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Forelimb Regeneration in Normal and Hypophysectomized Larvae of the salamander, *Hynobius retardatus*[1]

By

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(*With 1 plate*)

Regenerative processes of amphibian extremities have been studied by several investigators in relation to pituitary control. According to Schotté ('26), hypophysectomy results in an inhibition of forelimb regeneration in the larval *Salamandra* and the adult *Triturus*, although the same operation does not inhibit regeneration in the larval *Triturus*. Inhibition of forelimb regeneration by hypophysectomy was also confirmed histologically in the adult *Triturus* by Hall and Schotté ('51). Similar results have been reported in the adult *Triturus* by Richardson ('40, '45) and Wilkerson ('63).

On the other hand, pituitary control of regeneration by administration of pituitary extracts has been reported by others. Adams ('32) reported that limb regeneration in the adult *Triturus* was accelerated by injection of pituitary extract. Herrell ('34) and Puckett ('38) reported the effect of pituitary extracts on tail regeneration in frog tadpoles. They found that regeneration was retarded when the extracts were administered immediately after amputation, and that regeneration was accelerated when the extracts were administered a few days after amputation. All of these results seem to indicate that limb and tail regeneration in amphibians is hormonally controlled by the pituitary gland.

In order to analyse the underlying mechanism of regeneration in amphibians, histological studies were made of the regenerating forelimbs of normal and hypophysectomized larval salamanders, and the results are described below.

Materials and Methods

The larvae of the salamander, *Hynobius retardatus* Dunn, were used. They were reared in the laboratory from eggs collected from ponds in the vicinity of Sapporo. When the animals reached 30–45 mm in length, hypophysectomy was performed on 123 larvae. Sixty-eight larvae were used as controls.

Before the hypophysectomies were performed, the animals were anesthetized in 1.5% ethyl-urethane. Each experimental animal was fixed on a Petri dish on a base of paraffin

of about 3 mm in depth. Using a dissecting microscope, the hypophysectomies were performed with a pair of watchmaker's forceps through a small incision through the palatal epithelium and the parasphenoid bone. Injury of the larger blood vessels and the brain was carefully avoided, and more than 95% of the animals survived. Blanching in these animals indicated that the operation had been successful. Only animals showing albinism within 24 hours following the operation were used. In addition, when the experiment was completed, extirpation of the entire pituitary gland was verified histologically in all of the animals in which histological examinations were made on the regenerates.

The forelimbs of the animals were amputated with a pair of microscissors 3–5 days following the operation. In the majority of the larvae, both forelimbs were amputated through the distal humerus, but in some, one forelimb was amputated through the mid radius-ulna. Following amputation the animals were placed in groups of ten in small glasses containing tap water and maintained at room temperature (18°– 22°C). The water was changed every day and the animals were fed on large quantities of Tubifex worms.

The animals were inspected for regeneration of the forelimbs every day when the water was changed. For histological study of the early stages of regeneration, one group of larvae was fixed in Bouin's fixative at twelve hour intervals from 0 to 96 hours after amputation. Another group of larvae was fixed at three day intervals from 3 to 30 days after amputation. Regenerating limbs as well as heads were sectioned by the ordinary paraffin method. Serial, 7μ longitudinal sections were made of the regenerates and serial, 10μ sagittal sections of the heads. The sections were stained with Delafield's hematoxylin and eosin.

Results

Regeneration in normal animals: The regenerative processes are divided into the following five stages, which are based primarily upon the histological observations.

1) Wound healing stage (0–1 day after amputation). Severing a limb usually causes retraction of the soft tissues within a few minutes after amputation, thereby extruding the bony elements. Later, the wound surface, with the exception of the distal end of the amputated humerus, is gradually covered with epidermis which extends from the stump.

Immediately after amputation, there are many red corpuscles on the wound surface. Twelve hours after amputation, the severed end of the epidermis, which had been pulled slightly inward by the operative procedure, is extended toward the wound surface. At this stage, the basement membrane is not extended and lies under the stump epidermis, and there are no connective tissues on the wound surface.

Amputation produces muscle retraction and a loss of distinction in the cross striations, but no other changes. There is, however, a striking change in the staining property of the matrix of the amputated humerus, approximately 1 mm from the amputation surface (Fig. 1). In the more proximal part to this altered portion, the matrix is deeply stained with hematoxylin and the chondrocytes have spherical nuclei with clearly stained chromatins as is normal. In the more distal portion, the matrix is faintly stained with hematoxylin and the nuclei of the

chondrocytes are small and pycnotic. When the amputation is made at the level of mid radius-ulna, however, this change in the staining property of the cartilaginous matrix is not observed. In the later stages of wound healing, a group of cells, primarily pycnotic chondrocytes, accumulates on the distal border of the deeply stained portion of the humerus.

2) Dedifferentiation stage (1–3 days after amputation). The distal end of the amputated humerus, which is extruded from the wound surface early in this stage, is gradually covered with epidermis and is completely covered on the third day after amputation. There are no other detectable external changes in this stage.

The most characteristic changes in this stage are histological, i.e. dedifferentiation of tissue cells. In the cartilaginous skeleton, cells with enlarged nuclei are detached from the perichondrium (Fig. 2). The cells which accumulated in the stump humerus, flow out of the cut end, so that for about 1 mm proximal to the amputation level, the humerus loses almost all of its cells, with the exception of the thin, outer perichondrium. When amputation is made at a level distal to the humerus, however, the cells do not accumulate and do not flow out. In addition, there are several large multinucleated giant cells, near the cut end of the humerus. The nuclei of these cells are deeply stained with hematoxylin. These cells appear to be similar to the osteoclasts described by Hay and Fischman ('61). Dedifferentiation cannot be as clearly observed in the muscles which are disorganized in the distal portions, with indistinct cross striations, and liberated cells.

The wound epidermis, covering the wound surface, gradually increases in thickness until it reaches its maximum at the end of this stage and forms the "apical epidermal cap". The ordinary epidermis has 2–3 cell layers, but this cap contains 6–7 layers although it has fewer Leydig cells which are 2–3 times larger than other epidermal cells. This epidermal cap appears only slightly thicker than the stump epidermis, probably because of the smaller number of these cells. The inner layers of the cap have no Leydig cells at all. At the top of the cap, the inner layer contains three cell layers, but the number of layers decreases gradually toward the stump, and only a single layer is found in the proximal region of the cap. As in the preceding stage, there is no basement membrane under the apical epidermal cap.

Later in this stage, the blastema cells which result from dedifferentiation are first observed in the area slightly proximal to the wound surface.

3) Blastema stage (3–9 days after amputation). There are gradual external changes in the wound surface which was flat in the preceding stage and now gradually forms a mound. In the middle of this stage it forms the conical shape of a "conical blastema".

The blastema cells accumulate between the apical epidermal cap and the cut end of the skeleton to form a typical blastema (Fig. 3). Many blood capillaries are distributed among these cells. Each blastema cell is a kind of fibroblast. Its

cytoplasm is basophilic and its oval-shaped nucleus has distinct basophilic nucleoli (Fig. 4). The apical epidermal cap decreases slightly in thickness, and is composed of 5–6 layers of epidermal cells. There is no basement membrane in this area, and no differentiated tissues, with the exception of the remaining perichondrium.

4) Redifferentiation stage (9–15 days after amputation). In this stage, the regenerate continues its growth, and its distal end becomes pointed and soon flattened. In the middle of this stage the so-called "paddle-shaped regenerate" develops.

Histologically, this stage is characterized by the beginning of the differentiation of the new tissues. The cartilaginous skeleton and the muscles differentiate successively. With the progress of the differentiation, the blastema cells decrease in number. Early in this stage, condensation of the blastema cells begins at the cut end of the skeleton and extends in a distal direction. These cells have elongated nuclei and are arranged parallel to the cut surface of the skeleton. The intercellular spaces of this condensation are soon filled with matrix. The condensation of these cells, and of the matrix as a whole, form a "procartilaginous mass" (Fig. 5). With the progress of the differentiation, perichondrium appears around this mass. Surrounded by this perichondrium the new humerus and radius-ulna differentiate progressively from the mass, and later, the four cartilaginous digits differentiate. As for the differentiation of the muscles, blastema cells first accumulate on the sides of the stump humerus. These accumulated cells are elongated and are arranged parallel to the humerus. The cytoplasm of these cells gradually loses its affinity to hematoxylin and cross striations begin to be apparent.

The apical epidermal cap decreases in thickness and forms 2–3 layers of the epidermis. Later, the epidermis formed from this cap appears thinner, probably because of the fewer number of Leydig cells in these layers. The basement membrane is now observed under the regenerated epidermis. The membrane is thicker and more definite under the stump epidermis than under the regenerated epidermis. There are more blood vessels than in the preceding stage.

5) Digitiform stage (18–days after amputation). This stage is characterized by differentiation of the digits which is detectable by external observation. The first, second and third digits differentiate externally, early in this stage. The second digit is longer than the others. The fourth digit appears a few days later than the other three. Occasionally, the tip of the second digit is divided into two parts prior to the appearance of the fourth digit, but is soon fused.

Histologically, there are no significant changes in this stage. The only difference from the preceding stage is that the epidermis of the distal part of the regenerate becomes thickened in three areas which correspond to the interdigital regions. This thickened, interdigital epidermis later splits into four separate digits covered by epidermis of equal thickness. The basement membrane is similar to that of the preceding stage and is found under the entire epidermis.

There are a few blastema cells in the regenerate of this stage.

The histology of the regenerate suggests that regeneration of the limbs is almost completed at the beginning of this stage, *i.e.* at 18th day after amputation. On the 24th day after amputation, the regenerating limb has already completed differentiation and has formed four digits. The present investigation failed to disclose any histological differentiation other than growth, therefore, it seems safe to say that, from a histological view point, regeneration of the forelimb is completed within 24 days. Even in these fully differentiated limbs, the cartilaginous skeletons of the regenerate and the stump are distinguishable. As is clearly shown in Fig. 6, the perichondrium of the old humerus is embedded in the newly formed matrix of cartilage in the distal portion of the stump.

Regeneration in hypophysectomized animals: All of the histological observations were made on animals in which complete absence of the pituitary gland was verified histologically.

As previously described, the body of the hypophysectomized animal, with the exception of the tail fin, blanches white because of the contraction of melanophores resulting from the lack of MSH. In the control (non-hypophysectomized) animals, the tail fin and the external gills begin to degenerate at about the 15th day after amputation, indicating the beginning of metamorphosis, and, at the same time, the multicellular skin glands differentiate from the epidermis.

All of the hypophysectomized animals regenerate forelimbs bearing the normal four digits. No difference is observed in the manner or rate of regeneration in the normal and the hypophysectomized larvae. Figures 7 and 8 show complete forelimb regeneration in animals in which complete removal of the pituitary gland was histologically verified. It is concluded, therefore, that hypophysectomy in the *Hynobius* larvae has no effect on the rate or the processes of forelimb regeneration.

Discussion

In this paper, regenerative processes were divided into five stages. The first two stages are distinguishable only histologically, but the remaining three successive stages can be classified both by the external and the histological characteristics. The regenerative processes described are similar to those described in the larval *Ambystoma* (Thornton, '38) and the adult *Triturus* (Manner, '53; Schmidt, '58a, b).

Manner ('53) reported that, in the wound healing stage, the wound surface was covered with both epidermis and basement membrane, 24 hours after amputation, whereas Schmidt ('58b) stated that, 24 hours after amputation the wound surface was covered only by epidermis. The present observations support his description, that the basement membrane is not observed under the wound epidermis until differentiation of the new tissues begins.

Some earlier investigators considered that dedifferentiation is an active

phase.   Observing digit regeneration in larval salamander in the fresh state, Hay ('62) reported that the cartilaginous cells were enlarged at the time of dedifferentiation.   In forelimb regeneration in the adult newt, Manner ('53) and Chalkley ('54, '59) found a peak in the mitotic frequency in the dedifferentiation stage.   Hay and Fischman ('61) presented evidence to show that, in the same material, DNA synthesis proceeds most actively in the dedifferentiating tissues, suggesting that dedifferentiation is an essential and active process in the early phase of regeneration, and that without it, normal regeneration cannot ensue.

A remarkable change in staining property was observed in the matrix of the humerus at the wound healing stage.   Where the change occurred the cells of the humerus had rather small and pycnotic nuclei, and could not be regarded as active cells.   In addition, when amputation was performed at the level of mid radius-ulna, normal regeneration ensued without this change in staining property.   Thus, the change in the staining property of the cartilaginous matrix in the humerus may well be regarded as a degenerative, rather than a dedifferentiating process.

The effects of hypophysectomy on limb regeneration have been investigated in several urodelan species.   The results of experiments with larval *Salamandra* (Schotté, '26) and adult *Triturus* (Schotté, '26; Richardson, '40, '45; Hall and Schotté, '51; Wilkerson, '63) agree in showing that limb regeneration was effectively inhibited by hypophysectomy.   In contrast to these experiments, Schotté ('26, '61) reported that, in the larval *Triturus* and *Ambystoma*, hypophysectomy had no inhibitory effect on limb regeneration, although the rate of regeneration was slightly retarded.   This has also been reported in pectoral fin regeneration of the killifish, *Fundulus* (Liversage, '63).   In *Hynobius* larvae, hypophysectomy neither inhibited nor retarded the rate of regeneration.   It is concluded, therefore, that forelimb regeneration in the larval *Hynobius* is completely independent of pituitary control.

Summary

1.   Using the larval salamander, *Hynobius retardatus*, the processes of forelimb regeneration were studied in both normal and hypophysectomized animals.

2.   The regenerative processes could be divided into five stages: i) wound healing stage, ii) dedifferentiation stage, iii) blastema stage, iv) redifferentiation stage and v) digitiform stage.   Description based upon both external and histological observations was made for each of these five stages.

3.   At the wound healing stage, a remarkable change in staining property occurred in a wide area of the matrix of the amputated humerus.   This change occurred only when amputation was performed through the humerus.

4.   Differentiation took place first in the cartilaginous skeleton and later in the muscles.   The manner of their differentiation was described in detail.

5.   Hypophysectomy had no effect upon limb regeneration in the *Hynobius* larvae.

6.   Results obtained in this study were discussed in relation to those of the

other workers concerned with the effects of hypophysectomy on regeneration in some urodeles.

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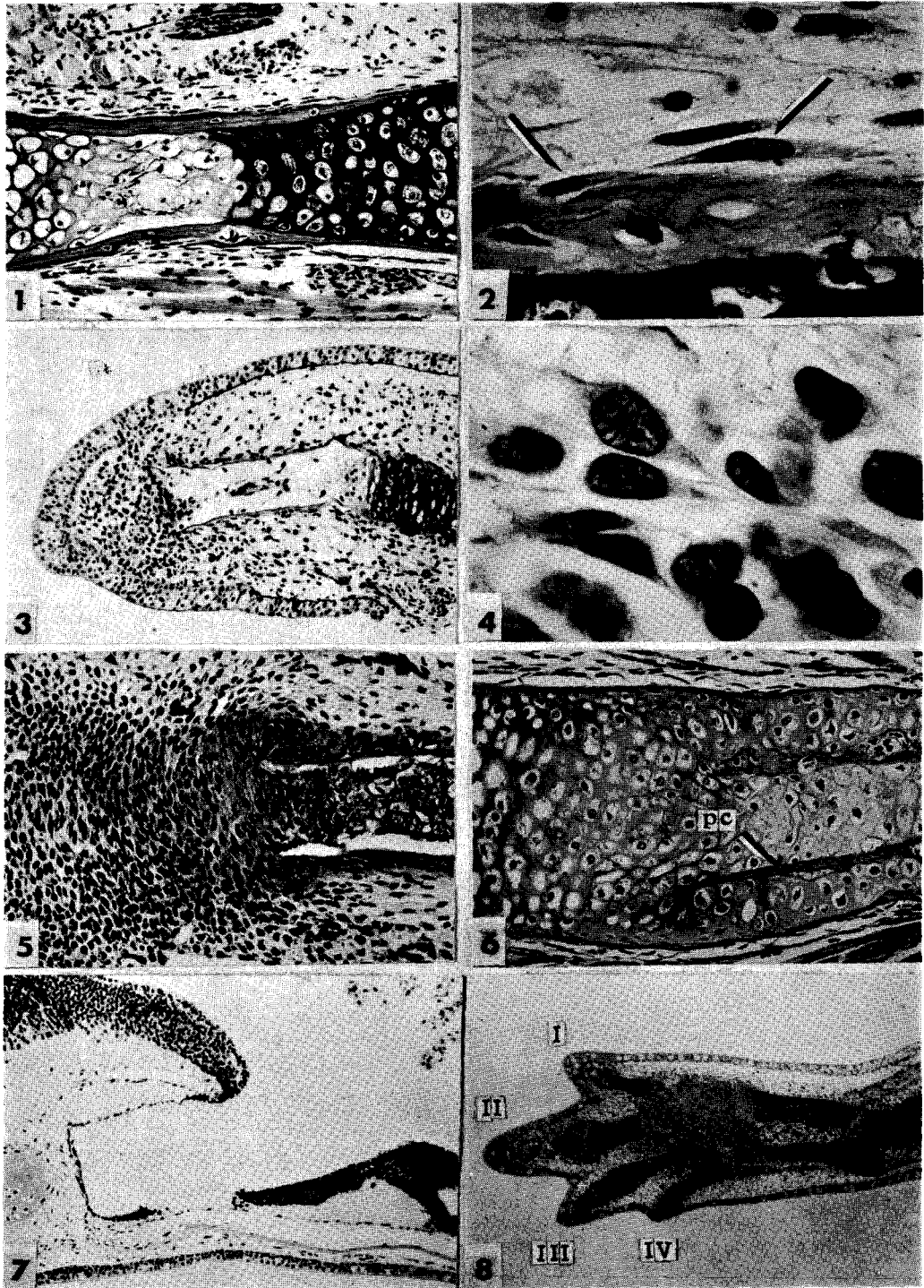
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Explanation of Plate XII

All of the figures are photomicrographs of specimens fixed in Bouin's fixative and stained with Delafield's hematoxylin and eosin.

Fig. 1. Section through the amputated humerus of a non-hypophysectomized animal, showing the change in staining property in the matrix. Matrix in the distal portion (left) decreases in affinity to hematoxylin, as compared with the proximal portion. Fixation 12 hrs. after amputation. ca. ×90

Fig. 2. Section through the limb in a non-hypophysectomized animal fixed 24 hrs. after amputation, showing initiation of dedifferentiation in humerus. Note cells (arrows) leaving perichondrium of the humerus. ca. × 475.

Fig. 3. Limb of non-hypophysectomized animal in blastema stage, showing a typical blastema. Fixation 6 days after amputation. ca. × 55.

Fig. 4. Higher magnification of the limb shown in Fig. 3, showing blastema cells. Note the distinct nucleoli. ca. × 900.

Fig. 5. Portion of limb in differentiation stage in non-hypophysectomized animal, showing differentiation of procartilaginous mass. Fixation 9 days after amputation. ca. × 90.

Fig. 6. A part of the original amputation level of regenerated limb at digitiform stage, showing old perichondrium (pc) of humerus embedded in newly differentiated matrix. ca. × 90.

Fig. 7. Median sagittal section of the head of hypophysectomized animal showing complete absence of pituitary fragment. Fixation 24 days after amputation. ca. × 55.

Fig. 8. Completely differentiated limb of hypophysectomzied animal shown in Fig. 7. Fixation 24 days after amputation. I, II, III and IV indicate the first, second, third and fourth digits, respectively. ca. × 20.