The effect of hydrocortisone on limb regeneration in the bullfrog tadpole, *Rana catesbeiana*

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**SUMMARY**

Tadpoles of the bullfrog *Rana catesbeiana* lose the ability to regenerate limbs amputated through the femur at a very early stage in development. Well-differentiated limbs which in other species can regenerate if amputated through the ankle, cannot in this species. Non-regenerating tadpole limbs either show rapid healing of the cut surface by connective tissue with no breakdown of skeletal structures, or the beginning of dedifferentiation in the cartilage. However, the cells of the rudimentary blastema that forms do not undergo proliferation; instead they become separated from the epidermis by the regrowth of the connective tissue layer which acts as a block to regeneration. This condition, termed the critical stage, is reached more often by animals that received injections of hydrocortisone than by control animals. Experimental animals furthermore, produced larger blastemata. No advanced regeneration could be obtained however.

**INTRODUCTION**

Anuran amphibians are able to regenerate limbs in the early stages but generally lose this ability during metamorphosis. Forsyth (1946) found that limbs of *R. sylvatica* amputated through the femur lost their regenerative capacity after the knee joint became recognizable. The loss of regenerative ability was gradual, and three states of regenerative capacity could be distinguished:

1. Complete regeneration – seen in early stage limbs.
2. A stage in which an ‘accumulation blastema’ forms as a result of de-differentiative activity, but which shows little cell proliferation and never undergoes differentiation and morphogenesis. This was termed the ‘critical stage’.
3. Limbs which show no blastema formation but undergo rapid epidermal and mesodermal healing involving the formation of a connective tissue pad at the wound surface.

The loss of regenerative capacity in the anuran tadpole occurs in a proximo-

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distal direction (Schotté & Harland, 1943). Animals with well-developed limbs that cannot regenerate if amputated through the femur may still be able to regenerate if amputated through the tarsals. Schotté & Harland also distinguished an intermediate stage, comparable to Forsyth’s ‘critical stage’, when their tadpoles were amputated through the tibia–fibula.

The loss of regenerative capacity is correlated with the rapid healing of the connective tissue which prevents the establishment of an intimate relationship of the epidermis and the cut mesodermal tissues of the stump (potential blastema cells); this relationship is essential for regeneration to occur (Forsyth, 1946).

The histological picture in non-regenerating anurans is similar to that seen in newts which have been hypophysectomized. Hall & Schotté (1951) describe early invasion of connective tissue under the epidermal cap with little visible dedifferentiation of skeletal structures. The situation in hypophysectomized newts may be modified by ACTH (Schotté & Chamberlain, 1955), which will enhance regeneration. It has also been shown that other anterior pituitary hormones, most notably prolactin, enhance regeneration in hypophysectomized newts (Tassava, 1969). Tassava furthermore suggested that the ACTH used by Schotté & Chamberlain (1955) had been contaminated with trace amounts of other anterior pituitary hormones which may have been responsible for the results. It has, however, been demonstrated that corticosteroids themselves can enhance regeneration in hypophysectomized newts (Schotté & Bierman, 1956). Similarly, transplantation of additional adrenal glands into post-metamorphic R. clamitans and R. pipiens will promote limb regeneration in these frogs (Schotté & Wilber, 1958).

It was hoped, therefore, that injections of hydrocortisone into tadpoles would prolong the period in which limbs could regenerate.

**MATERIALS AND METHODS**

Bullfrog tadpoles, *Rana catesbeiana*, 75–100 mm long from North Carolina, were used in this study. Animals were divided into four groups based on the external morphology of the hind limbs (see Fig. 1).

- **T (tiny)**. Early limb-bud or rudiment, no sign of knee joint, equivalent to Taylor & Kollros (1946) stage V; 5 experimental, 4 controls.
- **S (small)**. Knee joint not yet clearly recognizable, distal part of the limb is pad-like with toe rudiments beginning to appear (3–5 mm long), equivalent to Taylor & Kollros stage VIII; 14 experimental, 10 controls.
- **M (medium)**. Knee joint clearly visible, shank and thigh make an obtuse angle (6–10 mm long), equivalent to Taylor & Kollros stage X; 13 experimental, 10 controls.
- **L (large)**. Shank forming acute angle with thigh, foot well-developed (longer than 10 mm), equivalent to Taylor & Kollros stage XII; 12 experimental, 13 controls.
Limbs were amputated with iridectomy scissors under a dissecting microscope after animals were stunned by immersion in ice water. Animals in the L group were amputated through the ankle (tibiale-fibulare), all other groups were amputated through the distal part of the femur of the right limb. Experimental animals were injected three times weekly with 0.1 cm³ hydrocortisone acetate suspended in mineral oil (Light, White Blandol – Sonneborn Chemical and Refining Co., N.Y.C.). The site of injections was at the base of the tail, but was varied somewhat to avoid deleterious effects. Yale tuberculin syringes of 0.5 cm³ capacity and ½ in. (1.27 cm) 27-gauge needles were used for the injections. All animals were maintained in aquaria at 20 °C and were fed raw spinach regularly.

Hind limbs were examined periodically for signs of regeneration. Limbs were removed from the animal 3 weeks post amputation, fixed in Bouin’s fluid, sectioned longitudinally at 10 μm and stained with hematoxylin and eosin.

RESULTS

The only animals to show any visible external signs of regeneration were ones from the T group. When examined histologically, all experimental and control limbs were found to have well-formed blastemata (Fig. 2). When examined histologically, limbs of animals in the other groups were found to be in one of two possible conditions. In some, the connective tissue and basement membrane had healed rapidly over the wound surface and there was no visible dedifferentiation of the skeletal elements (Fig. 3). In others, breakdown of the cartilage matrix adjacent to the cut-tip had started and some dedifferentiated cells had accumulated, but the connective tissue layer had grown around and over the cut cartilage. This condition is analogous to Forsyth’s ‘critical stage’ and is seen in Fig. 4. Limbs were therefore classified as regenerating, critical, or non-regenerating.

In the T group, all 5 experimental and 4 control limbs were regenerating. In the S group, 10 experimental limbs were critical and 4 were non-regenerating, whereas 4 controls were critical and 5 were non-regenerating. In the M group, 8 experimental limbs were critical and 5 non-regenerating; 3 control limbs were
Figures 2-4
All limbs 3 weeks after amputation.

Fig. 2. Regenerating limb of control T-stage animal. Well-formed blastema is present distal to the cartilage. \( \times 80 \).

Fig. 3. Non-regenerating control M-stage limb. Connective tissue (c) has healed over the cut end of the cartilage with no dedifferentiation visible. \( \times 35 \).

Fig. 4. Critical limb of experimental S stage. Although considerable dedifferentiation of cartilage has occurred, connective tissue (c) has healed around it, thus blocking any further regenerative events. \( \times 80 \).
Hydrocortisone and limb regeneration

Table 1. Hydrocortisone-injected and control tadpoles at different degrees of limb development showing regenerating, non-regenerating and critical limbs

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<tr>
<th></th>
<th>Regenerating</th>
<th>Critical</th>
<th>Non-regenerating</th>
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<tbody>
<tr>
<td><strong>T experimental</strong></td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>T control</strong></td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>S experimental</strong></td>
<td>0</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>S control</strong></td>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>M experimental</strong></td>
<td>0</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td><strong>M control</strong></td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td><strong>L experimental</strong></td>
<td>0</td>
<td>3</td>
<td>8</td>
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<tr>
<td><strong>L control</strong></td>
<td>0</td>
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critical and 6 non-regenerating. In the L group (amputated through the tibiale-fibulare) 3 experimental limbs were critical and 8 non-regenerating; 3 control limbs were critical and 10 non-regenerating. These data are summarized in Table 1.

If the data are examined in terms of the percentage of limbs which at least started the dedifferentiative processes, the following percentages are obtained:

<table>
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<tr>
<th></th>
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<tbody>
<tr>
<td><strong>T experimental</strong></td>
<td>100</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td><strong>S experimental</strong></td>
<td>71</td>
<td></td>
<td>44</td>
</tr>
<tr>
<td><strong>M experimental</strong></td>
<td>61</td>
<td></td>
<td>33</td>
</tr>
<tr>
<td><strong>L experimental</strong></td>
<td>27</td>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>

Blastema size of mid-sagittal sections of S and M limbs was measured by calibrated ocular micrometer. Experimental S blastemata measured $250 \pm 40 \mu m$ (s.e.) whereas control S blastemata measured $145 \pm 22 \mu m$, a difference almost significant to the $P = 0.01$ level. Experimental M blastemata were $252 \pm 14 \mu m$ whereas controls measured $175 \pm 14 \mu m$, a difference significant to the 0.005 level.

**DISCUSSION**

From the control data one can observe that the ability of *R. catesbeiana* to regenerate a limb is confined to the T stage, and that by the S stage the limbs can, at most, form an ‘accumulation’ blastema. This is supported by control data from another study of *R. catesbeiana* limb regeneration (Weis, 1972). The loss of regenerative capacity in this species occurs earlier than in *R. sylvatica* (Forsyth, 1946), and in *R. pipiens* (Fry, 1966), which lost it at a somewhat later stage. Furthermore, the well-developed (group L) limbs in this species do not regenerate after amputation through the long tarsals although *R. clamitans* with comparable limb development can still regenerate if amputated at this level (Schotte & Harland, 1943). Other species differences have been found among ranids. Michael & Sammak (1970) have found that *R. ridibunda ridibunda* adults can
regenerate forelimbs hetermorphically. Adults of other groups of anurans are also able to produce heteromorphic regenerates (Goode, 1967).

The ability of the hind limb to dedifferentiate and form an accumulation blastema (critical stage) gradually decreases as the developmental age of the limb increases. Treatment with hydrocortisone, however, is seen to cause an increase in the percentage of limbs in the $S$ and $M$ groups which can reach the critical stage, as opposed to those which show no dedifferentiative processes. One can view the events in these limbs as a 'race' between the dedifferentiative processes and the regrowth of the connective tissue over the wound surface which then prevents regeneration. Presumably, the corticoid treatment was able to slow down the growth of connective tissue and thereby some skeletal de-differentiation took place, but subsequently the connective tissue closed over the amputation surface and thus prevented any true regeneration from taking place. Glucocorticoids are known to interfere with wound healing by diminishing cellular migration, inhibiting fibroblast formation and fibrin deposition (Cameron, 1953) and thereby could prevent the connective tissue from healing over the stump. Other methods which repress these wound-healing processes have also been effective in promoting limb regeneration in anurans. Rose (1944) treated the stump with salt solutions which prevented wound healing to the extent that some adult frogs could regenerate limbs. Polezhaiev (1945) demonstrated that repeated trauma to the limb stump was sufficient to promote regeneration in many cases.

In the present experiments hydrocortisone injections were not adequate to produce advanced regeneration, but could only increase the percentage of critical stage limbs and increase the size of the accumulation blastemata formed. The larger-size blastemata are probably due to a delay in the healing of the connective tissue, attributable to the hydrocortisone. The hormonal situation in any animal is one of delicate balance and precise timing. For best results one should have the precise quantity of hormone present at the time when the tissues are most susceptible to its action. Schotte & Lindberg (1954) have found that there is a transitory period of corticoid activity associated with regeneration in newts. The failure to achieve advanced regeneration in these experiments, therefore, may have been due to the failure to achieve the proper hormonal balance at the precise time when it was needed.

This research was supported by a grant from the Rutgers University Research Council.

REFERENCES


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(Manuscript received 16 May 1972, revised 20 July 1972)