydrostatic pressure in animals lacking circulation. Bodenstein ('48) and Bodenstein and Goldin ('48) have published photographs showing collapsed vitreous chambers in the eyes of Amblystoma larvae subjected to nitrogen mustard. It has been suggested (Kemp, op. cit.) that these and many other abnormalities induced by exposure of embryos to various drugs or radiations may well be secondary effects resulting from impaired circulation.

SUMMARY

1. Larvae of Amblystoma punctatum rendered circulationless by excision of heart at Harrison stage 40, 41 or 42 survived at room temperature up to 15 days.

2. Gross examination revealed a gradual regression of gills and only slight further development of limbs after cardiectomy. Eyes remained small and immovable and the head became microcephalic. Melanophores became contracted so that heartless animals were "albino." Internal swelling was pronounced in the gastroduodenal region. Differentiation of digestive tract continued but was considerably slower than normal.

3. Microscopic examinations revealed that the vitreous chamber of the eye became collapsed in circulationless animals. Pronephric tubules, postcardinal sinuses, dorsal aorta, dorsal mesentery, spaces beneath the mesodermal sheath of the gut, and the coelom in the gastroduodenal region became swollen. Utilization of yolk was conspicuously retarded in the intestine and the spleen stopped developing after circulation ceased. Cartilage in the limbs, skull and vertebral primordia differentiated only slightly after the heart was removed.

4. Circulating blood is important for regulating hydrostatic pressure in Amblystoma larvae, as well as for supplying oxy-

gen and other materials needed for the continual synthetic activity of differentiation and growth.

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PLATE 1

EXPLANATION OF FIGURES

1 Dorsal views of control (left) and experimental larvae one day after operation. Contraction of most of the melanophores of the heartless larva is apparent. Those farthest ventral along the flank are contracted most. Eye of experimental larva is smaller than that of control. × 8.

2 Eye at stage of operation. Central part of retina is separated into ganglionic layer and nuclear layer. Rods are growing out into space between retina and tapetum. Sclerosis of fibers evident in center of lens. × 70.

3 Eye of heartless larva one day after operation. Collapse of vitreous chamber has brought lens and retina into direct contact. × 70.

4 Eye of control larva one day after stage of operation. Rods are slightly longer and more numerous than on previous day. × 70.

5 Intestine and limb of larva at stage of operation. Yolk platelets are closely packed throughout the intestine. Limb shows central blood vessel within mesodermal core. × 70.


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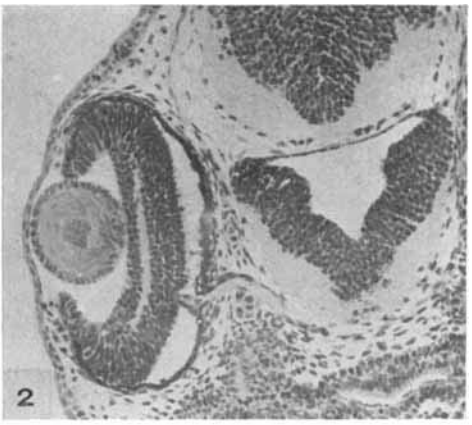
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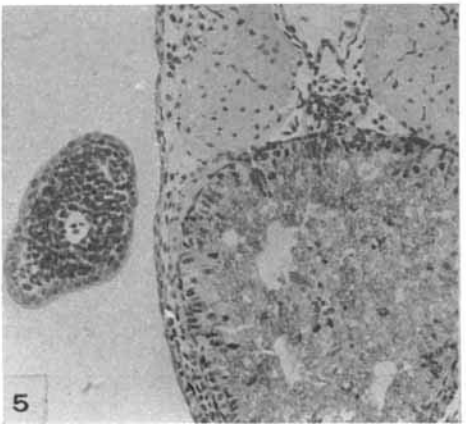
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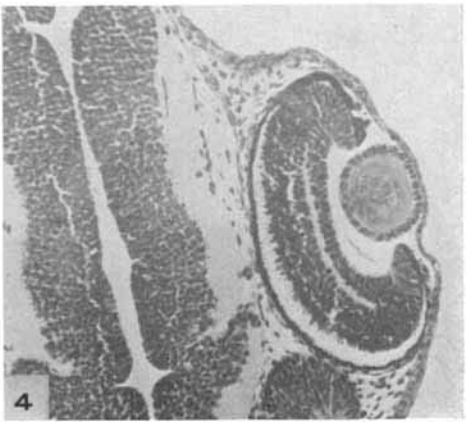
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PLATE 2

EXPLANATION OF FIGURES

6 Six-day experimental larva showing distention of pronephric tubules (pr), postcardinal sinus (ps), dorsal aorta (a), dorsal mesentery (m), space beneath mesodermal sheath of stomach (s), and coelom (c). × 70.

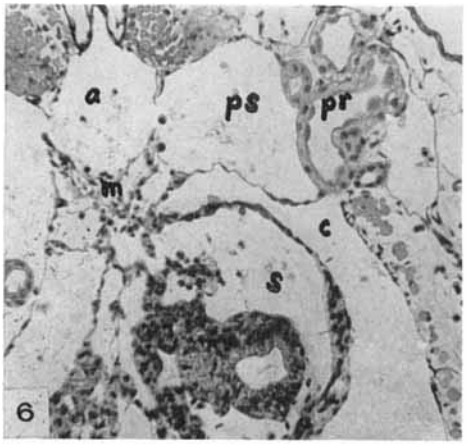
7 Six-day control larva at level of pronephric tubules (pr), stomach (st) and lung buds (l). × 70.

8 Intestine of 6-day experimental larva. Note ballooning of dorsal mesentery. Partially digested yolk occludes lumen and intestinal epithelium is packed with yolk platelets. × 70.

9 Intestine of 6-day control larva. Yolk digested from lumen but platelets still abundant in epithelium. × 70.

10 Section through auditory vesicle of 14-day heartless larva. Parachordal cartilages (pc), palatoquadrate cartilage (pq), and cartilage of auditory capsule (a) can be recognized but are poorly developed. × 70.

11 Fourteen-day control larva. Basilar plate of trabecular cartilages (b), palatoquadrate (pq), and auditory capsule (a) are well developed. × 70.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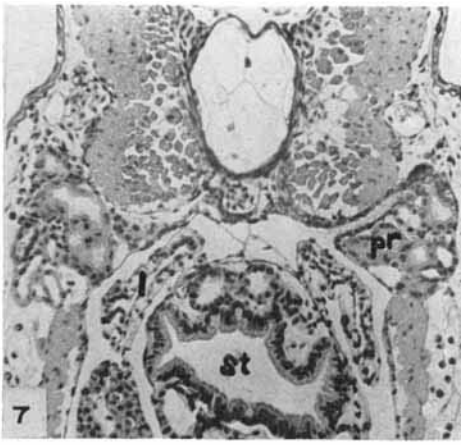
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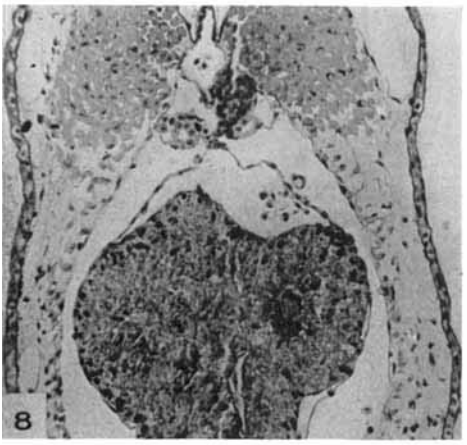
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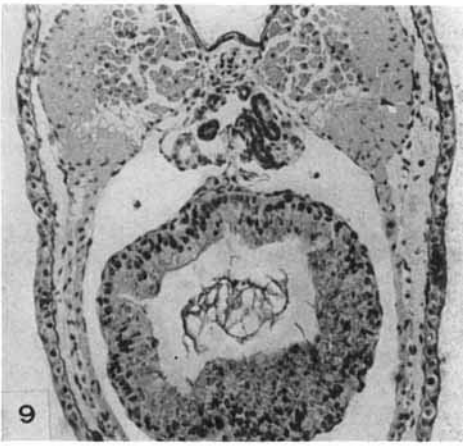
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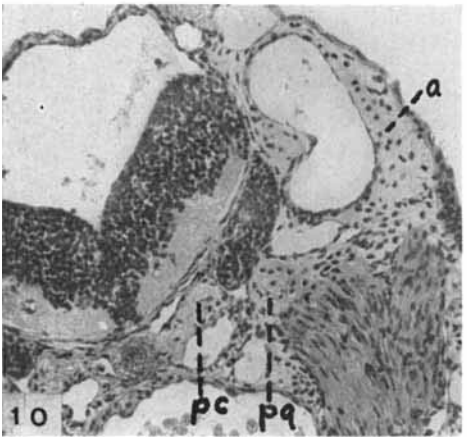
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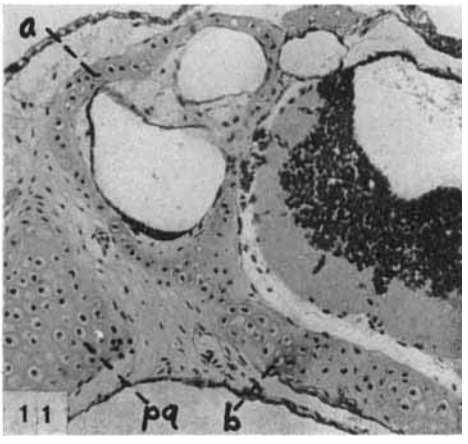
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