Tissue Culture Studies on Amphibian Metamorphosis

I. Growth pattern of tadpole tissue*

By

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Introduction

The phenomenon of metamorphosis of amphibians has been the subject of a large number of investigations because it involves a striking alteration of form and structure in a relatively short period of time. Since the atrophy of the larval anuran tail presents the most remarkable element among the events during the metamorphosis, much work has been done regarding the cause of this phenomenon. Barfurth (1887) ascribed the atrophy of the tail to the occlusion of the dorsal aorta by the growing urostyle. Bataillon (1891) studied the blood vessels of normal and atrophying tails and described a change in vascular distribution in the latter. Helff (1930) had extirpated the anlage of the urostyle prior to metamorphosis and found that typical tail atrophy was exhibited during subsequent metamorphosis. Thus it was revealed that the growth of the urostyle could not be a fundamental factor inducing tail atrophy. In fact the tail which was transplanted heterotopically underwent characteristic histolysis at the same stage of metamorphosis at which the tail normally degenerates. On the other hand, limbs which were grafted to the tail remained without any degeneration while the tail itself underwent degenerative changes at the time of metamorphosis. So it is now evident that the degenerative changes of the anuran tail during metamorphosis are not dependent upon such extrinsic factor as the urostyle, but are based on a kind

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of self-differentiation. The feeding experiment of Gudernatsch ('12) demonstrated that the thyroid gland is of great importance in amphibian metamorphosis. The evidence implies, though it does not definitely prove, that the thyroid hormone acts directly without the mediation of other organs in the initiation of metamorphosis. As reactions of tissues to the thyroid hormone differ according to the part of the body, the metamorphic pattern must be the product of an interaction of two basic factors, the influence of the hormonal agent and an inherent pattern of tissue sensitivity. The same kinds of tissue from different parts of the body react differently. In some cases the tissue response is one of growth, in others one of histolysis. For example, the leg muscular tissue is stimulated by the thyroid hormone to grow, while that of the tail responds by histolysis. However, little is known as to the nature of such differential sensitivity of one and the same kind of tissue to the metamorphic stimulant.

In this connection the present work was undertaken in order to contribute to the understanding of the mechanism by which metamorphosis is governed. So far as is known to the author the tissue culture method has not been used in this field of research. The author will here confine himself to a description of growth patterns of several kinds of tissues in vitro from amphibian tadpoles. Other experimental studies with thyroid substance are in progress.

Materials and Methods

Materials used were mostly larvae of Rana catesbiana and partly those of Rana pipiens. Rana catesbiana selected for use consisted of three groups. Individuals in the first group ranged from 4.5 to 5.0 cm trunk length, from 7.0 to 7.2 cm tail length with hindlimb of about 3 cm length (thigh 1 cm, leg 0.8 cm, foot 1.2 cm) (fig. 1). The second group consisted of tadpoles at the late stadium of metamorphosis whose trunk length measured 4.2 cm, tail length 1.3 cm, thigh 1.8 cm, leg 1.5 cm, foot 2.3 cm (fig. 2). The third group was made up of younger tadpoles whose total body length was 8.0 cm.

Cultured tissue were: 1. cut pieces of tail, 2. tail skin, 3. stroma of the fin, 4. tail muscle, 5. back skin, 6. back muscle, 7. leg skin, 8. leg muscle.

The roller tube method of culture on double cover slips was employed. The solid medium which held the tissue to the cover slip consisted of one part chicken plasma containing 1 mg neomycin sulfate.
per ml. and one part embryonic extract from 8-day chicks, the fluid medium was composed of equal parts of the usual nutrient fluid used in this laboratory (45 parts Gey’s balanced salt solution, 50 parts human ascitic fluid of malignant origin, 5 parts chick embryonic extract, 1000 units of penicillin per ml.) and Holtfreter’s solution to which 0.15 mg of neomycin sulfate per ml. was added in a few experiments. As the external surface of tadpoles is exposed and therefore contaminated, it must be cleaned with the utmost care. First the animals were washed repeatedly with tap water, and then with distilled water and finally with sterilized Holtfreter’s solution. Tissue to be cultured were taken from the body with fine scissors and forceps, cut in pieces of about 1–2 mm square with knives made from razor blades.

The roller tube cultures were kept at a room temperature of 22–24°C. Observations were made at low magnification every day on all cultures. Almost every day some of the cultures were selected for more precise observation with phase contrast microscopy and still pictures were made to record significant events. Time-lapse cinematography was also employed to record cellular activity.

The culture were fixed and stained after various periods of cultivation from 1 day to nearly two weeks. After washing in Holtfreter’s solution for 15 minutes, the cultures were fixed in methyl alcohol, 10% formalin or Helly’s fluid. They were stained by Jacobson’s method, iron hematoxylin or Azan according to Heidenhain, silver impregnation (Lillie) and periodic acid-Schiff’s reagent.

Observations

1. Culture of piece of tail
   a. Premetamorphic material

In explants including skin tissue the epithelial outgrowth was exceptionally well seen (fig. 3). Migration of epithelial tissue usually began in a few hours. An epithelial sheet around the explant with a margin 700 micra from the explant was observed in as little as five hours after the culture was prepared. The rate of spread of the epithelial sheet measured from a moving picture sequence was revealed to be about 100 micra per hour. As there may have been a restraining influence due to the closed chamber, and as a result of the illumination used for cinematography, the actual speed of outgrowth in the roller tube might have been somewhat greater than seen in the moving picture sequences. Pigment cells were scattered in the epithelial sheet
Macrophages were also observed in the epithelial sheet or in those areas where epithelial proliferation failed. In a few days, however, outgrowth generally stopped and a shedding off of the epithelial cells from the central part of the sheet took place. Frequent changes of fluid nutrient could sometimes prevent precocious degeneration of the epithelial outgrowth for approximately two weeks. A fibroblastic type of outgrowth appeared in the zone of degeneration of the epithelial cells and in those cultures that had failed to produce a sheet of epithelial cells.

Cytological features of epithelial cells in migrating sheets are shown in figures 4 to 8. At the free edge the cytoplasm formed a thin, wavy undulating membrane which showed very active movement during the outgrowth (fig. 4). Nuclei had sharply defined nuclear membranes and 1 or 2 nucleoli (figs. 5 & 6). Around the nuclei peculiar dark halos were observed with phase contrast microscopy in most of the cells (fig. 5). These halos were sometimes thin but occasionally they were fairly thick (figs. 5 & 6). When extremely thin the nuclei appeared to consist of a double layered membrane (fig. 22). The halos often sent out straight or curving branches stretching in the cytoplasm and ending in a sharp cone or coiling up near the halo (fig. 6). In fixed and stained preparations various kinds of dyes did not stain this structure with the exception that it became pale grey with osmium tetroxide. Fine granules were discernible around nuclei (figs. 4 & 9). In thick outgrowths the epithelial cells were compact in the superficial layer. The boundaries of such elements described straight lines and consequently the cells were polygonal (fig. 9). In contrast, cells in the deeper layers were looser and fairly wide intercellular spaces were recognizable (fig. 10). On the surface contacting the glass slide straight lined boundaries were not discernible. Filamentous mitochondria were numerous in epidermal cells in vitro (fig. 7). With careful focusing of phase objectives these could be separated from delicate, actively motile folds of the superficial cytoplasm (fig. 8).

Connective tissue elements seen after the epithelial cells were shed, or, in the part that had failed to produce a sheet of epithelial cells, consisted of mesenchymal cell types and macrophages as seen in the culture of the fin stroma. The movement of the macrophages appeared very rapid in film sequences.

b. Epithelial outgrowth from the skin of degenerating tail at the late stadium of metamorphosis

Epithelial migration from explants of degenerating tail tended to
start a little later than tissue obtained in premetamorphosis. The process required 10–12 hours. No peculiarities of outgrowth were recognizable, except that the epithelial cells usually contained brown pigment granules and sometimes fat droplets and were accompanied by many macrophages.

2. **Culture of fin stroma**

The stroma of the tail fin of the tadpoles consists of jelly-like loose connective tissue including blood vessels and peripheral nerves. After removing the skin of the fin, the stroma was cut away from the axial musculature of the tail, cut into pieces and explanted.

Cell outgrowth began from the most of the explants 2 or 3 days after they were set up in culture. The outgrowing cells were of mesenchymal and macrophagic type. The mesenchymal cells showed very complicated shapes the contour of which were usually obscure because of low contrast between cytoplasm and environment (fig. 11). Nuclei were of oval form and had two or three nucleoli. Often the nuclei were hardly visible. They were usually surrounded by minute granules which appeared optically dense with dark phase microscopy and stained a dark violet or purple color with Jacobson's method. The cells stretched out thin, wing-shaped processes in various directions which became associated with neighboring cells. A few fatty droplets were visible in older cultures (fig. 11). No ordinary fibroblasts were discernible.

3. **Culture of tail muscle**

   a. Premetamorphic material

Cell outgrowth began in as little as two days after culture, but in some cases several days were required. The cell types seen were mostly mesenchymal just as those seen in the culture of fin stroma (figs. 12, 14 & 15), but sometimes spindle shaped cells appeared which usually were arranged in cords and provided with fine longitudinal striations of the cytoplasm. The border of the cytoplasm was much more distinct (fig. 16) than that of other star-shaped mesenchymal cells (compare with fig. 12). These appeared to be somewhat different from ordinary fibroblasts. Such cells were not seen in any of the cultures of tail fin stroma. They probably represented endothelial cells in a stage of fibroblastic transformation. Among the outgrowing connective tissue elements slender cells containing longitudinal fibrils were occasionally observed (fig. 15). These were identified to be muscle fibers since they were often multinuclear (fig. 18), although they lacked cross striations (fig. 17).
b. Culture of tail muscle after onset of metamorphosis

Cell outgrowth was slight in these preparations. Some cultures did not show any proliferation even after ten days. Most of the outgrowing cells were of obscure mesenchymal type. Pigment granules were often included in the cytoplasm. Macrophages were also sometimes seen in abundance. Figure 13 shows typical mesenchymal cells grown from muscle explant from the root of the tail at the late stage of metamorphosis. They were provided with great amounts of perinuclear granules which stained deep purple with Jacobson's method. Macrophages often contained brown pigment. From the proximal end of the tail root, however, well defined spindles probably representing ordinary fibroblasts were intermingled. There was no outgrowth of muscle fibers.

4. Culture of skin from the dorsal surface

There was little difference in the character of the outgrowth of epithelial tissue as compared to that of tail skin before as well as after onset of metamorphosis. The superficial layer consisted of polygonal cells (fig. 22 & 23), while deeper layers showed cells of irregular shape with relatively wide intercellular spaces (fig. 24). The rate of outgrowth of the epithelial sheet was measured to be about 150 micra per hour during the initial stage of outgrowth.

5. Culture of muscle from the dorsal region

Cell outgrowth was observed in as little as two days. In the area of outgrowing cells there were well defined, fibroblast-like cells besides elements of obscure mesenchymal type which were also seen in the tail muscle culture. Young muscle fibers were also seen both in the cell outgrowth and in the explants proper. The fact that many muscle fibers were seen in the explants was one of the most obvious features of the cultures of the dorsal muscle culture (fig. 20 & 21). The young muscle fibers were much thinner than those within the explant and showed no cross striations. The conclusion that these were definitely muscle fibers was based on their similarity to young muscle fibers seen in tadpoles of the early stage of development, for example, in the rectus abdominis, and the fact that they were multinuclear. They appeared only after more than ten days in culture.

6. Culture of leg skin

Concerning the features of outgrowth and morphology of the epithelial sheet nothing was found which warrants special description. However, the rate of spread of the epithelial sheet was measured in a moving picture sequence. In a 9 hour culture of leg skin during
metamorphosis, the rate of movement of cells measured on basis of ten nuclei selected at random was; 80, 109, 63, 100, 113, 120, 100, 150, 128, 94 micra per hour. The average was 105.7 micra per hour. The rate of migration of each individual cell was variable. In one and the same cell the speed changed from time to time.

7. Culture of leg muscle

Hind limb muscle was cultivated after removing the skin, after onset of metamorphosis. Outgrown cells consisted exclusively of well defined spindle cells (figs. 25 to 27). They were arranged in longitudinal rows (figs. 25 & 26) and were therefore suggestive of an endothelial origin. Mesenchymal cells of the type described in the culture of fin stroma or of tail muscle were never seen. Differentiation of newly formed muscle fibers was not observed.

Discussion

In culture of anuran larval tissues it was found that epithelial outgrowth is always predominant when skin is involved in explants. Cell outgrowth began in a few hours and a sheet spread at a rate that we have not seen for various mammalian or avian tissues. It was expected that the tissue of the tail after the onset of metamorphosis might show no outgrowth or at least give evidence of a marked retardation. However, the epidermis of the metamorphosing tail was found to have almost the same potency of proliferation as that of the pre-metamorphic epidermis. The only difference between the outgrown epithelial cells taken before and after the onset of metamorphosis was the existence of pigment granules and fat-droplets in the cytoplasm of the latter. However, this was not a constant feature. There were cells which were lacking in pigment granules in postmetamorphic material and cells which had fat-droplets in older cultures of pre-metamorphic tissue. Therefore we can say that there was no critical difference in the outgrowth-patterns of epithelial tissues taken from the skin before and after the onset of metamorphosis.

The nature of the perinuclear halo in epidermal cells requires further study. With dark phase contrast microscopy these halos appeared optically dense, they were not stained by a variety of dyes, reacted negatively to periodic acid-Schiff's reagent, unimpregnable with silver, while osmium tetroxide made them grey. Although most of them appeared around nuclei, some of them were found apart from the nuclei. In rings which were seen to be thin, they were always
peri-nuclear. It is probable that the nuclei were involved in their production.

In contrast to the fact that there is no apparent difference in the characteristics of outgrowth of the epithelial tissues according to their source of origin, there were important differences in cell outgrowth from the musculature of the tail, trunk and leg under the conditions of these experiments. The only connective tissue element which was seen to grow from tail muscle was mesenchymal except for macrophages. Mesenchymal cells characteristically had extensive protoplasmic processes of a very complicated nature. They were regarded as the chief components of the jelly-like connective tissue of the tail and were found not only in the fin stroma but also in the intermuscular spaces. It was revealed by observations on cultures of dissociated muscle fiber of the tail that the mesenchymal cells were derived from the endomysium (fig. 19). In contrast, muscle of the leg yielded spindle cells, while from trunk muscle both kinds of cells emerged. The contrast between these was distinct (compare figs. 14 & 25 or figs. 15 & 26). The fact that connective tissue cells from tail and leg are different in nature corresponds to the difference in response of these tissues during metamorphosis. One shows growth, another histolysis and resorption, other tissues involve a combination of the two processes. It should also be mentioned that outgrowth of connective tissue cells from tail muscle is relatively delayed if it is explanted during metamorphosis.

Outgrowth and differentiation of muscle fibers in the cultures was observed in dorsal and tail muscle. In the latter instance it was found only in cultures of tadpoles in the younger stages of development. These slender muscle fibers appeared after about 10 days of culture and were never seen in earlier stages of cultivation even in the explants. Therefore, it appears certain that the fibers must have differentiated from some undifferentiated myoblastic cells involved with the muscular tissue during cultivation.

Summary

Cultures of skin and muscle of tail, dorsum and leg and stroma of tail fin of tadpoles of Rana catesbiana and Rana pipiens before and during metamorphosis were carried out by means of the roller tube method.

When skin was involved in explants, epithelial outgrowth was
always predominant. The rate of emigration of the epithelial sheet was extraordinarily fast. The characteristic feature of the epithelial cells was the presence of a perinuclear halo whose nature requires further study. In the mode of epithelial outgrowth there were no significant difference between materials in relation to their origin and stage of metamorphic development.

From explants of tail muscle peculiar connective tissue cells were observed having very complicated cytoplasmic processes whose boundaries were quite indistinct. These cells were quite similar to the cells outgrown from stroma of tail fin. Muscles of hind limbs produced only well defined spindle cells. These had cytoplasmic margins which were distinct and therefore in sharp contrast to those of the cells from tail muscle. Muscle from the dorsal region gave rise to both types of cells.

The difference in pattern of outgrowth from muscles of these three areas appears to correspond to the difference in response to the inducing agent of metamorphosis.

Outgrowth of myoblasts and differentiation of muscle fibers were observed in cultures of tail muscle of the younger stage of development and of dorsal muscle.

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References

Explanation of figures

Plate I

Fig. 1. A tadpole of *Rana catesbiana* before onset of metamorphosis (group 1). 1.2×

Fig. 2. A tadpole of *Rana catesbiana* at the late stage of metamorphosis (group 2). 1.2×

Plate II

Fig. 3. An epithelial sheet from an explant of tail before onset of metamorphosis. Twenty-four hour culture. Jacobson's stain. 19×

Fig. 4. Marginal region of a growing epithelial sheet from an explant of tail before onset of metamorphosis. Three day culture. Prepared with the use of an 8mm AO dark phase contrast objective. 270×

Fig. 5. Cells with perinuclear halos in an epithelial sheet from tail before metamorphosis. Four day culture. Prepared with 1.8 mm dark phase contrast objective. 1300×

Fig. 6. Cells in an epithelial sheet from an explant of tail before metamorphosis. Perinuclear halos. Phase contrast microscopy. 1300×

Fig. 7. Mitochondrial pattern in the epithelial cells outgrown from an explant of tail. Four day culture. Phase contrast microscopy. 1300×

Fig. 8. Folds on the cell surface of an epithelial sheet from an explant of tail. Three day culture. Phase contrast microscopy. 1300×

Plate III

Fig. 9. Superficial layer of an epithelial sheet from tail skin of a tadpole before metamorphosis. One day culture. Phase contrast microscopy. 500×

Fig. 10. Deeper layer of the same field as fig. 9. 500×

Fig. 11. Mesenchymal cells from an explant of fin stroma of tadpole tail. A macrophage is seen in the left upper area. Thirteen day culture. Phase contrast microscopy. 500×

Plate IV

Fig. 12. Mesenchymal cells from tail muscle. Thirteen day culture. Phase contrast microscopy. 400×

Fig. 13. Mesenchymal cells from muscle of tail root at the late stage of metamorphosis. Thirteen day culture. Phase contrast microscopy. 400×

Plate V

Fig. 14. Culture of tail muscle before metamorphosis. Twelve day culture. Jacobson's stain. 18×

Fig. 15. An enlarged sector of the square area in fig. 14 showing outgrown muscle fibers (m). 150×

Fig. 16. Spindle cells outgrown from tail muscle. Twelve day culture. Phase contrast microscopy. 400×

Fig. 17. An enlarged sector of the square area 1 in fig. 15 showing muscle fiber containing longitudinal fibrils. Jacobson's stain. 1080×
Plate VI

Fig. 18. An enlarged sector of the square area 2 in fig. 15 showing multinuclearity of a muscle fiber. 430 x

Fig. 19. Cell outgrowth from endomysium of a single fiber of tail muscle. Fourteen day culture. Jacobson's stain. 93 x

Fig. 20. An entire explant of dorsal muscle from a tadpole before metamorphosis shown together with surrounding zone of emigrating cells. Slender, newly differentiated muscle fibers are seen in the left bottom portion of the explant. Twelve day culture. Jacobson's stain. 15 x

Fig. 21. An enlarged sector of the square in fig. 20 showing fine muscle fibers differentiated in the explant. 93 x

Plate VII

Fig. 22. Superficial layer of an epithelial sheet from the skin of dorsal region of a tadpole. Three day culture. Phase contrast microscopy. 400 x

Fig. 23. A polygonal cell in the epithelial sheet from the skin of a dorsal region. The nucleus is surrounded by a thin halo. Three day culture. Phase contrast microscopy. 1270 x

Fig. 24. Deeper layer of the same field as in fig. 22. 400 x

Plate VIII

Fig. 25. An entire explant of leg muscle with surrounding emigrating spindle cells. Nine day culture. Jacobson's stain. 19 x

Fig. 26. An enlarged sector of the square area shown in fig. 25. 78 x

Fig. 27. Spindle cells from leg muscle at the late stage of metamorphosis. Seven day culture. Phase contrast microscopy. 420 x