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## The Interaction between the Blastema and Stump in the Establishment of the Anterior-Posterior and Proximal-Distal Organization of the Limb Regenerate

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Interactions between the limb stump and the developing regenerate were studied in the limbs of adult newts, *Notophthalmus viridescens*. Forelimb blastemas at various stages were transplanted to the contralateral forelimb such that the anterior-posterior axes of stump and blastema were opposed. The blastemas were transplanted either from a proximal to distal, distal to proximal, proximal to proximal, or distal to distal level limb stump. The results indicate that at the earliest stage studied the anterior-posterior axis of the blastema is established but is not stable. An interaction between the stump and blastema at this early stage results in the production of a variety of limbs intermediate in polarity between the graft and the stump. At all later stages, the original anterior-posterior axis of the blastema can be retained, although under certain grafting conditions the stump can still exert considerable influence over the anterior-posterior organization of the final regenerate. In those circumstances in which the blastema retains its original handedness, the interaction between stump and blastema results in the production of separate anterior and posterior supernumerary regenerates.

The results of transplanting proximal blastemas to a distal limb level indicate that the proximal boundary of the blastema has been established by the earliest stage studied, leading to the production of limbs with serially duplicated segments. However, irrespective of the stage of a blastema transplanted from a distal to proximal level, there are no deleted structures in the proximal-distal axis of the resulting limb. From both histological examination of transplanted regenerates and the arrangement of skeletal elements of the resulting limbs, it is postulated that the stump plays an important role in the production of the intercalary regenerate.

### INTRODUCTION

Previous experiments have shown that a single early-stage limb blastema transplanted to a nonlimb ("indifferent") region of an animal is capable of forming almost all structures it would have formed if it had remained on the limb stump (Stocum, 1968; de Both, 1970; Stocum and Dearlove, 1972; Faber, 1971). This capacity of the blastema to undergo autonomous development has been termed selfdifferentiation and is similar to the capacity for autonomous development of transplanted early embryonic amphibian (Harrison, 1918; Detwiler, 1929) or chick (Rudnick, 1945) limb buds. Results such as these indicate that the limb blastema, at least at the stages examined, is a self-contained system,

in the sense that it can develop normally without contact with the stump from which it was derived. However, they leave unanswered the question of the extent to which the blastema and stump interact when they are adjacent to each other, as in the normal condition *in situ*. The experiments described in this paper were designed to investigate the interaction between the stump and the blastema in order to understand the factors involved in ensuring that the regenerate and stump together form a harmonious structure. The two major developmental events investigated were the establishment within the blastema of the appropriate elements in the proximal-distal and anterior-posterior axes.

Little is known about how the anterior-posterior axis of the limb regenerate is established. Lodyženskaja (1928, 1930) concluded from transplantation studies that both the anterior-posterior and the dorsal-ventral axes of the regenerate are fixed at the time of initial blastema formation, whereas Milojević (1924) and Schwidefsky (1935), from similar studies, concluded that these axes become fixed only after a certain stage in regeneration. More recently, Droin (1959), Rahmani (1960), Settles (1967), Lheureux (1972), and Carlson (1974) have shown that the axial polarity of the adjacent stump tissues influences the axial polarity of the regenerate. Carlson (personal communication) has further dissected the role played by various stump tissues and has shown that it is the dermis and muscle of the limb stump, rather than the epidermis or skeleton which influence the axial polarity of the regenerate. More is known about the establishment and control of axial polarity in developing limb buds than in regenerating amphibian limbs. The extensive work of Harrison (1917, 1921) and the later work of Takaya (1941) showed that the anterior-posterior and dorsal-ventral axes of amphibian limb buds are established sequentially, the anterior-posterior being the first. A similar sequence of axis establishment has been described for chick limb buds by Chaube (1959). In the chick, a region of the posterior limb mesenchyme, known as the "zone of polarizing activity" can influence the anterior-posterior axis after its initial establishment (Saunders and Gasseling, 1968; MacCabe *et al.*, 1973) and the limb ectoderm can similarly influence the dorsal-ventral polarity (Pautou and Kieny, 1973; MacCabe *et al.*, 1973, 1974). In the transplantation studies reported here we have investigated the interaction between the regeneration blastema and its adjacent limb stump in the establishment of the anterior-posterior axis of the regenerate.

During normal amphibian limb regeneration new structures differentiate in a proximal-distal sequence, and the structures that are formed are exactly those necessary to complete the appendage. Although the mechanism by which the proximal-distal sequence of structures becomes specified within a growing blastema is unknown, Stocum and Dearlove (1972) have shown that young blastemas transplanted to a nonlimb ("indifferent") region of the body under conditions which prevent further distal outgrowth form only proximal limb elements. They have further shown that the more advanced the blastema is at the time of transplantation, the more distally complete is the resulting regenerate. These results are comparable to those obtained in developing amphibian limbs (Tschumi, 1957) and chick limbs (Saunders, 1948; Saunders *et al.*, 1957) and show that, during vertebrate limb morphogenesis, the capacity for differentiation of limb structures is acquired in a proximal-distal sequence over time. However, a major difference exists between developing and regenerating limbs. In the former, the most proximal structures to be laid down are always the same, whereas, in regenerating limbs, the most proximal structures to be laid down will vary depending on the level of amputation. In the experiments reported here we have studied the degree to which the proximal-distal level of the stump influences the proximal-distal organization of structures formed by the blastema.

#### MATERIALS 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 hr light cycle. They were fed two or three times per week with either *Tubifex* or *Artemia*. Chloretone (Parke, Davis and Co.) was used as an anesthetic when amputations and operations were performed.

A total of 411 newts were used in these experiments and, of these, 116 died before the limbs were mature enough to be analyzed. Forelimbs were amputated at either a proximal level (through the proximal half of the humerus) or a distal level (through the distal half of the radius and ulna). The tissues at the amputation site were trimmed to give a flat wound surface and to provide a clean line between the stump and the ensuing regenerate. The developing regenerates were staged according to the criteria described by Iten and Bryant (1973). Transplantations were performed at the stages of early bud, medium bud, late bud, and early digits, and all transplants consisted of mesodermal and epidermal tissues. Each operated animal was placed on its side on damp cotton so that the recipient stump did not come into contact with either the cotton or the flank of the animal. After transplantation, all animals were kept at 10°C for 24 hr where they remained anesthetized and immobile during the critical phase of wound healing. At all other times, the animals were kept at 25°C. All transplants were made between the forelimbs of the same animals in order to avoid any possible immune rejection of the blastema (Cohen, 1971). The following four types of transplant operations were performed (see Fig. 1):

*Proximal to distal transplants.* The distal (right) limb blastema was removed and discarded. The proximal (left) limb blastema was removed and transplanted to the distal (right) stump.

*Distal to proximal transplants.* The proximal (left) limb blastema was removed and discarded. The distal (right) limb blastema was removed and transplanted to the proximal (left) stump.

*Proximal to proximal transplants.* The proximal (right) limb blastema was removed and discarded. The proximal (left) limb blastema was removed and transplanted to the proximal (right) stump.

*Distal to distal transplants.* The distal

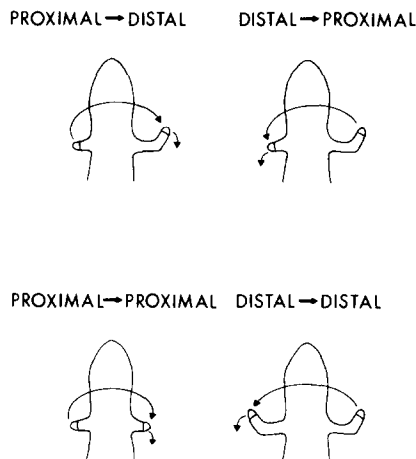


FIG. 1. Diagram of the four different types of transplant operations performed.

(left) limb blastema was removed and discarded. The distal (right) blastema was removed and transplanted to the distal (left) stump.

The results reported in this paper refer solely to the above types of transplants made to limb stumps from which blastemas had been removed. However, an additional small series of experiments was performed where late bud blastemas were transplanted from proximal to distal and distal to proximal, on freshly amputated limb stumps. The results of these experiments were similar to those reported here, indicating that the presence or absence of a blastema on a stump prior to the time of transplantation has little relevance for the outcome of the experiments.

In all transplantation operations care was taken not to include any stump tissue with the transplanted blastema. At all stages used, the original site of amputation is distinct due to pigmentary differences between the regenerate and the stump. However, due to the fact that dedifferentiation extends proximally from the site of amputation for 0.5–1.0 mm, in all cases regenerate tissue remains on the recipient stump. Hence, transplants made at early bud and medium bud are placed in contact with undifferentiated cells at the original



site of amputation. Transplants made at late bud and early digits are placed in contact with newly differentiated cells at the site of amputation. Since the cross-sectional area of a limb stump at the proximal level is almost the same as that of a limb at a distal level ( $1.48 \pm 0.46 \text{ mm}^2$ , proximal stump;  $1.43 \pm 0.31 \text{ mm}^2$ , distal stump;  $P = 0.7$ ),<sup>1</sup> the cut surface of a transplanted blastema matched the exposed surface of the stump, leaving little if any uncovered stump tissue. All transplants were made such that the dorsal-ventral axes of the graft and stump were aligned and their anterior-posterior axes were opposed. In most cases a carbon mark was placed on the middorsal edge of the transplant to ensure that the transplant was properly situated on the limb stump. The limbs with their transplants were staged and examined closely for signs of resorption every day for the first week after the operation and then three times per week until they reached the stage of late digits and no further changes were apparent. The resulting limbs with their regenerates were placed in Bouin's fixative and then stained as whole mounts with Victoria Blue (Bryant and Iten, 1974) in order to examine the skeletal arrangement of the limbs.

A histological study was also made to examine the survival of the transplanted blastema as well as the histological changes occurring in the transplanted regenerate and the adjacent stump tissues. Regenerates at the stages of early bud, late bud, and early digits were transplanted from a distal to a proximal level, and regenerates at the stage of early digits were transplanted from a distal to a distal level in the manner described above. At various intervals after the operation, the limbs with their transplanted regenerates were placed in Bouin's fixative. Serial longitudi-

nal sections of these limbs were stained with either Mayer's hematoxylin and eosin or Mallory's triple strain for connective tissue.

## RESULTS

Following the transplantation of a regenerate from one forelimb to the contralateral limb stump of the same animal, the transplanted regenerate was delayed in its development by several days. However, the delay was nowhere near what it would have been if the transplant had regressed and had been replaced by a blastema from the stump. This is shown by a comparison between the time taken for the contralateral limb (from which the transplanted blastema had been removed) to reach the same stage (Table 1). On the average, transplants became vascularized or revascularized by 5 days after transplantation.

TABLE 1

COMPARISON BETWEEN TIME TAKEN FOR TRANSPLANT TO REACH NEXT STAGE AFTER OPERATION AND TIME TAKEN FOR REGENERATING CONTRALATERAL LIMB TO REACH THE SAME STAGE

Stage of transplant	Type of operation <sup>a</sup>	No. of cases	Mean no. of days to next stage ( $\pm$ SEM)	
			Transplant	Contra-lateral limb
Early bud	P to D	21	7.0 ( $\pm$ 0.5)	8.9 ( $\pm$ 0.3)
	D to P	13	5.2 ( $\pm$ 0.3)	9.9 ( $\pm$ 0.5)
	P to P	15	6.5 ( $\pm$ 0.8)	9.8 ( $\pm$ 0.7)
	D to D	14	7.1 ( $\pm$ 0.8)	13.3 ( $\pm$ 1.3)
Medium bud	P to D	13	11.8 ( $\pm$ 1.6)	12.2 ( $\pm$ 0.6)
	D to P	14	8.7 ( $\pm$ 1.0)	15.4 ( $\pm$ 1.5)
	P to P	15	7.9 ( $\pm$ 0.8)	11.9 ( $\pm$ 0.5)
	D to D	12	8.2 ( $\pm$ 1.3)	15.1 ( $\pm$ 0.6)
Late bud	P to D	13	9.1 ( $\pm$ 2.0)	16.4 ( $\pm$ 0.7)
	D to P	12	14.4 ( $\pm$ 1.0)	17.3 ( $\pm$ 0.9)
	P to P	13	9.7 ( $\pm$ 1.3)	16.3 ( $\pm$ 0.4)
	D to D	12	7.6 ( $\pm$ 1.5)	16.3 ( $\pm$ 0.8)
Early dig-its	P to D	12	19.5 ( $\pm$ 3.2)	27.0 ( $\pm$ 1.6)
	D to P	13	16.0 ( $\pm$ 3.4)	24.5 ( $\pm$ 1.0)
	P to P	12	26.1 ( $\pm$ 3.6)	26.0 ( $\pm$ 1.5)
	D to D	10	16.4 ( $\pm$ 4.4)	25.9 ( $\pm$ 2.2)

<sup>1</sup> 5  $\mu\text{m}$  cross sections of 12 limbs were used to calculate the cross-sectional area at levels equivalent to the proximal and distal amputation sites used in this experiment.

<sup>a</sup> P, proximal; D, distal.

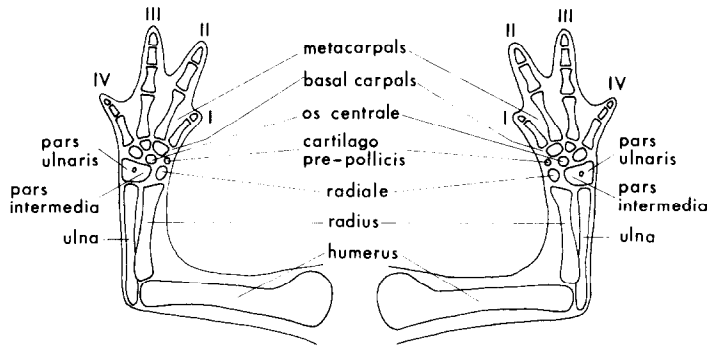


FIG. 2. Camera lucida drawings of skeletal elements of normal left and right regenerated forelimbs.

Although the transplantations were autoplasmic, about 24% of the transplants which survived to be analyzed showed either a visible reduction in size or an accumulation of fluid beneath the epidermis of the transplant which distorted its shape. These signs of poor survival during the first few days are probably attributable to a failure of these transplants to become reinnervated, revascularized, or both. Transplanted regenerates showing these signs are not included in the results. Although the distal to proximal transplants performed at the stage of early digits became revascularized with 3–6 days, the majority of the grafts in this series showed loss of some or all of their external digital morphology. This loss occurred 6–9 days after complete revascularization and at about the same time that the regenerate began to elongate. Regenerates at the same stage but transplanted to a distal stump, seldom showed this loss of digital morphology.

#### *Skeletal Organization of Transplanted Regenerates*

All limbs described below were prepared as Victoria Blue-stained whole mounts for detailed skeletal analysis at the end of the experiment. This procedure stains cartilaginous elements dark blue and bone either very faintly blue or not at all. Therefore in all cases, the regenerated skeletal elements, which are cartilaginous,

could be clearly distinguished from mature stump elements. Forelimbs were amputated at either a proximal level (through the proximal half of the humerus) or at a distal level (through the distal half of the radius and ulna). Under normal undisturbed conditions, a regenerate completes the proximal–distal sequence of skeletal elements of the limb; therefore, a regenerate from a proximal level forms a distal portion of a stylopodium (continuous with the stump stylopodium), a zeugopodium, and an autopodium, while a regenerate from a distal level forms a distal portion of a zeugopodium (continuous with the stump zeugopodium) and an autopodium.

Many of the skeletal elements of a regenerated forelimb have characteristic shapes and arrangements which allow them to be accurately identified<sup>2</sup>. This pattern of skeletal elements permits an analysis to be made of the anterior–posterior and proximal–distal skeletal organization of the regenerate (see Fig. 2). The stylopodium consists of a single element, the humerus, with a large, round distal end. In the zeugopodium, the anterior (preaxial) radius has an expanded distal end and the posterior (postaxial) ulna has an olecranon process at its proximal end. In the autopodium, the proximal anterior (preaxial) carpals are the small round radiale and cartilago pre-pollicis which are sometimes

<sup>2</sup>Nomenclature for skeletal elements from Francis (1934).

fused in the regenerate. The proximal posterior (postaxial) carpals are the large rectangular-shaped *pars ulnaris* and *pars intermedia* which are either fused or closely associated in the regenerate. When these two elements are fused, a central hole, the *foramen perforans carpi* remains. The central carpal is the *os centrale*. The distal carpals are the single oval basal carpal of digits I and II, the round basal carpal of digit III, and the round basal carpal of digit IV. The metacarpals and the phalanges are dumbbell shaped and the digital formula (number of phalanges) is usually 1.2.3.2; however, a formula of 1.2.2.2 or 1.2.2.1 is not unusual. In the descriptions which follow, the interpretation of the handedness of a particular regenerate is based on the relative positions of the radius and ulna, the relative positions of the *radiale* and *cartilago pre-pollicis* carpals and the *pars intermedia* and *pars ulnaris* carpals, the arrangement of the digits with respect to the distal carpals and the digital formula. All of the limbs resulting from the various transplantation operations were analyzed, first, in terms of their organization in the anterior-posterior axis and, second, in terms of their organization in the proximal-distal axis.

#### *Anterior-Posterior Organization*

All of the transplant operations reported in this paper were made from right to left limbs or vice versa, and all were made such

that the dorsal-ventral axes of the stump and graft coincided and the anterior-posterior axes were opposed.

The following are definitions of the types of skeletal arrangements encountered in these experiments, in addition to normal left or right limbs. Limbs with normal asymmetry (left or right) but with just one or two additional carpals or digits were classified as "normal."

*Expanded right limb.* A limb in which both the anterior and posterior edges of the autopodium are appropriately positioned for a right hand but in which the central region of the autopodium contains a variable number of extra skeletal elements (Fig. 3).

*Expanded left limb.* A limb in which both anterior and posterior edges of the autopodium are appropriately positioned for a left hand but in which the central region of the autopodium contains a variable number of extra skeletal elements (Fig. 4).

*Double anterior limb.* A limb in which both the preaxial and postaxial margins of the autopodium consist of anterior structures. Between these anterior structures are variable numbers of extra skeletal elements, leading in the most extreme cases to almost two complete autopodia, joined in the center by their posterior edges (Fig. 5). In many of these double anterior limbs, there are three zeugopodial elements.

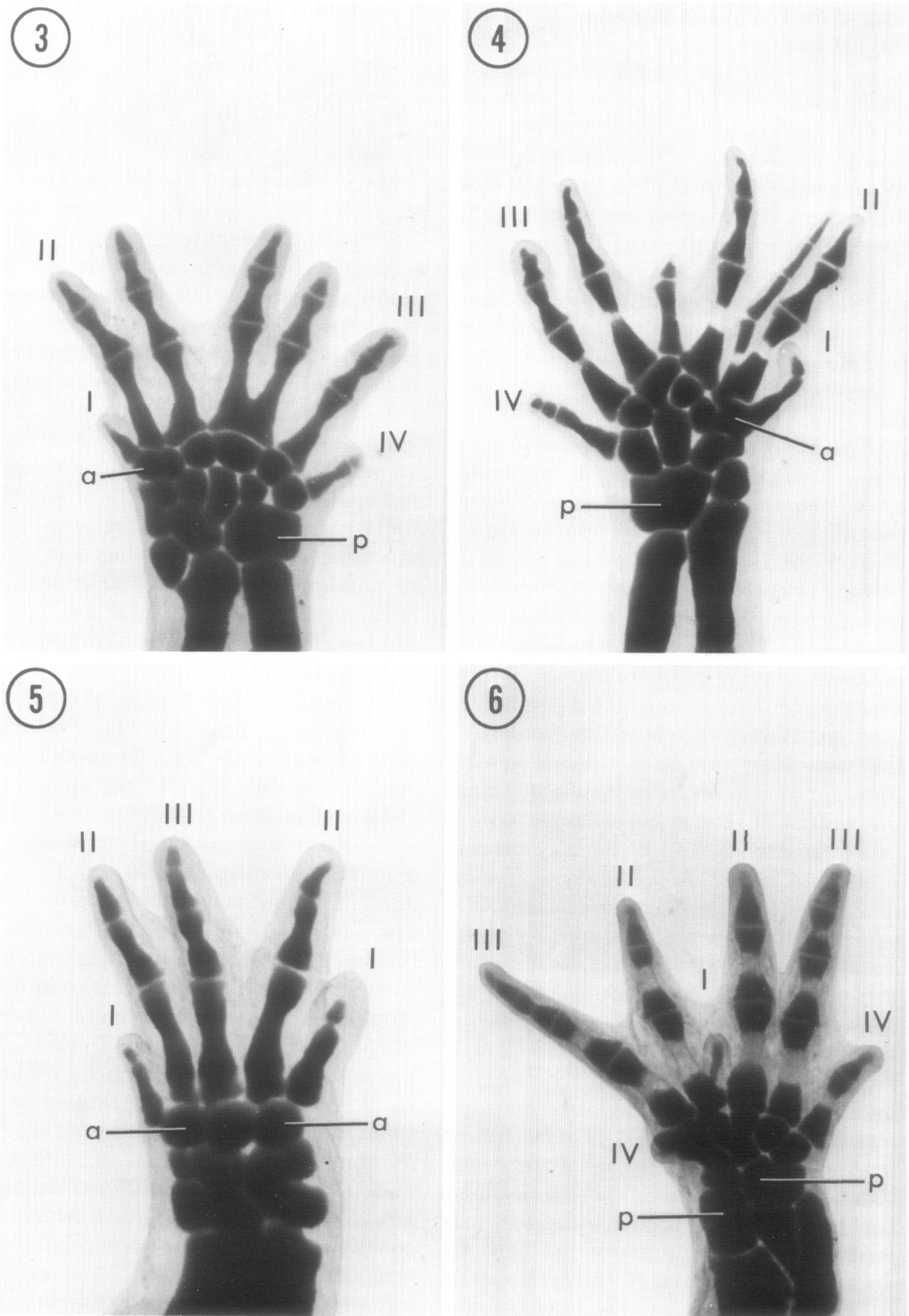
*Double posterior limb.* A limb in which

FIG. 3. Skeletal preparation of an expanded right limb formed after transplantation of an early bud blastema from left to right (proximal to distal). Anterior and posterior carpals and digits are separated by supernumerary centrally located carpals and digits. a, Single anterior basal carpal of digits I and II; p, single large proximal posterior carpal (fused *pars ulnaris* and *pars intermedia*).  $\times 25$ .

FIG. 4. Skeletal preparation of an expanded left limb formed after transplantation of a late bud regenerate from right to left (distal to distal). Anterior and posterior carpals and digits are separated by supernumerary, centrally located carpals and digits. Abbreviations as in Fig. 3.  $\times 20$ .

FIG. 5. Skeletal preparation of a double anterior autopodium formed after transplantation of an early bud blastema from left to right (proximal to distal). Both pre- and postaxial margins of the autopodium consist of anterior digits and carpals. Posterior digit and carpals are located in the center of the autopodium. Abbreviations as in Fig. 3.  $\times 30$ .

FIG. 6. Skeletal preparation of a double posterior autopodium formed after transplantation of a medium bud blastema from left to right (proximal to distal). Both pre- and postaxial margins of the autopodium consist of posterior digits and carpals. Anterior digits and carpals are located in the center of the autopodium. There are also three zeugopodial elements proximal to the posterior carpals (p). Abbreviations as in Fig. 3.  $\times 16$ .



FIGS. 3-6

both the preaxial and postaxial margins of the autopodium consist of posterior structures. Between these posterior structures are variable numbers of extra skeletal elements, leading in the most extreme cases to almost two complete autopodia, joined in the center by their anterior edges (Fig. 6). In many of these double posterior limbs, there are three zeugopodial elements.

*Indeterminate limb.* A limb in which the skeletal elements, usually of the autopodium, are so arranged that an interpretation of handedness is impossible.

*Supernumerary regenerate.* A regenerate that arises as a new and separate outgrowth distinct from the transplanted regenerate. A supernumerary regenerate develops in the vicinity of the border between the transplanted regenerate and the stump (Figs. 7-11).

None of the types of limbs listed above has ever been found during normal regeneration in our laboratory. Furthermore, in a separate experiment where blastemas have been transplanted from a hindlimb to a forelimb and vice versa without changing the anterior-posterior or dorsal-ventral axes, none of the above types of abnormalities occurs, although occasionally one or two extra carpals and digits may develop (Gritz and Bryant, in preparation). Simple removal and replacement of a blastema *in situ* likewise does not produce any of the above types of limbs. The results described below can therefore be attributed to the opposition of the anterior-posterior axes of the stump and transplant. In general, early bud blastemas do not retain their original handedness upon transplantation, but blastemas at later stages of regeneration tend to do so, depending upon the conditions of transplantation. Those transplants that do not retain their handedness may form completely in conformance with the anterior-posterior axis of the limb stump, or they may show various intermediates between the handedness of their site of origin and their site of grafting. The results

also show that the retention of handedness by the graft is closely correlated with the production of supernumerary regenerates of the handedness of the stump.

The results for all four different types of transplant operations are presented in Table 2. Significant variations occur between the results of these different groups, despite the common opposition of the anterior-posterior axes in all groups, and possible reasons for this will be considered in the Discussion.

As can be seen from Table 2, none of the limbs resulting from the transplantation of early bud regenerates to contralateral limb stumps retained their original handedness. At medium bud, 30% of all proximal to proximal transplants and 23% of all proximal to distal transplants retained their handedness. These figures increase to 85% for proximal to proximal transplants and 100% for proximal to distal transplants by late bud, and to 100% for both groups by early digits.

The results of all groups indicate that the original handedness of the transplanted regenerate can be modified depending on the type of transplant performed. The greatest modification occurs in the distal to proximal group, where only a small percentage (15%) of the limbs resulting from transplants made at the advanced stage of early digits retain the original handedness. Less modification occurs in the distal and proximal to proximal groups and the least modification of all in the proximal to distal group. In those cases where the blastema did not retain its handedness, limbs were formed which could be classified as double anterior (Fig. 5), double posterior (Fig. 6), indeterminate, expanded right (Fig. 3), or expanded left (Fig. 4), as well as limbs which were completely normal and appropriate to the host stump. These latter limbs occasionally possessed one or two extra digits and carpals. The overall incidence of double anterior and double posterior limbs was the

same, 6% for each. However, not all groups had a similar incidence of these mirror-image limbs, as can be seen from Table 2. In the proximal to proximal and proximal to distal groups, all six double posterior limbs and four of the five double anterior limbs developed a third zeugopodial element. In most cases this could not be further identified as a radius or an ulna. All but one of the limbs produced in the distal to distal to proximal groups failed to produce any extra zeugopodial elements. In

this one case, only a small distal portion of a third zeugopodial element was formed.

Supernumerary regenerates were produced in all the groups. They occurred with approximately the same frequency in proximal to distal (43% of the transplants), proximal to proximal (44% of the transplants), and distal to distal groups (40% of the transplants). However, in the distal to proximal group, only 4% of the transplants formed supernumerary regenerates. The supernumeraries arose close to the graft

TABLE 2  
ANTERIOR-POSTERIOR ORGANIZATION OF TRANSPLANTED REGENERATES

Type of operation <sup>a</sup>	Stage of transplant	No. of cases	Handedness of regenerate arising from transplant as a percentage of the total number of cases							Supernumerary regenerates as a percentage of the total number of cases			
			Left (%)	Ex-panded left (%)	Right (%)	Ex-panded right (%)	Dou-ble poste-rior (%)	Dou-ble ante-rior (%)	Inde-termi-nate (%)	Both ante-rior and poste-rior	Ante-rior only	Poste-rior only	No super-numer-aries
P to D (L to R)	Early bud	20	—	—	40	25	5	10	20	—	—	10	90
	Medium bud	13	23	—	30	8	8	23	8	15	8	—	77
	Late bud	13	100	—	—	—	—	—	—	31	31	38	—
	Early dig-its	12	100	—	—	—	—	—	—	50	8	—	42
P to P (L to R)	Early bud	14	—	—	79	—	7	—	14	—	—	—	100
	Medium bud	13	30	—	8	30	8	—	23	15	—	8	77
	Late bud	13	85	—	—	—	15	—	—	54	15	8	23
	Early dig-its	10	100	—	—	—	—	—	—	60	10	20	10
D to P (R to L)	Early bud	12	100	—	—	—	—	—	—	—	—	—	100
	Medium bud	12	42	33	—	—	8	—	17	—	—	—	100
	Late bud	12	17	50	—	—	17	—	17	—	—	—	100
	Early dig-its	13	8	62	15	—	8	—	8	15	—	—	85
D to D (R to L)	Early bud	13	38	8	—	—	8	23	23	—	—	8	92
	Medium bud	10	—	30	—	—	10	20	40	—	—	40	60
	Late bud	11	9	36	18	—	—	9	27	18	9	—	73
	Early dig-its	9	—	—	78	—	—	—	22	44	44	12	—

<sup>a</sup> P, proximal; D, distal; L, left; R, right.

margin at either the anterior or posterior limb surface or at both surfaces but never at dorsal or ventral surfaces (Fig. 7). The incidence of anterior supernumerary regenerates was similar to that of posterior supernumerary regenerates (49 anterior and 52 posterior). Thirty-five of the 66 limbs that developed supernumerary regenerates developed both an anterior and posterior supernumerary regenerate. In 17 of these limbs both supernumerary regenerates arose simultaneously, whereas in 14 limbs the anterior supernumerary began its development first, and in four cases the posterior supernumerary began its development first. Overall, the average time after transplantation before the appearance of a supernumerary regenerate was 15 days for anterior supernumeraries and 18 days for posterior supernumeraries.

The ability to form supernumeraries is closely correlated with the retention of the original handedness of the graft (see Figs. 8-11). Sixty-four regenerates developed in which the transplant retained its original handedness and, of these, 55 (86%) formed one or two supernumerary regenerates. In

only two of the limbs that formed supernumerary regenerates did the handedness of the regenerate arising directly from the transplant clearly correspond to the stump type handedness. Wherever handedness could be determined in the supernumerary regenerate (80 out of the total of 101 cases), it was appropriate to the stump type and opposite to the graft type (see Fig. 10).

#### *Proximal-Distal Organization*

The transplant operations reported in this paper were made either between similar limb levels (proximal to proximal, distal to distal) or between different limb levels (proximal to distal, distal to proximal). In general, the results show that when blastemas are transplanted from a proximal to a distal level, they behave autonomously, leading to the production of limbs with extra skeletal elements in the proximal-distal axis, whereas, when blastemas are transplanted from a distal to a proximal level, intercalary regeneration occurs so that the resulting limbs never have missing elements in the proximal-distal axis. Supernumerary regenerates that arise

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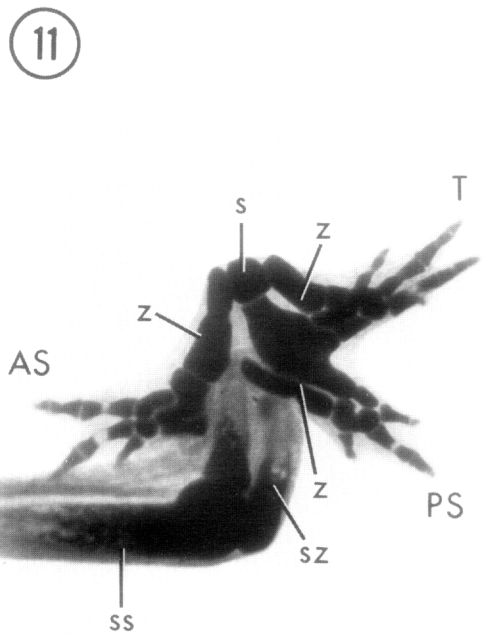
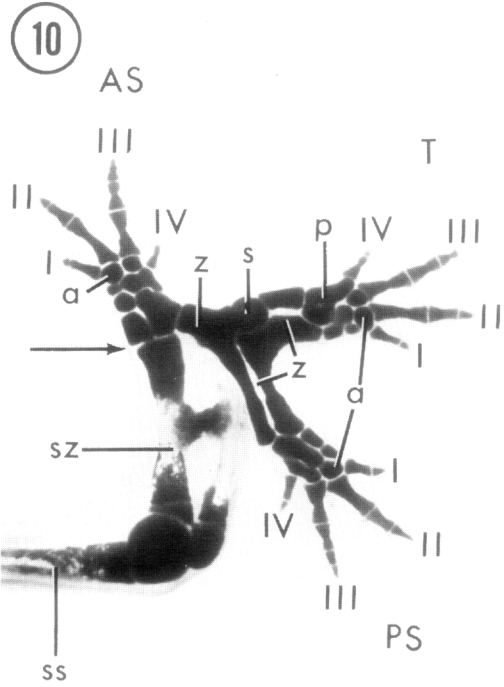
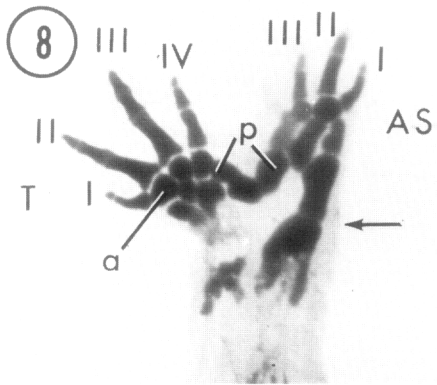
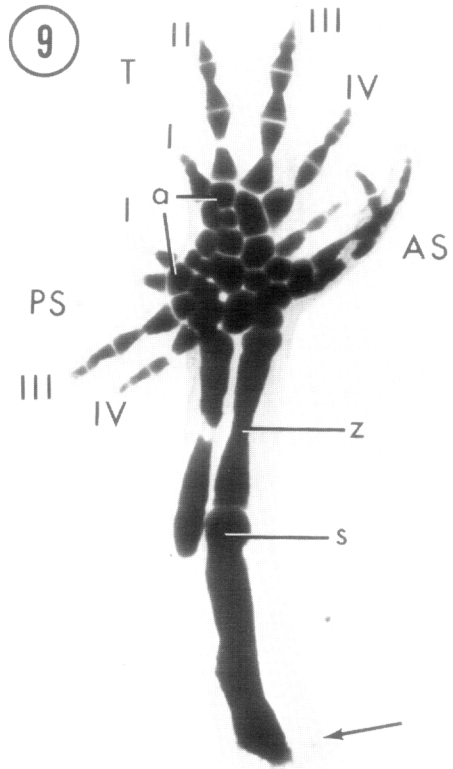
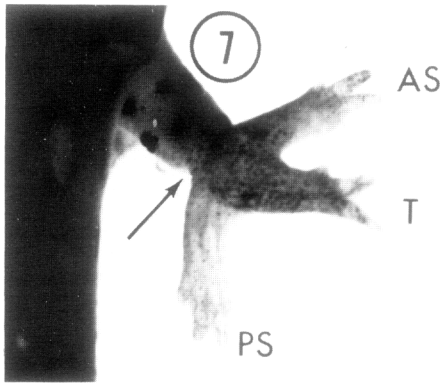
FIG. 7. Limb with two supernumerary regenerates formed after transplantation of a regenerate at the stage of early digits from left to right (proximal to proximal). The central limb is a left limb that developed directly from the transplant. Both anterior and posterior supernumerary regenerates developed close to the graft junction (arrow). AS, anterior supernumerary regenerate; PS, posterior supernumerary regenerate; T, transplant.  $\times 10$ .

FIG. 8. Skeletal preparation of a limb showing a single anterior supernumerary regenerate. A regenerate at the stage of early digits was transplanted from a right to a left limb (distal to distal). The autopodium developing from the transplant retained its right handedness. The anterior supernumerary regenerate is a left hand. Arrow indicates graft junction. Abbreviations as in Figs. 3 and 7.  $\times 15$ .

FIG. 9. Skeletal preparation of a limb showing anterior and posterior supernumerary regenerates. A regenerate at the stage of early digits was transplanted from right to left (distal to proximal). A single humerus as well as a radius and ulna have regenerated with left (stump-type) asymmetry. The transplant retained its right handedness. Arrow indicates level to which transplant was made. z, Regenerated zeugopodium; s, regenerated head of the stylopodium; other abbreviations as in Figs. 3 and 7.  $\times 13$ .

FIG. 10. Skeletal preparation of a limb showing both anterior and posterior supernumerary regenerates. A regenerate at the stage of early digits was transplanted from left to right (proximal to distal). The autopodium developing from the transplant retained its left handedness. Both anterior and posterior supernumeraries are right limbs. All three regenerates possess zeugopodial elements which articulate with the duplicated humeral head. Arrow indicates graft junction. ss, Stump stylopodium; sz, stump zeugopodium; other abbreviations as in Figs. 3, 7, and 9.  $\times 13$ .

FIG. 11. Skeletal preparation of a limb with two supernumerary regenerates. A regenerate at the stage of late bud was transplanted from left to right (proximal to distal). Both the anterior and posterior supernumerary regenerates, as well as the regenerate developing directly from the transplant possess zeugopodia that articulate with the duplicated head of the humerus. Abbreviations as in Figs. 7, 9, and 10.  $\times 15$ .



**Figs. 7-11**



close to the border between stump and graft appear to form a proximal-distal sequence of elements appropriate to the graft rather than the stump (compare Figs. 8 and 9 with Figs. 10 and 11).

As can be seen from Table 3, in all cases where blastemas at any stage are transplanted between similar limb levels, either proximal to proximal or distal to distal, the resulting limbs have a normal proximal-distal sequence of elements (stylopodium, zeugopodium, autopodium); skeletal elements are neither missing nor added. However, when transplants are made from a proximal to a distal level, even at the stage of early bud, serial duplications of skeletal structures in the proximal-distal axis are formed. Thus, one limb resulting from an early bud transplant (Fig. 12) had the following sequence of elements: stylopodium, proximal half of zeugopodium, distal portion of stylopodium, complete zeugopodium, and autopodium (S, Z<sup>P</sup>/S<sup>D</sup>, Z, A). Another five specimens had a stylopodium, proximal half of zeugopodium, complete zeugopodium, and autopodium (S, Z<sup>P</sup>/Z<sup>D</sup>,

A), making a total of 30% of the limbs resulting from early bud transplants in which serially duplicated structures were present. This percentage increases to 77% by medium bud (see Fig. 13) and 100% by late bud (see Fig. 14) and early digits (see Fig. 15). None of the six early bud transplants in which these extra skeletal elements were formed retained their original handedness: Two formed right stump-type limbs, two formed double anterior limbs, one formed an expanded right limb, and one was indeterminate. Similarly, at medium bud, of the ten limbs which developed extra skeletal elements in the proximal-distal axis, only three retained their original handedness. The medium bud transplant illustrated in Fig. 13 has extra elements in the proximal-distal axis, but it has stump (right) handedness.

In contrast to the results of the proximal to distal transplants, where limbs are formed containing serial duplications of some skeletal elements, distal to proximal transplants never gave limbs in which particular limb segments were deleted. Out

TABLE 3  
PROXIMAL-DISTAL SKELETAL ORGANIZATION OF LIMB STUMPS AND THEIR TRANSPLANTS

Type of operation <sup>a</sup>	Proximal-distal organization of stump and regenerate <sup>b</sup>	Stage of transplant			
		Early bud	Medium bud	Late bud	Early digits
P to D	No. of cases	20	13	13	12
	% S, Z <sup>P</sup> /S <sup>D</sup> , Z, A	5	23	54	58
	% S, Z <sup>P</sup> /Z, A	25	54	46	42
	% S, Z <sup>P</sup> /Z <sup>D</sup> , A	70	23	—	—
D to P	No. of cases	12	12	12	13
	% S <sup>P</sup> /S <sup>D</sup> , Z, A	100	100	100	100
	% S <sup>P</sup> /Z, A	—	—	—	—
	% S <sup>P</sup> /Z <sup>D</sup> , A	—	—	—	—
P to P	No. of cases	14	13	13	10
	% S <sup>P</sup> /S <sup>D</sup> , Z, A	100	100	100	100
D to D	No. of Cases	13	10	11	9
	% S, Z <sup>P</sup> /Z <sup>D</sup> , A	100	100	100	100

<sup>a</sup> P, proximal; D, distal

<sup>b</sup> /, Division between stump and transplant; S, S<sup>P</sup>, S<sup>D</sup>, stylopodium, proximal stylopodium, distal stylopodium; Z, Z<sup>P</sup>, Z<sup>D</sup>, zeugopodium, proximal zeugopodium, distal zeugopodium; A, autopodium.

of the total of 49 transplants at all stages, only three were defective in having just one zeugopodial element instead of the normal two. In all cases, irrespective of the handedness of the autopodium, the zeugopodial elements were arranged in conformity to the anterior-posterior axis of the stump (Fig. 9). Comparisons were made of the lengths of the zeugopodial elements in normal distal regenerates, normal proximal regenerates and regenerates resulting from distal to proximal transplants. The results of these comparisons show that the zeugopodial elements of the distal to proximal transplants are about the same length (radius,  $2.4 \pm 0.1$  mm; ulna,  $3.1 \pm 0.2$  mm) as zeugopodial elements in proximal regenerates (radius,  $2.1 \pm 0.1$  mm; ulna,  $2.6 \pm 0.2$  mm) and significantly longer ( $P < 0.001$ ) than the regenerated distal portion of these elements in normal distal regenerates (radius,  $1.6 \pm 0.1$  mm; ulna,  $1.7 \pm 0.2$  mm).

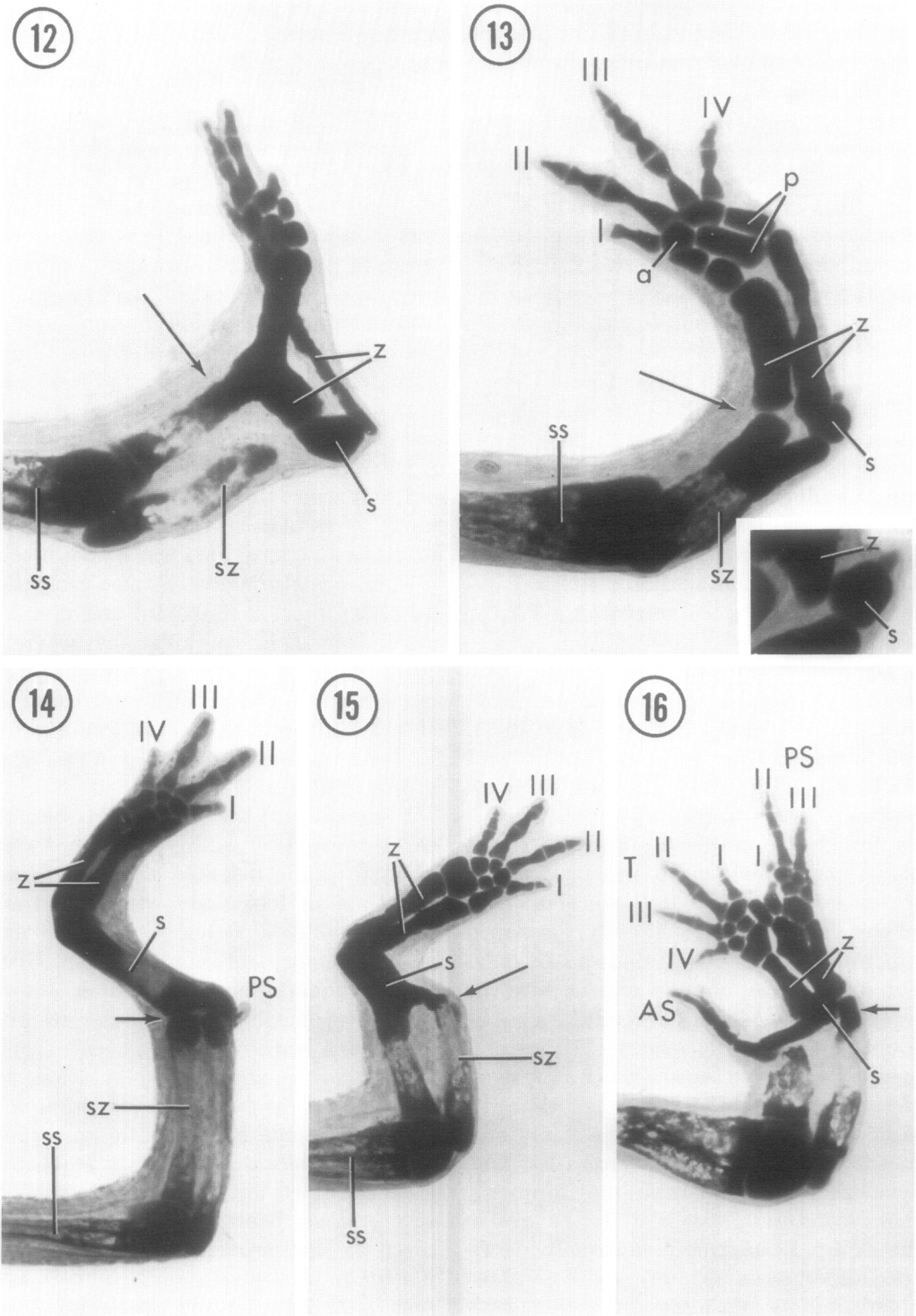
The supernumerary regenerates which formed in these experiments varied in completeness along the proximal-distal axis from almost entire limbs (Figs. 10 and 11) to single digits (Fig. 14; see Table 4). The maximally developed supernumeraries forming in proximal to distal transplants and proximal to proximal transplants are appropriate to the level of origin of the graft. Such maximally developed supernumeraries usually share an articulation on the head of the humerus with the regenerate developing directly from the transplant (Figs. 10 and 11). A supernumerary regenerate is usually not more complete in the proximal-distal axis than the regenerate developing from the transplant. In the proximal to distal group less well developed supernumeraries may form only those structures appropriate to the level of the stump. Furthermore, maximally developed supernumeraries forming after transplants of distal blastemas either to a proximal (Fig. 9) or a distal (Fig. 8) level were appropriate to the graft, and, in these groups, there were no instances of super-

numeraries that appeared to be appropriate to the stump.

#### *Histological Organization of Transplanted Regenerates*

From the results described above on the proximal-distal organization of the different graft combinations, it is apparent that grafts from a proximal to a distal level survived and formed structures appropriate to the level of origin of the graft. On the other hand, one possible interpretation of the distal to proximal grafts, where limbs with deleted segments were never formed, is that the transplants did not survive and the resulting regenerates were simply re-regenerates from the proximal stump. In order to assess the likelihood of this possibility, a histological examination of distal to proximal transplants was made. A comparison was also made between early digital stage distal to proximal and distal to distal transplant combinations, where in the former case, regression of the digits usually occurs at about the time that limb elongation begins. A total of 45 graft combinations have been analyzed in this histological study.

Regenerates at the stage of early bud, the earliest stage used in this study were transplanted from a distal to a proximal level, and three or four limbs with their transplants were fixed at 1, 2, 3, 4, 6, or 10 days after the transplantation operation. Histological examination of these limbs showed that the transplant was neither resorbed nor overgrown by the adjacent stump. Within 24 hr after transplantation, the epidermis of the transplant and stump had fused. An epidermal indentation formed, probably where the transplant and stump epidermis met, and it remained visible for several days. Scattered dead cells (recognized by their pycnotic nuclei) as well as blastema cells undergoing mitosis were seen in the transplanted blastema 1-2 days after transplantation (see Fig. 18) and there were also a few scattered phagocytes within the transplant. By 4 days, many



FIGS. 12-16

TABLE 4  
PROXIMAL-DISTAL SKELETAL ORGANIZATION OF SUPERNUMERARY REGENERATES

Type of operation <sup>a</sup>	Proximal-distal organization of supernumerary regenerates <sup>b</sup>	Stage of transplant			
		Early bud	Medium bud	Late bud	Early digits
P to D	No. of cases	2	5	17	13
	% S <sup>D</sup> , Z, A	—	40	24	54
	% Z, A	—	60	34	23
	% Z <sup>D</sup> , A	—	—	18	23
	% A	100	—	24	—
D to P	No. of cases	0	0	0	4
	% A	—	—	—	100
P to P	No. of cases	0	5	17	15
	% S <sup>D</sup> , Z, A	—	—	35	87
	% Z, A	—	80	47	—
	% Z <sup>D</sup> , A	—	—	—	7
	% A	—	20	18	7
D to D	No. of Cases	1	4	5	13
	% Z <sup>D</sup> , A	—	—	—	15
	% A	100	100	100	85

<sup>a</sup> P, proximal; D = distal.

<sup>b</sup> Abbreviations as in footnote *b* of Table 3.

mitotic figures were seen in the transplant but very few dead cells or phagocytes. For the first 1-2 days after transplantation, the junction between the transplant and stump was visible as a small space containing tissue exudate, trapped erythrocytes, and tissue debris (Fig. 17). This space disappeared 3-4 days after transplanta-

tion, but the junction between the transplant and stump remained visible as a distinct discontinuity between the mesenchyme of the stump and the transplant, since the density of cells in the transplant was greater than in the adjacent stump. The epidermal indentations were still visible at this time. By 6 days after transplan-

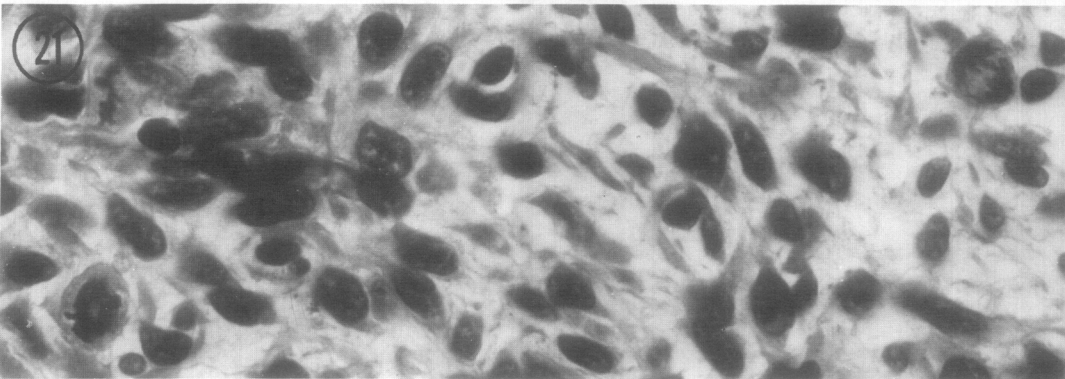
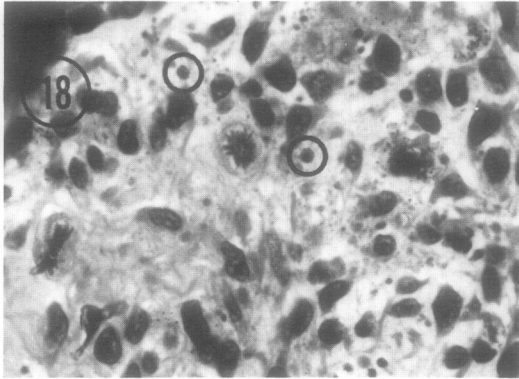
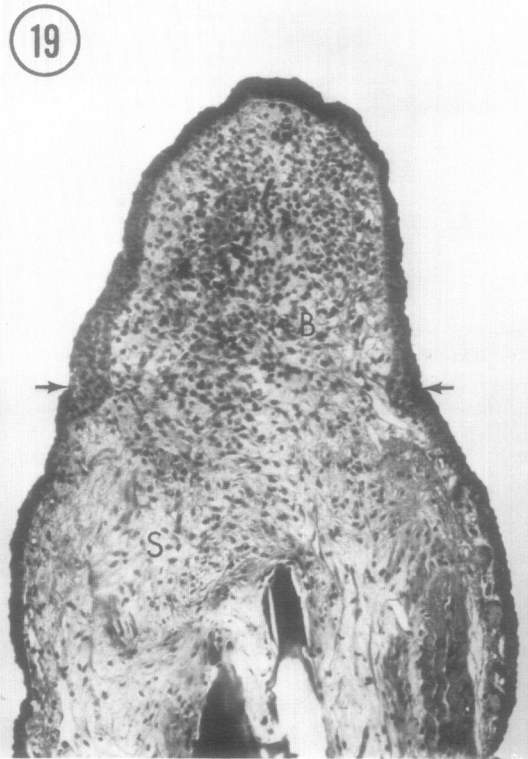
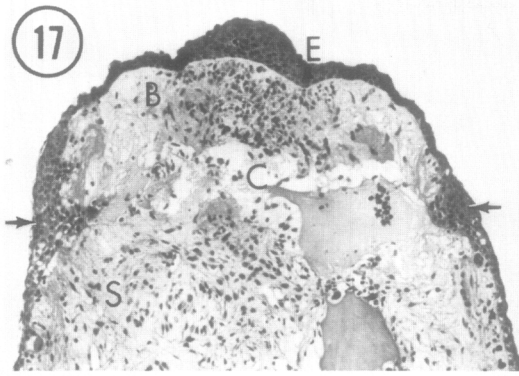
FIG. 12. Skeletal preparation of a limb showing serial duplication of structures in the proximal-distal axis. A blastema at early bud was transplanted from proximal to distal (left to right). In addition to the stump stylopodium and zeugopodium, an extra stylopodium and zeugopodium are formed. Arrow indicates the site of grafting. Abbreviations as in Figs. 9 and 10.  $\times 20$ .

FIG. 13. Skeletal preparation of a limb showing serial duplications of structures in the proximal-distal axis. A blastema at medium bud was transplanted from proximal to distal (left to right). The transplant is a right limb. Arrow indicates site of grafting.  $\times 13$ . Insert: Duplicated elbow region showing separate stylopodium;  $\times 36$ . Abbreviations as in Figs. 3, 9, and 10.

FIG. 14. Skeletal preparation of a limb showing serial duplication of structures in the proximal-distal axis. A blastema at late bud was transplanted from proximal to distal (left to right). PS: A small posterior supernumerary regenerate consisting of a single digit. Arrow indicates site of grafting. Abbreviations as in Figs. 3, 7, and 9.  $\times 13$ .

FIG. 15. Skeletal preparation of a limb showing serial duplication of structures in the proximal-distal axis. A blastema at the stage of early digits was transplanted from proximal to distal (left to right). Arrow indicates site of grafting. Abbreviations as in Figs. 3 and 9.  $\times 10$ .

FIG. 16. Skeletal preparation of a limb showing serial duplication of some skeletal elements. This transplant had shown signs of severe regression and was classified as a nonsurvivor. Arrow indicates site of grafting. Abbreviations as in Figs. 3, 7 and 9.  $\times 13$ .



FIGS. 17-21

tation, the mesenchyme of the transplant and stump had become so well integrated that no discontinuity could be seen between them, and only the epidermal indentations marking the probable junction remained (see Fig. 19). This disappearance of the border between the transplant and stump also seemed to coincide with the onset of visible outgrowth of the regenerate. Chondrogenesis was observed in the regenerate at 10 days after the transplantation operation.

Since it appeared that early bud transplants survived and were not overgrown by stump tissues, we next examined the histological change occurring in older regenerates following transplantation. Regenerates at the stage of late bud, the time of onset of differentiation in the regenerate, were transplanted from a distal to a proximal level, and three limbs with their transplants were fixed at either 2, 4, or 6 days after transplantation. Histological examination of these limbs indicated again that there was good survival of the transplanted regenerate and that the few dead cells seen in the transplanted regenerate were not localized. Cartilage seen in the transplanted regenerate at 2 days after transplantation had dedifferentiated by 4-6 days. Furthermore, by 6 days the adjacent stump cartilage had dedifferentiated, and numerous mitotic figures could be seen near the stump-regenerate border.

In order to examine the histological

changes associated with the disappearance of external digital morphology in the distal to proximal series, distal level regenerates at the stage of early digits were transplanted to a proximal level and four or five limbs with their transplants were fixed at either 6 or 11-14 days after transplantation, i.e., before and after the disappearance of external digit morphology and before and after visible outgrowth of the regenerate. These limbs showed dramatic histological changes both in the transplanted regenerate and the adjacent stump tissue. At 6 days after transplantation, there were few signs of dedifferentiation of either the transplant or the adjacent stump (see Fig. 22), but, by 11-14 days following transplantation, both the transplant and adjacent stump had dedifferentiated (Fig. 23) and numerous mitotic figures were found both in the proximal portion of the transplant (Fig. 20) and the adjacent stump tissue (Fig. 21). There were also cases where only the proximal portion of the early digit transplant dedifferentiated along with the differentiation of the adjacent stump. This dedifferentiation was not accompanied by cell death in either the graft or the stump.

We next examined whether these histological changes occurring in the early digit transplants and adjacent stump were a result of the trauma of the operation or a result of the discrepancy in level between transplant and stump. Regenerates at the

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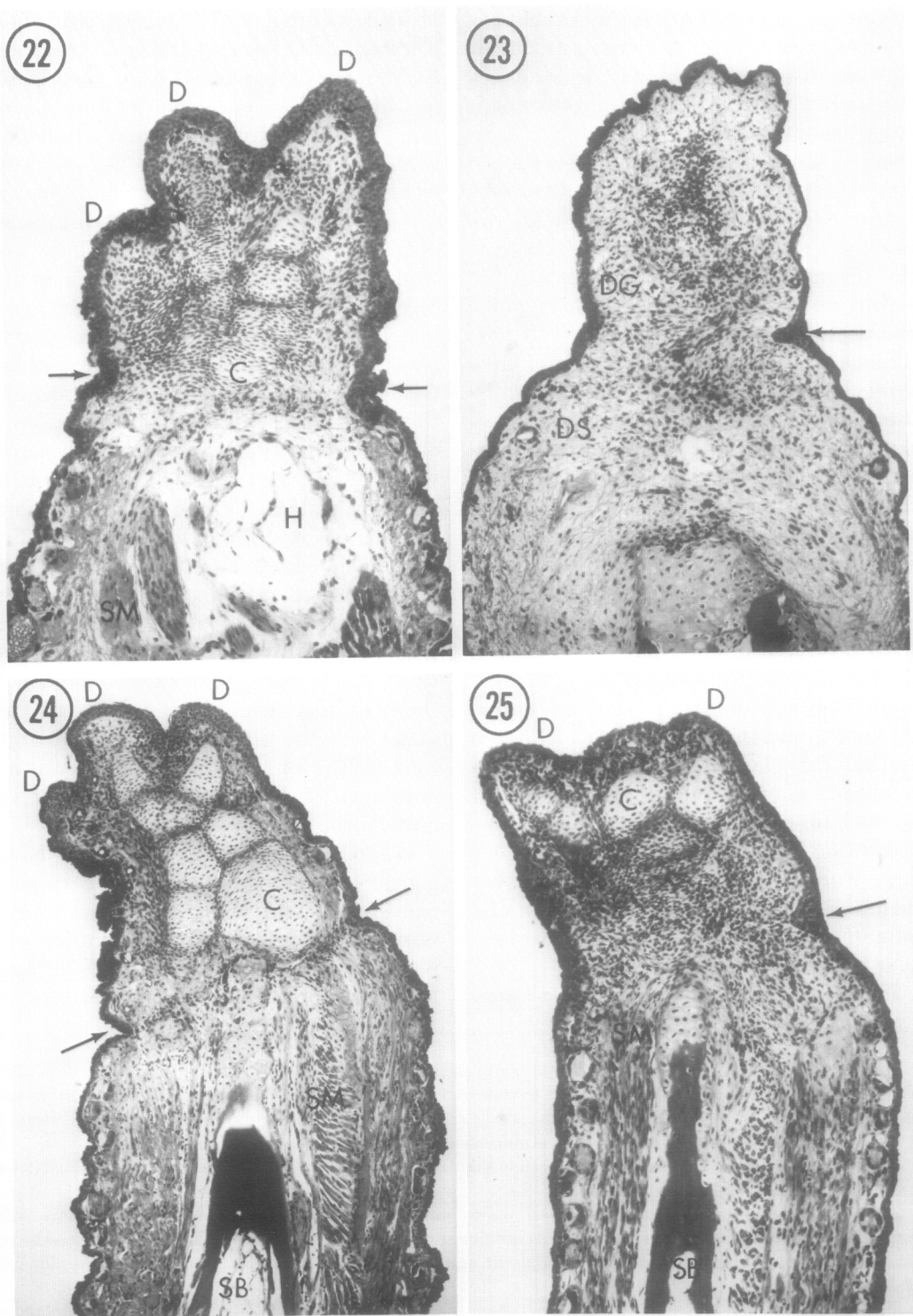
FIG. 17. Longitudinal section of a distal early bud blastema on a proximal stump, two days after transplantation. Epidermal indentations at arrows indicates probable graft junction. Although mature skin glands are not seen up to the distal edge of the stump skin in this section, they can be seen in adjacent sections. E, epidermis; B, transplanted blastema; S, stump tissue; C, cleft between transplant and stump.  $\times 60$ .

FIG. 18. Higher magnification of the transplanted blastema shown in Fig. 17. Scattered dead cells (circles) and mitotic figures can be seen.  $\times 370$ .

FIG. 19. Longitudinal section of a distal early bud blastema on a proximal stump, 6 days after transplantation. Although mature skin glands are not seen up to the distal edge of the stump skin on the left side of this section, they can be seen in adjacent sections. Epidermal indentations at the arrows indicate probable graft junction. Abbreviations as in Fig. 17.  $\times 60$ .

FIG. 20. Higher magnification of dedifferentiated transplant shown in Fig. 23. Many mitotic figures and no dead cells are seen.  $\times 670$ .

FIG. 21. Higher magnification of dedifferentiated stump shown in Fig. 23. Many mitotic figures and no dead cells are seen.  $\times 600$ .



FIGS. 22-25



stage of early digits were transplanted from a distal to a distal level and three to five limbs with their transplants were fixed at 6-7 days or 12-15 days after transplantation. These limbs by 6-7 days after transplantation showed no signs of dedifferentiation of either the transplant or the adjacent stump (Fig. 24); by 12-15 days after transplantation there was some dedifferentiation of the proximal portion of the transplant and only the very distal portion of the stump (Fig. 25). In some cases the transplant dedifferentiated, but the extent of dedifferentiation of the adjacent stump was usually the same or less than that illustrated in Fig. 25. Therefore, it appears that a distal graft placed onto a proximal stump results in the dedifferentiation of both the graft and the adjacent stump, whereas a distal graft placed onto a stump of the same level results in some dedifferentiation of the graft, but only minimal dedifferentiation of the stump (cf. Figs. 23 and 25).

#### DISCUSSION

The results presented here show that, despite the capacity of limb blastemas to undergo selfdifferentiation (Stocum, 1968; de Both, 1970; Stocum and Dearlove, 1972; Faber, 1971), the stump and blastema do interact when they are adjacent to each other. This interaction leads to modification of both the anterior-posterior and proximal-distal organization of the resulting limb regenerate.

The question of survival of the transplant is one to which we gave considerable attention. We are confident, within the limits of the techniques used, both from the histological controls and from the daily observations of all transplants, that the transplanted regenerates described here as survivors neither regressed nor died to be replaced by stump regenerates. Although dead cells were observed within the early bud and late bud transplants, they were scattered and relatively few in number. Many of the healthy blastema cells were in mitosis. The survival of more than 70% of all transplants performed is probably attributable to rapid revascularization and reinnervation of the grafts. Furthermore, there were no indications that the transplants became overgrown by new regeneration from the stump. An analysis of the times taken to reach the next stage after transplantation shows that, although the blastemas are somewhat delayed, they are not delayed enough to make re-regeneration a serious possibility. Complete re-regeneration and overgrowth is very unlikely because the two limb levels used as transplantation sites were very similar in cross-sectional area, leading in almost all cases to a very good fit between transplant and stump. However, it is not unlikely that some mixing of cells from the stump with those of the transplanted blastema occurred. This seems particularly likely in the case of transplants made at the stages of early bud and medium bud, where cells

FIG. 22. Longitudinal section of a distal early digits regenerate on a proximal stump, 6 days after transplantation. Arrows mark the probable graft junction. Both stump and graft are differentiated. D, digits; C, graft cartilage; SM, stump muscle; H, hole caused by loss of humerus in sectioning.  $\times 60$ .

FIG. 23. Longitudinal section of a distal early digits regenerate on a proximal stump, 11 days after transplantation. Arrow marks epidermal indentation at probable graft junction. Digital morphology is lost, and both stump and graft are extensively dedifferentiated. DG, dedifferentiated graft; DS, dedifferentiated stump.  $\times 60$ .

FIG. 24. Longitudinal section of a distal early digits regenerate transplanted to a distal stump, 7 days after transplantation. All tissues are fully differentiated. Arrows indicate probable graft junction. SB, stump bone; other abbreviations as in Fig. 22.  $\times 50$ .

FIG. 25. Longitudinal section of a distal early digits regenerate transplanted to a distal stump, 12 days after transplantation. A limited amount of dedifferentiation has occurred at the level of the site of grafting (marked by an arrow). Although mature skin glands are not seen up to the distal edge of the stump skin in this section, they can be seen in adjacent sections. Abbreviations as in Figs. 22 and 24.  $\times 60$ .



at the site of transplantation were still undifferentiated. The results of the proximal to distal transplants in themselves provide additional evidence for graft survival. Therefore, we believe that the results presented here are the products of an interaction between the transplant and the stump. Stocum (submitted for publication) has also concluded from similar studies in larval axolotls that transplanted regenerates survive well.

#### *Anterior-Posterior Organization*

After transplanting blastemas to limb stumps in such a manner that the anterior-posterior axes of the transplant and stump were opposed, Milojević (1924), using *Triturus cristatus*, and Schwidefsky (1935), using a number of species of newts, found that the anterior-posterior axis of a blastema becomes fixed only after a certain stage in regeneration. Lodyženskaja (1928, 1930), on the other hand, from similar studies using axolotls concluded that this axis is established when the blastema first forms. The results of similar experiments presented here show that the anterior-posterior axis of the transplanted regenerate may indeed be established at an early stage but that it is capable of being modified to a greater or lesser extent by contact with the adjacent stump, depending upon the conditions of transplantation. The transplant operation leading to maximal maintenance of the anterior-posterior organization is the one where a proximal blastema was grafted to a distal stump. In these operations, 23% of medium bud and 100% of late bud and early digit transplants retained their original handedness. These results contrast markedly to those in which distal blastemas were transplanted to proximal limb levels. In these operations only two transplants made at the stage of early digits retained their original handedness. These seemingly contradictory results are more easily reconciled when it is realized that distal to proximal transplants undergo rad-

ical dedifferentiation following revascularization and integration with the stump. It seems likely that in this reorganization process, which precedes further elongation of the limb, most of the original axial information which the graft might have possessed may be abolished. With this in mind, the discussion which follows refers primarily to the proximal to distal transplants in which disturbance of the graft appears to be at a minimum.

Transplants that underwent a change of handedness sometimes formed limbs appropriate to the host stump but also often formed a spectrum of limb types intermediate between site of origin and site of grafting. These were classified as expanded right or left limbs, double anterior limbs, and double posterior limbs; they appear to be similar to limbs described by Milojević (1924), Lodyženskaja (1930), and Schwidefsky (1935) following transplantation and axial reversal of blastemas. Furthermore, such limbs were also found by Harrison (1921) following axial reversal of amphibian limb buds. Harrison (1969) also reported intermediate forms from his studies on the development of axial polarity of the amphibian ear. The intermediate limb types formed in our experiments are interpreted as stages in the transition of the anterior-posterior organization of the transplant to that of the stump. That these results are attributable to opposition of the anterior-posterior axes of the stump and transplant is clear from other experiments (Gritz and Bryant, unpublished) where regenerates were transplanted from forelimbs to hindlimbs and vice versa without opposition of either the anterior-posterior or dorsal-ventral axes of the transplant and the stump. Limbs such as those described here as intermediates were never found. Similarly, simple undercutting of a blastema and its replacement on the stump in the correct orientation did not produce any disturbances in the anterior-posterior organization of the regenerate.

When regenerates at more advanced stages (late bud or early digits) were transplanted from a proximal to distal level, they retained their original anterior-posterior organization in all cases. In 80% of these cases, supernumerary regenerates formed in addition to the regenerate arising directly from the transplant. These results indicate that even though the anterior-posterior organization of the transplanted regenerate is stable, an interaction occurs between the opposed anterior-posterior axes of the stump and the transplant, resulting in the production of supernumerary regenerates. All supernumerary regenerates formed either in the anterior or the posterior position and ranged in completeness from a single digit to one or two complete limb regenerates. Well-developed supernumerary regenerates could be identified as mirror images of the transplanted regenerate. These supernumerary regenerates are almost identical to the "reduplications" Harrison (1921) and Swett (1927) obtained from experiments where the axes had been opposed in developing amphibian limb buds. Furthermore, the formation of supernumerary limbs resulting from the opposition of limb axes is not unique to vertebrates. Grafting a distal portion of an insect appendage onto a stump with changed orientation either by combining contralateral stumps and grafts or by rotation through 90 or 180° results in the formation of supernumerary regenerates, usually where axial disharmony is at a maximum (Bullière, 1970; Bohn, 1965, 1972; Shaw and Bryant, 1974a).

The results of analogous experiments in chick embryos show some similarities and some differences to those reported here. It has been shown that when the distal portion of a developing chick limb bud is either rotated 180° or exchanged with the distal portion of the contralateral limb bud such that the anterior-posterior and dorsal-ventral axes or just the anterior-posterior axis have been reversed, either the

distal tip of the limb forms a mirror-image duplicate, or the reversed portion of the limb bud simply retains its original axial polarity (Saunders *et al.*, 1958; Saunders and Gasseling, 1959; Amprino and Camosso, 1959; Amprino, 1968). The production of these mirror-image duplicates led to the discovery of the "zone of polarizing activity" (ZPA), located in the posterior portion of the limb bud that has subsequently been implicated in the control of the anterior-posterior polarity of the developing avian limb (Saunders and Gasseling, 1968; MacCabe *et al.*, 1973). The duplicated chick limbs were usually what we have designated as double posterior limbs; neither double anterior limbs, expanded right or left limbs, nor separate anterior or posterior supernumeraries were formed. In our results, on the other hand, 8% of all limbs that did not retain their original handedness formed double anterior limbs and a similar percentage formed double posterior limbs. Similarly, when supernumerary regenerates formed, an equal number formed in the anterior of the limb as formed in the posterior. These basic differences between the results of chick limb-bud axial reversal or rotation and those of amphibian limb-bud axial reversal or rotation (Harrison, 1921; Swett, 1927) in combination with the axial reversals of limb regenerates reported here suggest that the regenerating or developing amphibian limb is not controlled by a single zone homologous to the ZPA of the avian limb bud. Carlson (1974) came to the same conclusion for the regenerating amphibian limb from the results of experiments in which the axial organization of adjacent stump tissue had been altered. Similar experiments, from which similar conclusions could be drawn, have been performed by Droin (1959), Rahmani (1960), Settles (1967), and Lheureux (1972). These conclusions imply that the anterior-posterior organization of regenerating amphibian limbs is controlled in a different man-

ner from that of the developing chick limb.

The simplest model to describe the results observed in this study is illustrated in Fig. 26. It is proposed that the blastema has its anterior-posterior axis specified at the time of blastema formation. This initial polarity may be acquired either by influence of the stump on the blastema or by virtue of the stump's contribution of cells to the blastema. Under conditions in which the anterior-posterior axis of the blastema is unstable, such as in the early bud blastema, or in any stage blastema transplanted from a distal to a proximal level, a spectrum of limbs with handedness intermediate between that of the limb of origin and the site of grafting is produced. The production of these intermediates may result from a number of possible factors, such as anterior and posterior polarizing influences from the stump, a radical reorganization of the blastema as a result of a discrepancy in level between blastema and

stump, or a relatively large contribution of new cells to the blastema from the adjacent stump. Under conditions in which the anterior-posterior axis is stable, such as in advanced regenerates, where no stump-type or intermediate limbs are formed, an interaction between the opposing axes of the stump and blastema results in the stimulation of outgrowth in the form of supernumerary regenerates at the positions of maximum disharmony: the anterior and the posterior. The anterior-posterior axis of the supernumerary limbs appears to be governed by the adjacent anterior and posterior surfaces of the stump and graft. The supernumerary regenerates can be thought of as "intercalary" regenerates in the sense that appropriate structures are interposed between adjacent anterior and posterior surfaces of stump and transplant. This explanation does not attempt to formulate the mechanisms by which the anterior-posterior axis is established, by which it can be modified, or by which supernumerary regenerates are produced, but experiments are in progress to investigate further the interaction between the stump and regenerate.

### Proximal-Distal Organization

Spallanzani (1768) was perhaps the first to note that irrespective of the level of amputation of a regenerating amphibian limb, the regenerate that formed was almost an exact replica of that which it replaced. The mechanisms by which a regenerate is able to complete appropriately the proximal-distal sequence of structures in an appendage are unknown. The experiments reported here attempt to assess the degree to which stump-blastema interactions play a role in ensuring that a regenerate will normally form structures appropriate to its level of origin. In these experiments, blastemas originating at one limb level were transplanted to a different limb level at various developmental stages. All control limbs, where blastemas were

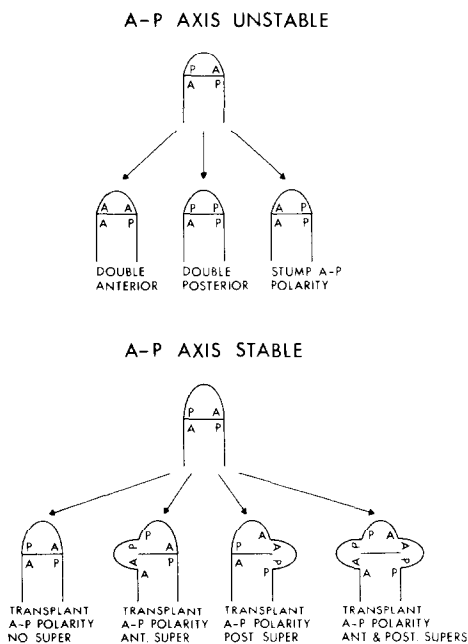


FIG. 26. Diagram of the interaction between stump and blastema when the anterior-posterior (A-P) axis of the blastema is unstable and when it is stable. A, anterior; P, posterior; SUPER(S), supernumerary regenerate(s).

transplanted at various stages of development to the same limb level, were completely normal in that they showed neither deleted nor added structures in the proximal-distal axis.

When a blastema originates at a proximal level and is transplanted at the stages of early bud or medium bud to a distal level, 30 and 70%, respectively, of the resulting limbs with their regenerates show serial duplication of some skeletal elements in the proximal-distal axis. Typically, two complete sets of zeugopodial elements will be represented, and in some cases the distal end of the stylopodial element is represented twice. Similar transplantations performed at late bud and early digits give the same results in 100% of the cases. These results indicate that by the stage of medium bud, a stage which is still completely undifferentiated, the proximal boundary of the blastema is firmly specified and that this specification is not modified by contact between the blastema and a stump of a more distal level. At the earliest stage tested, early bud, only 30% of the resulting limbs showed serial duplication. The fact that 70% of the cases formed a normal sequence of elements along the proximal-distal axis can be interpreted in several ways. First, it is possible that damage to the very small blastema (<0.5 mm in length) results in deletion of presumptive elements; second, it is possible that cells from the stump are added to this small blastema and become a dominant influence in the resulting regenerate; third, it is possible that the proximal boundary of the blastema is not firmly fixed by the stage of early bud and can be modified by contact with the stump, resulting in a limb with the normal proximal-distal sequence of skeletal elements. Recent experiments by Stocum (submitted for publication), using *Ambystoma*, have yielded essentially the same results as those reported here. The ability of undifferentiated as well as more advanced blastemas to develop au-

tonomously is consistent with the findings of Stocum (1968) and de Both (1970) following the transplantation of limb blastemas to nonlimb regions of the animal. These authors report that such blastemas form almost all structures distal to the level of amputation from which the transplanted blastema arose. Further evidence of blastema autonomy, possibly even of autonomy of the individual cells of the blastema, comes from recent work by Wallace and Madon (personal communication) and Wallace (1972). In experiments performed using axolotls in connection with a rather different problem, blastemas were caused to develop on distal limb stumps but were composed of cells that had migrated from or originated in more proximal limb regions. In several instances (one is shown in Wallace, 1972, Fig. 4a), serially duplicated limb parts were produced. Dinsmore (1974) in a recent experiment reported the production of proximal limb elements from distal limb blastemas transplanted to the orbit and supplemented in mass by minced whole limb muscles. It is possible that these results are attributable to the fact that cells derived from proximal muscle cells provided the most proximal boundary of the supplemented blastema, rather than being attributable to the effect of increased cell mass. de Both (1970) has postulated that the proximo-distal sequence of structures formed by a blastema is dependent upon the mass of the blastema, i.e., the greater the mass of the blastema, the more proximal the structures which form. This hypothesis does not seem to be applicable to adult newt limb regeneration where no significant differences in the size of limb blastemas from proximal and distal levels of amputation are found until the stage at which differentiation begins (Iten and Bryant, 1973; see also Carlson, 1974). Furthermore, attempts to repeat experiments in which the mass of the blastema is increased have not led to the formation of more proximal limb ele-

ments (Stocum, submitted for publication).

The results of the experiments reported here in which proximally derived blastemas were transplanted to distal limb levels are consistent with but do not prove the idea that the most proximal stump cells which contribute to the blastema determine the most proximal boundary of the regenerate. However, a few of the transplants that were not considered in the results because they had shown signs of extensive regression of the distal portion of the transplant nevertheless went on to form structures appropriate to the original level of the blastema (see Fig. 16).

All blastemas transplanted from a proximal to distal level were situated in such a manner that the anterior-posterior axis was reversed with respect to the anterior-posterior axis of the stump, and supernumerary regenerates arose along the anterior and/or posterior edge of the limb, as discussed in the previous section. The proximal-distal organization of the supernumerary regenerates ranged from single digits to regenerates consisting of the distal portion of the stylopodium, complete zeugopodium, and autopodium. When two supernumerary regenerates were formed, in many cases one of them was appropriate to the level of origin of the transplant and the other consisted of a proximal-distal sequence of skeletal elements appropriate to the level of the stump; however, in a small number of cases (six) both supernumeraries consisted of structures appropriate to the level of origin of the transplanted regenerate. From the proximal-distal organization of the supernumerary regenerates it appears that either they are derived from the transplanted regenerate or they are derived from the adjacent stump but are influenced by the transplanted regenerate. These possibilities are currently being investigated in our laboratory.

The results of the proximal to distal transplantation experiments show that in

the undifferentiated blastema the proximal boundary of the regenerate is firmly fixed and that proximity to a more distal limb stump does not modify this boundary, with the possible exception of early bud-stage regenerates. However, somewhat different results are obtained for the reciprocal (distal to proximal) transplantation. In distal to proximal transplants the resulting limb regenerates have neither deficiencies nor duplications along their proximal-distal axis. Furthermore, the lengths of the intercalated zeugopodial elements are the same as those in normal regenerates from a proximal level amputation. The results are the same for various stages of regeneration including the cases where well differentiated digital stage regenerates were transplanted. They suggest either that the transplanted blastema has become respecified to form structures appropriate to its new level along the limb or that intercalary regeneration from the stump or the transplant has occurred. Intercalary regeneration from the stump appears to be the most plausible explanation for three reasons. First, the results of transplantation from a proximal to distal limb level indicate an inability of the blastema to respecify its proximal boundary. Second, histological examination of transplanted regenerates has shown that, when a regenerate at the stage of early digits is transplanted from a distal to a proximal level, both the transplant and adjacent stump undergo dedifferentiation, whereas, when a regenerate of the same stage is transplanted to the same level limb stump, very little dedifferentiation of the adjacent stump occurs. Schotté *et al.* (1941) also found that regenerates transplanted to the same level in the limb could inhibit dedifferentiation in denervated larval limb stumps. Third, many of the limbs resulting from distal to proximal transplantation operations possess extra skeletal elements or separate supernumerary regenerates as a result of the opposition of the anterior-posterior axes of stump and

blastema. These supernumerary structures were found only in the distal portion of the zeugopodium and autopodium of the resulting regenerate, that is, the supernumerary structures were all appropriate to the level of origin of the transplanted blastema. There were never any supernumerary elements within the intercalated stylopodium and zeugopodium. Furthermore, the asymmetry of these intercalated elements was always appropriate to the stump.

The relevance of these results to the findings of others working with the chick limb bud must be considered. Summerbell *et al.* (1973) (see also Wolpert *et al.*, 1974) have performed experiments on developing chick limbs in which either portions of the proximal-distal axis were deleted or extra portions were added. They report that very little regulation occurred, so that the resulting limbs possessed either serial deletions or serial duplications. These results led Summerbell *et al.* (1973) to propose a model for the establishment of the proximal-distal limb sequence that postulates a progress zone at the distal tip of the limb. Cells acquire information about their relative position in the limb as they leave the progress zone, based on the amount of time they have spent in the zone. This model predicts that no regulation can take place outside of the progress zone for either intercalary excesses or intercalary defects. Although it has been suggested that this hypothesis may account for the specification of the proximal-distal axis during limb regeneration as well as limb development (Summerbell *et al.*, 1973; Smith *et al.*, 1974; Wolpert *et al.*, 1974), our results do not support this model. Taken on their own, the results of transplantation from a proximal to a distal limb level are consistent with the "progress zone" model, in that little or no regulation occurs in these cases and limbs with serially duplicated elements are formed. In contrast, the results of the distal to proximal transplants clearly

show that regulation for intercalary defects, however this occurs, can take place without the involvement of a distal progress zone. Again we must emphasize that for a variety of reasons discussed previously, particularly the production of supernumerary structures and the histological evidence, we discount the idea that these results are attributable to the death of the transplanted blastemas. Similar results have been obtained by Stocum (submitted for publication) using regenerating limbs of *Ambystoma* larvae. Furthermore, experiments similar to those performed by Summerbell *et al.* (1973) have also been performed in chick limbs by Hampé (1959) and Kieny (1964a-c, 1967; see also Sengel, 1974). These authors report that chick limbs possess considerable powers of regulation for both excesses and deletions in the proximal-distal sequence.

In considering models to describe the way in which the proximal-distal sequence of limb elements might be laid down, one is tempted to find a model that will account for the results in chick limbs as well as the results reported here. However, the results of similar experiments in the two systems are different enough to make a parsimonious but comprehensive model impossible. With the evidence presently available, the simplest model to account for our results is based on the ones proposed by Rose (1962) (Rose's rule of distal transformation) from studies on amphibian limb regeneration, P. Bryant (1971, 1974) from studies on regeneration of imaginal disc fragments of *Drosophila*, and Bohn (1965) from studies on cockroach leg regeneration. In this interpretation it is postulated that the limb possesses a proximal-distal gradient of developmental capacity (Fig. 27a). This capacity is expressed when a portion of the limb gradient is removed (Fig. 27b). A blastema forms at the cut surface and is capable of forming all the missing distal levels of the limb gradient but not the more proximal levels. In order to account for our

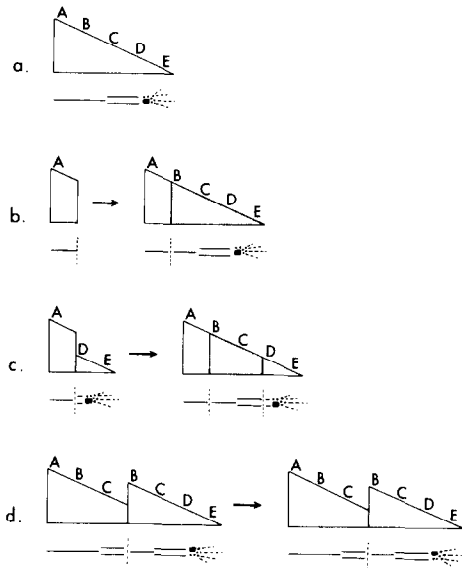


FIG. 27. Diagram of the gradient of development capacity in the regenerating newt limb. Values A, B, C etc. correspond to particular portions of the limb, as shown beneath the gradients. a, A complete gradient, representing the entire appendage; b, normal regeneration from a proximal level; c, distal to proximal transplant leading to intercalary regeneration; d, proximal to distal transplant leading to tandem duplication of limb elements.

results we have to postulate that the stimulus to produce a blastema at any level along the limb gradient is dependent upon that surface being free or being in contact with more distal limb levels, with a "step-down" in the gradient (Fig. 27c). No blastema is formed when a given limb level is in contact with the same or a more proximal level (Fig. 27d). Formation of a blastema in the distal to proximal transplants will result in the production of the missing levels in the gradient. The presence of the most distal parts of the limb gradient (in the transplant) will inhibit such an intercalary blastema from forming all the limb levels distal to its site of origin. It should be pointed out that similar experiments in insects (Bohn, 1970a, b; 1971, Bullière, 1971; Shaw and Bryant, 1974b) lead to intercalary regeneration in both proximal

to distal and distal to proximal combinations, the former involving reversal of polarity in the intercalary regenerate. The difference in the results of similar experiments in cockroaches and newts may be due to the overall polarity which nerves exert on the stump-graft combinations in newts, but this idea has not yet been tested. Alternatively, it is possible that the recognition of such a step in the proposed gradient requires that the interacting tissues both be undifferentiated. Some support for this latter idea comes from other experiments (Gritz and Bryant, unpublished) where a low frequency of intercalary duplications with reversed polarity is found in cases where different levels within the blastema are brought into contact with one another.

From the experiments reported in this paper we can conclude that both the proximal-distal and the anterior-posterior organization of the blastema become established early in the regeneration process. However, the two axes are not irrevocably linked, since some proximal to distal transplants formed serially duplicated structures in the proximal-distal axis (indicating establishment of the proximal boundary of the blastema) but failed to show retention of their original handedness (indicating instability in the anterior-posterior axis). The reciprocal result, where a transplant retained its handedness but failed to show specification in the proximal-distal axis, was never obtained. Experiments are being conducted to define further the interaction that occurs between stump and blastema during regeneration and to investigate the cellular origin of supernumerary and intercalary regenerates.

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