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The Regulative Ability of the Limb Regeneration Blastema of *Notophthalmus viridescens*: Experiments *in situ*

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Summary. 1. The regulative ability of the regeneration blastema of the newt limb (*Notophthalmus viridescens*) was tested by operations *in situ*. Either the anterior, posterior, dorsal, or ventral half of the blastema was removed at various stages during regeneration.

2. All blastemas operated on prior to the stage of four early digits showed a delay in reaching the subsequent stages of regeneration.

3. The blastema is capable of extensive regulation in the anterior-posterior and dorso-ventral axes even after many of its cells have begun to differentiate.

4. Early digital stages of regeneration were found to be defective in regulative ability. Additional skeletal elements were present in limbs which had been operated on at the stage of three early digits. Supernumerary digits as well as additional skeletal elements were present in limbs which had been operated on at the stage of four early digits. Removal of the posterior half of the regenerate at one of these late stages resulted in more severe abnormalities than did removal of the anterior half.

5. Either the anterior or the posterior half of a mature limb was removed back to the level of the wrist. In several cases, an almost complete autopodium developed alongside the remaining half autopodium.

6. Removal of half of a regenerate at digital stages gave results similar to those obtained following removal of half of a mature limb.

7. The results are discussed in the context of other experiments on regenerating limbs, and of experiments on other developing systems. It is concluded that amphibian blastemas in common with a number of other systems can develop according to the presumptive fates of their cells, or they can regulate when they are given the opportunity for growth and cell division.

The regeneration blastema of an amphibian limb is derived from tissues in the adjacent stump, and is destined to give rise only to those structures which lie distal to the level of amputation. To approach an understanding of the mechanisms by which the regenerate and the stump together form a harmonious structure, the developmental capabilities of the blastema cells are being examined. In this study we have investigated the regulative ability of the blastema in response to defects made *in situ* at various stages of regeneration.

There are many similarities between regeneration blastemas and embryonic limb buds (see Faber, 1971) and it is not unexpected to find that these similarities include the ability to regulate. A number of studies have shown that the embryonic limb buds of both chicks and amphibians are capable of extensive regulation (see review by Zwilling, 1961). For example, a large portion of a chick limb bud can be removed without causing defects in the resulting limb (Wolff and Hampé, 1954; Amprino and Camosso, 1955; Hampé, 1957, 1958; Barasa, 1964; Dubey

and Patil, 1971; Hansborough, 1954)¹. Conversely, when one chick limb bud is grafted onto the distal tip of another bud, the two combine to form a normal limb (Kieny, 1959; Hampé, 1957). When a portion of the proximal mesoderm from a chick leg bud is grafted into a more distal region of a wing bud, leg structures will form which are appropriate to the new position within the appendage (Saunders *et al.*, 1957). It has even been shown that chick limb bud mesenchyme can be dissociated into cells, reaggregated, recombined with ectoderm, and still produce a relatively normal limb (Zwilling, 1964; MacCabe *et al.*, 1973). Experiments performed on amphibian limb buds have shown that they also have extensive powers of regulation. A half limb bud is capable of forming a whole limb (Harrison, 1918, 1921); a divided limb bud can form two whole limbs (Swett, 1926); and two superimposed limb buds can give rise to a single, normal limb (Harrison, 1921).

Several studies have indicated that the regeneration blastema is also capable of regulation (see Faber, 1971). Two or more blastemas, transplanted in random orientation with respect to each other to a neutral area of the body, are capable of combining to form a single regenerate (de Both 1970; Michael and Faber, 1971). A proximal half of a blastema, transplanted under conditions which encourage further outgrowth and reorganization, forms both distal and proximal limb structures (Stocum, 1968). Some early experiments by Milojevic and Vlatkovic (1926) and Swett (1928) on young blastemas split into two halves, showed that each half was capable of forming a whole limb. However, apart from these experiments little is known of the ability of blastemas *in situ* to regulate for experimentally produced defects.

Material and Methods

Male and female adult newts, *Notophthalmus (Triturus) viridescens*, were maintained in spring water at a constant temperature of 25°C and a 12 hour light cycle. They were fed two to three times per week with *Tubifex*. Chloretone (Parke, Davis and Co.) was used as an anesthetic when amputations and operations were performed.

The forearms of 115 newts were bilaterally amputated through the distal third of the humerus. Operations were performed on the left limbs of these animals at different stages of regeneration. No operations were performed on the right regenerating limbs which served as controls. The regenerating limbs were staged according to the criteria described by Iten and Bryant (1973). Operations were performed on limbs at each of the following stages of regeneration (see Table 1): early dedifferentiation (9 animals); moderate early bud (35 animals); medium bud (9 animals); late bud (25 animals); palette (12 animals), and early digits (12 at three early digits, and 13 at four early digits)².

The experimental operation consisted of removing one half of the blastema back to the original level of amputation. Either the anterior, posterior, dorsal, or ventral half of the blastema was removed. Both the experimental limb and the control limb of each animal were observed and staged three times per week until they reached the stage of late digits.

1 A paper has appeared since this article was submitted which indicates that chick limb buds may be more restricted in their ability to regulate for intercalary defects than these early experiments would suggest (Summerbell *et al.*, 1973).

2 Our descriptions (Iten and Bryant, 1973) of each stage of limb regeneration were based on observable changes in both the external appearance and the internal histological organization. The stage of early digits can be divided into two substages, three early digits and four early digits, since there is a change in the external appearance. However, the change in the internal histological organization is not significant enough, in our opinion, to warrant the establishment of two completely separate stages.

Operations were also performed on unamputated mature limbs. In 12 animals, half of the hand, either the anterior half or the posterior half, was removed proximally to the region of the wrist.

At the end of the experiment both experimental and control limbs were removed and placed in Bouin's fixative. The majority of the limbs were prepared as whole mounts by the following method: Treatment over a 2 day period with several changes of 2% ammonium hydroxide to remove the fixative; treatment over a 2 day period with 10% hydrogen peroxide to remove pigmentation; dehydration for two hours in 50% alcohol; dehydration for two hours in acid alcohol; staining for two hours in 1% Victoria Blue B (Matheson, Coleman and Bell) in acid alcohol; destaining in 70% alcohol; dehydration in 95% and 100% alcohol; clearing and storage in methyl salicylate (technique according to Pat Tonge, personal communication).

Results

Following the removal of either the anterior, posterior, dorsal, or ventral half of a blastema, the remaining portion always reorganized to occupy the entire stump area except in those cases in which the operations were performed at the stages of three early digits or four early digits. After an operation at one of the latter stages, a new regenerate arose from the exposed distal surface of the stump and became partially integrated with the remaining half blastema. All blastemas which were operated on prior to the stage of four early digits, were delayed in reaching the next stage of regeneration (Figs. 1, 2, 3) when compared to controls, but thereafter regenerated at a normal rate. Furthermore, the extent of this delay was correlated with the stage of the regenerate at the time of operation. When operations were performed at the stage of moderate early bud, the blastemas were delayed by an average of 2.2 days in reaching each of the subsequent stages; when the operations were performed at late bud, they were delayed by 7.3 days; and when the operations were performed at three early digits, they were delayed by 10.2 days. However, when blastemas were manipulated at four early digits, the remaining halves reached maturity at the normal time.

When the regenerating limbs had developed to the stage of late digits, they were examined externally, and then fixed and prepared as whole mounts. A number of the regenerates developed three instead of the usual four digits. However, since the frequency of occurrence of this abnormality was similar in control (15%) and experimental (10%) regenerates, we have not considered it in our analysis of the results. All 115 control limbs formed regenerates of normal external appearance. Two of 85 skeletal preparations of control regenerates possessed supernumerary skeletal elements.

Of the 90 limbs operated on prior to the stage of three early digits, all but one of the regenerates were of normal appearance and size; of 71 skeletal preparations examined all but one had normal skeletons (Fig. 4). The one exception showed a supernumerary digit. None of the limbs operated on at three early digits showed any external evidence of abnormality, but they did show certain skeletal abnormalities. Limbs in which the anterior half of the blastema had been removed at this stage showed in three of the six cases abnormal arrangements of the carpals (see Fig. 5a). Limbs in which the posterior half of the blastema had been removed at this stage showed in one case an extra distal portion of the radius; in another case an extra carpal element; and in two cases, abnormally arranged carpal elements (see Fig. 5d).

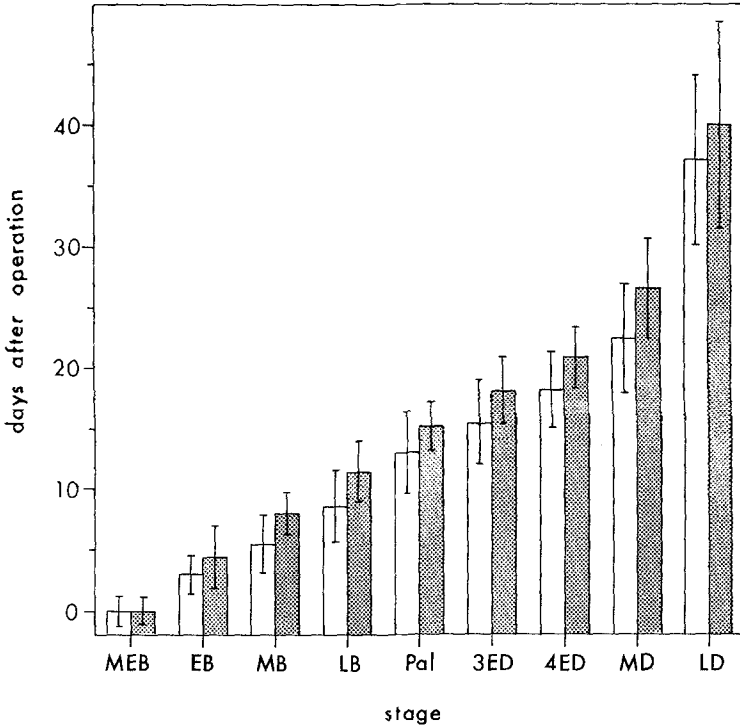


Fig. 1. Comparison of the time taken to reach each stage of regeneration by blastemas operated on at moderate early bud with the time taken by control, unoperated blastemas. Average difference = 2.2 days. Stippled bars represent experimental limbs; clear bars represent control limbs. Two standard deviations about the mean are shown for each bar. Stage abbreviations: *MEB* moderate early bud, *EB* early bud, *MB* medium bud, *LB* late bud, *Pal* Palette, *3 ED* three early digits, *4 ED* four early digits, *MD* medium digits, *LD* late digits

Operations at four early digits gave rise to much more frequent and more severe abnormalities. In the six cases where the anterior half of the regenerate was removed at this stage, no supernumerary digits were formed (Table 1). However, one limb was found to possess a small supernumerary ulna, one was found to have an extra carpal element, one showed abnormally arranged digits, and one showed abnormally arranged radius, ulna, and carpals (see Table 2 and Fig. 5b). When the posterior half of the regenerate was removed at this stage, all seven regenerates produced supernumerary digits (Table 1). The skeletons of all these limbs were found to possess additional phalanges; five were found to have extra carpals; and one was found to have a second ulna and an abnormal radius (Table 2). An extreme example is illustrated in Fig. 5e.

In 12 animals, half of a mature forelimb was removed back to the level of the wrist. These limbs were examined when no further development was apparent. Of the six cases where the anterior half of the autopodium had been removed, two regenerated three new digits, bringing the total number of digits to five; one regenerated two digits; two, one digit; and the last, no digits (Table 1). All of

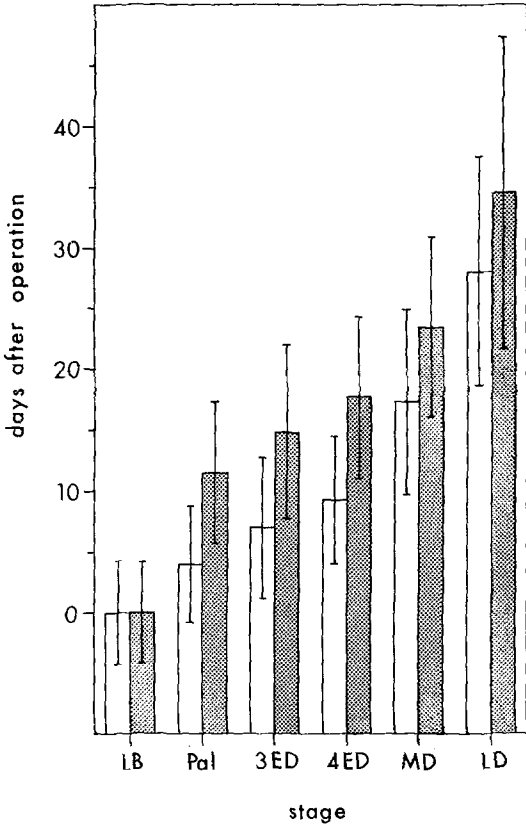


Fig. 2

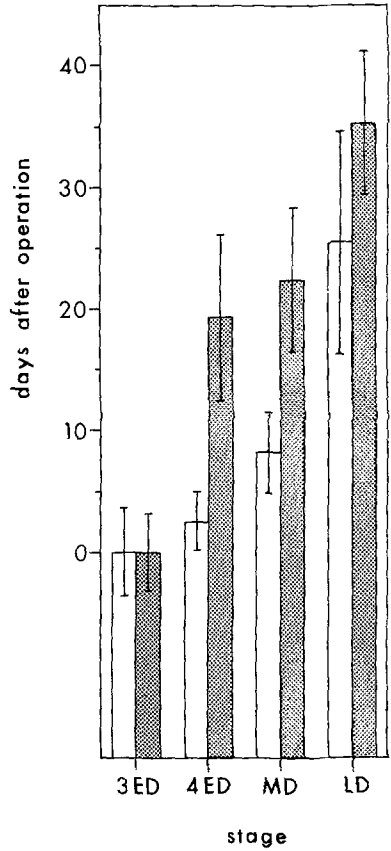


Fig. 3

Fig. 2. Comparison of the times taken to reach each stage of regeneration by blastemas operated on at late bud with the time taken by control, unoperated blastemas. Average difference = 7.3 days. Stippled bars represent experimental limbs; clear bars represent control limbs. Two standard deviations about the mean are shown for each bar. Abbreviations as in Fig. 1

Fig. 3. Comparison of the times taken to reach each stage of regeneration by blastemas operated on at three early digits with the time taken by control, unoperated blastemas. Average difference = 10.2 days. Stippled bars represent experimental limbs; clear bars represent control limbs. Two standard deviations about the mean are shown for each bar. Abbreviations as in Fig. 1

the skeletal elements of these limbs were found to be abnormal (Table 2). In four of the six cases, supernumerary carpal elements were found to be present; in one other case, extra phalanges but no extra carpals were found. In the one remaining limb, the arrangement of the carpal elements was abnormal, although no extra structures were present. In the six cases where the posterior half of the autopodium was removed, three limbs regenerated four new digits, bringing the total to six; one regenerated three new digits; one, two new digits; and the last, one new digit (Table 1). Three limbs had regenerated supernumerary carpal

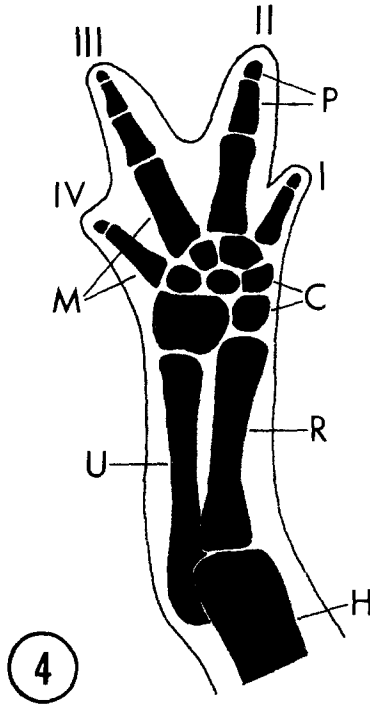


Fig. 4. Camera lucida drawing of a whole mount of a normal regenerated left limb stained with Victoria Blue B. Roman numerals indicate the digits. Abbreviations: *P* phalanges, *M* metacarpals, *C* carpals, *R* radius, *U* ulna, *H* humerus

elements, and one limb regenerated supernumerary phalanges but no additional carpal elements. The two remaining limbs showed abnormally arranged parts but no supernumerary structures (Table 2). In a total of nine out of the 12 limbs in which either the anterior or the posterior half of the autopodium had been removed, individual carpal elements which were damaged by the operation had regenerated (see Fig. 5c and f).

Discussion

The results presented in this paper show that the regeneration blastema of the amphibian limb is capable of extensive lateral regulation *in situ*, where the opportunities for growth and reorganization are maximal. This regulative ability persists beyond the stage at which some of the cells begin to differentiate (Iten and Bryant, 1973). Irrespective of which half of the blastema is removed, a complete limb is produced, and never a defective or double limb. Thus, the blastema possesses the same ability to regulate for laterally located defects as the limb buds of embryonic amphibians (Harrison, 1918, 1921) and chicks (Amprino and Camosso, 1955; Barasa, 1964; Dubey and Patil, 1971; Hansborough, 1954). In contrast to the complete regulative ability of the blastema in the dorso-ventral and anterior-posterior axes, there is some evidence which suggests that there

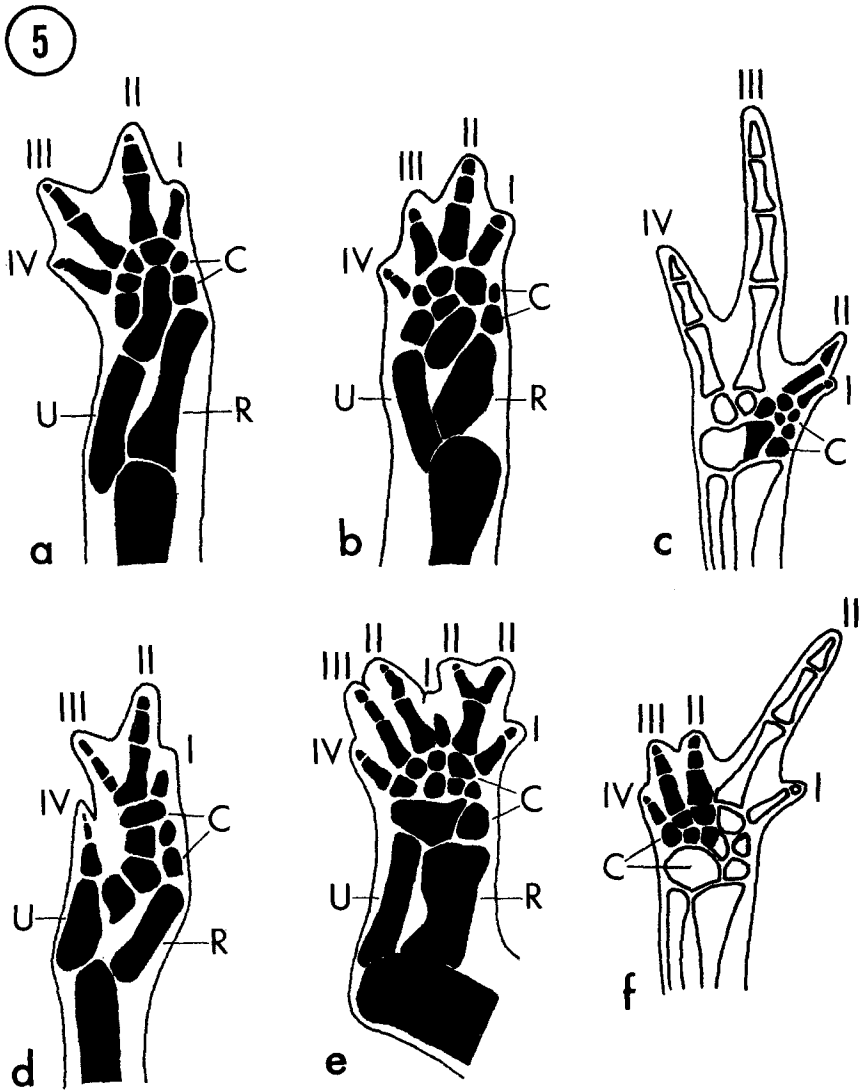


Fig. 5a—f. Camera lucida drawings of whole mounts of experimental left limbs stained with Victoria Blue B. a) Anterior half of the blastema removed at the stage of three early digits. Carpals are abnormal in appearance and abnormally arranged. b) Anterior half of the blastema removed at the stage of four early digits. Carpals are abnormal in number and arrangement; ulna and radius are abnormal in arrangement. c) Anterior half of the hand removed from a mature limb. Supernumerary carpal elements are present, and a damaged carpal has regenerated. Blackened structures are regenerated elements. d) Posterior half of the blastema removed at the stage of three early digits. Carpals are abnormal in appearance and arrangement. Ulna and radius are abnormal in arrangement. e) Posterior half of the blastema removed at the stage of four early digits. Supernumerary digits and carpals have formed. f) Posterior half of the hand removed from a mature limb. A supernumerary digit and supernumerary carpals have formed, and a damaged carpal has regenerated. Blackened structures are regenerated elements. Abbreviations as in Fig. 4

Table 1. External appearance of regenerated limbs

Stage at operation	Half of blastema removed	Total no. of limbs	No. normal limbs	No. limbs with supernumerary digits
Early dedifferentiation	Anterior	4	4	0
	Posterior	5	5	0
Moderate early bud	Anterior	9	9	0
	Posterior	11	11	0
	Dorsal	9	9	0
	Ventral	6	6	0
Medium bud	Anterior	4	4	0
	Posterior	5	5	0
Late bud	Anterior	7	7	0
	Posterior	6	6	0
	Dorsal	6	5	1
	Ventral	6	6	0
Palette	Anterior	4	4	0
	Posterior	8	8	0
Three early digits	Anterior	6	6	0
	Posterior	6	6	0
Four early digits	Anterior	6	6	0
	Posterior	7	0	7
Mature limb	Anterior	6	4	2
	Posterior	6	2	4
Control regenerates	—	115	115	0

may be certain limitations on this ability in the proximo-distal axis. Proximal halves of blastemas are capable of forming appropriate distal, as well as proximal, limb structures (Stocum, 1968) but distal halves only produce distal structures (Faber, 1965; Stocum, 1968). However, de Both (1970) claims to have shown some regulation in a proximal direction. He transplanted several blastemas to a neutral territory and describes the formation of limb structures appropriate to a more proximal level than the original level of amputation. Other evidence indicates that a blastema is normally not capable of forming more proximal structures. It is possible to reverse the proximo-distal axis of a newt limb (Dent, 1954; Butler, 1955) and to observe regeneration from an originally proximal-facing surface. In such experiments, a blastema forms but does not give rise to any more proximal limb structures; instead it produces a typical distal regenerate. This result indicates that a blastema is normally only able to develop structures which are distal to the level of amputation, and it is part of the evidence for the "rule of distal transformation" (Rose, 1962). Similar phenomena have recently been found in the imaginal discs of *Drosophila* (Bryant, 1971; Schubiger, 1971), and in this system the rule can be applied equally well to axes other than the proximo-distal axis (Bryant, 1971, 1973).

Despite a considerable ability to regulate throughout most of its development, the blastema eventually reaches a stage where this ability becomes impaired.

Table 2. Skeletal analysis of regenerated limbs

Stage at operation	Half of blastema removed	Total no. of limbs examined	No. normal limbs	No. abnormal limbs		% abnormal limbs
				Limbs with extra skeletal elements	Limbs with abnormally arranged skeletal elements	
Early dedifferentiation	Anterior	4	4	0	0	0
	Posterior	5	5	0	0	
Moderate early bud	Anterior	9	9	0	0	0
	Posterior	6	6	0	0	
	Dorsal	7	7	0	0	
	Ventral	6	6	0	0	
Medium bud	Anterior	4	4	0	0	0
	Posterior	5	5	0	0	
Late bud	Anterior	5	5	0	0	5
	Posterior	6	6	0	0	
	Dorsal	6	5	1	0	
	Ventral	3	3	0	0	
Palette	Anterior	3	3	0	0	0
	Posterior	2	2	0	0	
Three early digits	Anterior	6	3	0	3	58
	Posterior	6	2	2	2	
Four early digits	Anterior	6	2	2	2	85
	Posterior	7	0	7	0	
Mature limb	Anterior	6	0	6	0	100
	Posterior	6	0	4	2	
Control regenerates	—	85	83	2	0	2

When either posterior or anterior halves of blastemas were removed at three early digits, the resulting limbs, although apparently normal from the outside, were found to have an abnormal skeletal organization. The abnormalities ranged from atypical arrangement of the carpals and digits to extra skeletal elements. The operations performed on regenerates at four early digits resulted in much more severe abnormalities. In most cases additional carpal elements were produced, and in some cases, additional digits. At either three early digits or four early digits, removal of the posterior half of the limb resulted in more abnormalities than did removal of the anterior half of the limb. This result has a parallel in work on embryonic chick limb buds where Dubey and Patil (1971) have found that regulation following extirpations in the anterior (pre-axial) portion of limb bud is better than that which follows extirpations in the posterior (post-axial) part of the limb.

Although the limbs operated on at three and four early digits were somewhat variable in final appearance, examination of the most abnormal cases suggests the

following general explanation: After removal of one half of the blastema, the remaining half proceeds to develop autonomously, giving rise to a half limb. A second regenerate develops alongside the half limb, and independently produces an almost complete limb. Thus the final product in the most extreme cases is essentially one and a half limbs. This interpretation is consistent with the results obtained from those cases in which portions of mature limbs were removed. In many of these cases, an almost complete second hand developed alongside the remaining two digits. The findings of Weiss (1925, 1926) and Goss (1957) that a complete regenerate can form from half a limb stump, support this interpretation of our results. The change in the behavior of the blastema from regulative to nonregulative indicates a change in the ability of the cells involved to assess their relationship to other cells of the developing system and to respond appropriately. Both the influence of the stump tissues on the blastema, as well as cell contact and communication within the blastema will probably prove central to this change in behavior.

In our experiments, and in other experiments where the blastema has been shown to possess regulative ability (Stocum, 1968; de Both, 1970) the operations have been performed in a way which maximizes growth and reorganization. But when parts of the blastema are manipulated in a way which minimizes growth (Stocum, 1968; Stocum and Dearlove, 1972; Michael and Faber, 1971) they develop strictly according to their presumptive fates and show no regulation. The extensive regulative ability of the blastema following lateral defects *in situ*, as shown in our experiments, contrasts to the results of Stocum (1968) following the transplantation of anterior or posterior half blastemas to a neutral territory. In the latter experiments, the transplants were made in such a way as to minimize further growth, and the half blastemas developed according to their presumptive fates. The ability of transplanted lateral half blastemas to develop under conditions which maximize further growth has not been tested. It is clear that growth and cell division are essential for blastema regulation. Many other developing systems show the same duality of behavior: When there is an opportunity for growth and cell division, regulation can occur, but in the absence of such an opportunity development proceeds according to the presumptive fate of the tissue. This behavior is characteristic of amphibian limb buds (Tschumi, 1957), chick limb buds (Zwilling, 1961), chick somites (Keiny *et al.*, 1972), *Drosophila* imaginal discs (Bryant, 1973), and the insect integument (Lawrence, 1972).

The relationship between cell division and regulative ability as well as the role of the epidermis are presently being investigated in amphibian blastemas. In addition, an analysis is being made of the extent to which regulation can occur in blastemas which have been disorganized but in which the total mass remains the same.

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