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Authors

Krasner, Gary N
Bryant, Susan V

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Distal Transformation from Double-Half Forearms in the Axolotl, *Ambystoma mexicanum*

GARY N. KRASNER AND SUSAN V. BRYANT

*Center for Pathobiology and Department of Developmental and Cell Biology, University of California,
Irvine, California 92717*

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The capacity for distal transformation of surgically produced symmetrical forearms of the axolotl, *Ambystoma mexicanum*, was tested. The polar coordinate model for pattern regulation in epimorphic fields states that positional information is a property of cells which reflects their position in the field, specifically, by their location on a radius and a circumference of the field. The model proposes that for complete distal transformation to occur, a complete circumference of positional values must be present at the plane of amputation. Previous experiments have shown that surgically constructed, symmetrical upper arms of axolotls and newts are not capable of regenerating a distally complete pattern. In the present experiment, double-posterior and double-anterior symmetrical forearms were produced by exchanging the anterior half of the right forearm with the posterior portion of the left forearm. The grafts were allowed to heal for 5-35 days, at which time the limb was amputated through the center of the grafted region. Ninety-five percent of the double-anterior (left) forearms and 77% of the double-posterior (right) forearms either did not regenerate or regenerated symmetrical patterns. The regenerated patterns always converged, i.e., lost structures along the line of symmetry. The length of the healing time did not affect the number of elements regenerated. The remaining limbs regenerated asymmetrical structures, either normal, or parts of normal, hands. These results are compared with those of similar experiments previously performed on the upper arms of axolotls and newts and the hindlimbs of salamanders and are discussed in light of a model for distal transformation.

INTRODUCTION

The polar coordinate model for pattern regulation in epimorphic fields states that positional information is a property of cells which reflects their position in the field, specifically, by their location on a radius and a circumference of the field. The model proposes that complete distal transformation can only occur from a complete circumference of positional values at the plane of amputation (French *et al.*, 1976). Recent experiments in both newts and axolotls lend support to this idea. When surgically constructed symmetrical upper arms are amputated 1 or more months after grafting, distal transformation either does not occur or is defective (Bryant, 1976; Bryant and Baca, 1978; Tank, 1978a). The nature of these incomplete regenerates has suggested a hypothesis about the mechanism of distal

transformation (Bryant, 1978; Bryant and Baca, 1978; Bryant *et al.*, submitted). This hypothesis proposes that each successive level of the pattern distal to the plane of amputation is generated by intercalation across short arcs of the circumference. Hence, a complete circumference of positional values at one limb level generates a complete circumference at the next more distal level. Furthermore, this hypothesis predicts that when the circumference at the plane of amputation is symmetrical, the successive levels which are generated by intercalation across short arcs will also be symmetrical, but each level will lose positional values on either side of the plane of symmetry due to interactions across the midline. This loss of midline circumferential positional values (pattern contraction) leads to premature termination of the proc-

ess of distal transformation, since eventually so few circumferential positional values are present that intercalation ceases. Hence, it follows that some distal transformation can occur from incomplete circumferences of positional values and that a complete circumference of positional values is only necessary for *complete* regeneration, i.e., complete not only in the proximal-distal axis, but also around the circumference.

This model also predicts that the closer the amputation plane is to the distal end of the limb, the more likely it is that the distal tip of the pattern will be regenerated during distal transformation. Previous experiments by Stocum (1978) on the lower legs of salamanders have shown that the distal tip of the pattern can indeed be reached after amputation through a surgically constructed symmetrical shank. In this paper, we have investigated the prediction further by studying the behavior of symmetrical lower arms in axolotls. Our results are compared to those of Stocum (1978) in the lower leg of salamanders and those of Tank (1978a), Tank and Holder (1978), Bryant (1976), and Bryant and Baca (1978) in the upper arm of axolotls and newts.

MATERIALS AND METHODS

Male and female larval axolotls (*Ambystoma mexicanum*) ranging in size from 56 to 122 mm were utilized in these experiments. The animals were obtained from two spawnings of eggs raised in the laboratory at the University of California, Irvine. Animals were fed beef liver and maintained in individual plastic containers in 50% Holtfreter solution which was changed daily. They were maintained at room temperature ($18 \pm 2^\circ\text{C}$) except as noted below.

All animals were anesthetized by immersion in MS222 (ethyl *m*-aminobenzoate methanesulfonate, Eastman), diluted 1:1000 in 50% Holtfreter solution, before the grafting and amputation procedures.

Grafting procedure. Anesthetized animals were positioned so that their forelimbs were extended and at right angles to the

body. The forearm was split into anterior and posterior halves by cutting between digits II and III, continuing between the radius and ulna to the level of the elbow. On the right arm, the radius was disarticulated from the humerus and the soft tissue was cut perpendicular to the long axis of the arm at the elbow to separate completely the anterior half of the forearm (axial designations according to Carlson, 1974). On the left side, the posterior half was removed by disarticulating the ulna and cutting the soft tissue. Before contralateral exchange of the separated halves, the protruding proximal end of the radius or ulna was trimmed to improve the fit of the graft after transfer. The resulting double-half forearms were double-posterior on the right side (containing two ulnae and digits IV, III, III, and IV), and double-anterior on the left side (containing two radii and digits I, II, II, and I). This procedure is illustrated in Fig. 1. Sham-operated control limbs were prepared by removing one-half of the forearm, trimming the protruding proximal end of the bone, and replacing it *in situ*. Strips of lens paper were wrapped around the double-half forearms to support the grafts during healing. The animals were then returned to individual boxes containing 50% Holtfreter solution and placed in a refrigerator ($\sim 5^\circ\text{C}$) to reduce their activity. At the end of 4 days, the animals were removed from the refrigerator and maintained at room temperature for the duration of the experiment. The grafts were observed at 3- to 4-day intervals throughout the course of the experiment.

Amputation procedure. The grafts on experimental limbs were allowed to heal for 5 to 35 days. At the end of the specified period, the animals were anesthetized and amputated through the middle of the double-half forearm (Fig. 1). Control limbs were allowed to heal for 30 days prior to amputation. The protruding ends of the radii or ulnae were trimmed to provide a flat amputation surface.

Regeneration was allowed to proceed to

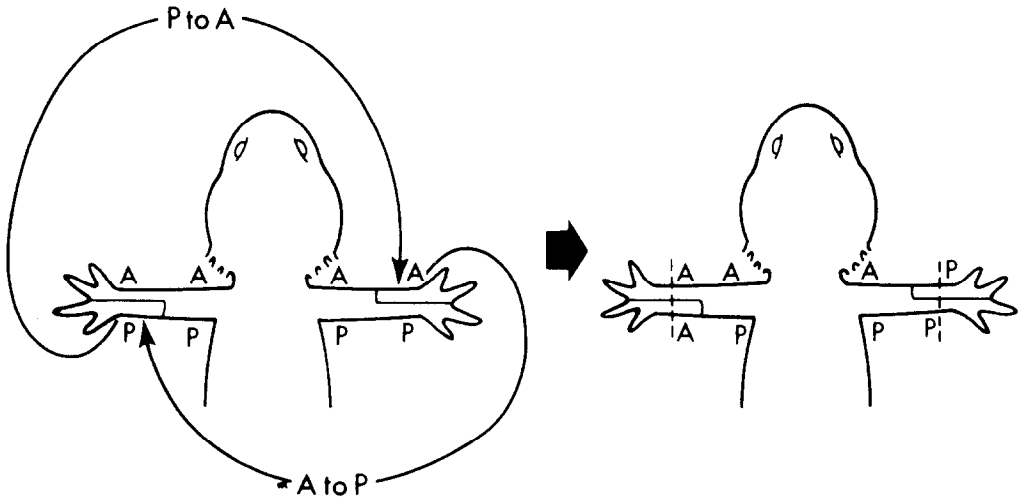


FIG. 1. Experimental design. The anesthetized animal was positioned with its arms extended. The anterior half of the right forearm was removed and exchanged with the posterior portion of the left forearm. This creates a double-anterior left forearm and a double-posterior right forearm. After allowing the graft to heal for different lengths of time, the limbs were amputated through the midforearm.

the stage of digital outgrowth (Tank *et al.*, 1976) or until no change was noted for a 14-day period. At that time, the limbs were drawn with the aid of a camera lucida, removed at the shoulder, photographed, and fixed in Bouin's solution. The limbs were stained as whole mounts with Victoria blue B (Bryant and Iten, 1974) to reveal the skeletal pattern.

Criteria for identifying skeletal elements. The normal hand of the axolotl has eight carpals, four metacarpals, and nine phalanges (Fig. 2). There are four digits, numbered from the radial (anterior) border I, II, III, and IV. Digits on symmetrical or incomplete hands are identified using the following morphological markers: Digit III has three phalanges, while the other digits have two; the metacarpals of digits I and II both articulate with the same basal carpal; the metacarpal of digit I articulate with this carpal and with the cartilago prepollicis; digits III and IV articulate separately with their basal carpals (nomenclature from Francis, 1934).

RESULTS

Controls. Five of seven control-anterior and three of four control-posterior limbs

regenerated normal asymmetrical limbs. The remaining limbs were also asymmetrical and had minor deviations from normal of the type often found in regenerated limbs. None of the controls developed supernumerary limbs.

Experimental groups. All forearms in which the graft became either detached or obviously necrotic were not considered. It should be noted that grafts of posterior halves were, in general, less successful than grafts of anterior tissue. The results of the remaining limbs are shown in Table 1. Of a total of 60 double-anterior forearms amputated at different times after grafting, 57 (95%) either failed to form any new separate skeletal elements or regenerated outgrowths which were symmetrical in the anterior-posterior plane. Of a total of 43 double-posterior forearms, 33 (77%) either failed to regenerate or formed symmetrical outgrowths. The remaining limbs in each group (5% of the double-anterior limbs and 23% of the double-posterior limbs) regenerated either normal limbs or limbs classified as partial regenerates based on their incomplete asymmetrical skeletal patterns.

Limbs with symmetrical regenerates and no regenerates. In both double-anterior

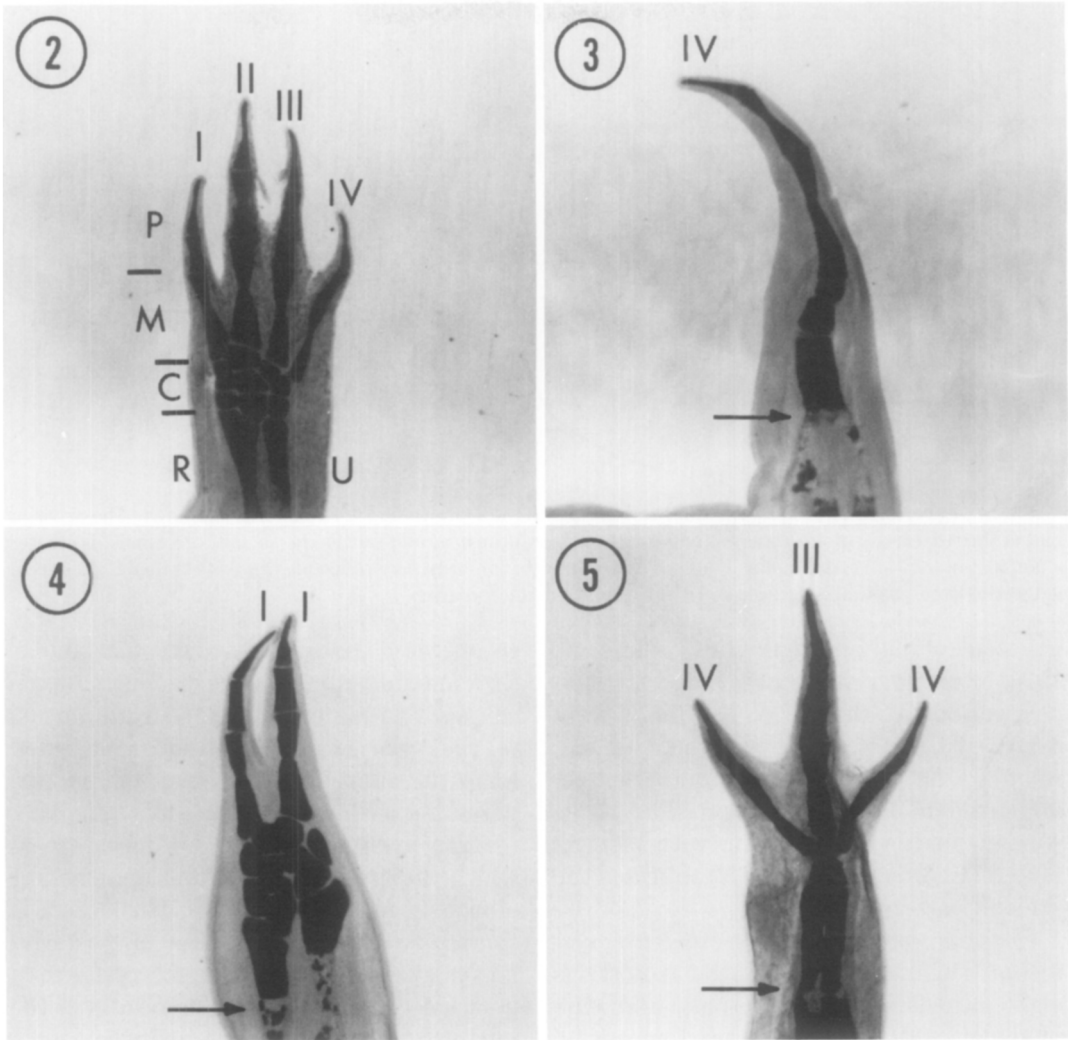


FIG. 2. A normal right forearm of an axolotl. Digits are numbered from anterior to posterior. Criteria for identifying digits: Digit III has three phalanges, while the other digits have two; metacarpals of digits I and II both articulate with the same basal carpal; metacarpal I articulates with an additional carpal; digits III and IV articulate separately with their basal carpals. Abbreviations: R—radius; U—ulna; C—carpals; M—metacarpals; P—phalanges. 8 \times .

FIG. 3. A one-digit symmetrical regenerate (digit IV) from a double-posterior forearm. Arrow indicates plane of amputation. 5 \times .

FIG. 4. A two-digit symmetrical regenerate (digits I and I) from a double-anterior forearm. Arrow indicates plane of amputation. 5 \times .

FIG. 5. A three-digit symmetrical regenerate (digits IV, III, and IV) from a double-posterior forearm. Arrow indicates plane of amputation. 8 \times .

and double-posterior limbs, symmetrical regenerates ranged from tapering structures without any digits to regenerates with one, two, or three digits (Figs. 3, 4, and 5). Despite the fact that each limb had four digits initially, none of the symmetrical regener-

ates developed four digits, and in fact, the majority of limbs regenerated fewer than three digits. Hence, in all cases where symmetrical outgrowths formed, pattern contraction occurred. Analysis of skeletons showed that in all cases there was a loss of

TABLE 1
TYPES OF LIMBS REGENERATED FOLLOWING AMPUTATION OF SYMMETRICAL FOREARMS

Type of limb	Time of amputation (days)	Total limbs	No regen. ^a	Symmetrical			Regenerates		Asymmetrical regenerates			
				Less than one digit ^b	One digit	Two digits	Three digits	Total digits	Mean number digits	Mean number elements	Normal regen.	Partial regen. ^c
2A	5-10	24	2	1	5	9	6	21	1.95	12.66	1	0
	20	10	0	0	6	3	1	10	1.5	10.2	0	0
	30-35	26	1	1	10	5	7	23	1.78	11.18	1	1
	Total	60	3	2	21	17	14	54	1.79	11.57	2	1
2P	5-10	15	6	1	4	0	0	5	0.8	6.24	1	3
	20	9	2	0	5	0	0	5	1.0	6.2	2	0
	30-35	19	4	2	5	0	4	11	1.54	7.97	4	0
	Total	43	12	3	14	0	4	21	1.23	7.13	7	3

^a No new separate skeletal elements regenerated.

^b Terminating at the level of the wrist.

^c This group consists of two 2-digit and two 1-digit asymmetrical regenerates.

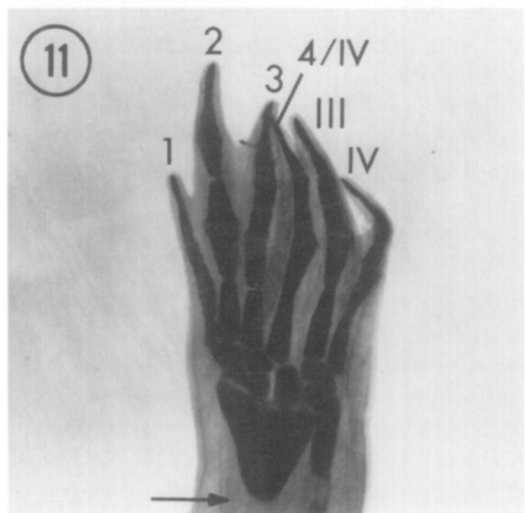
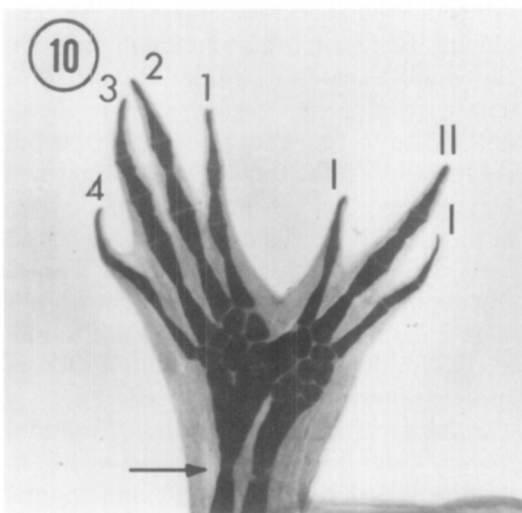
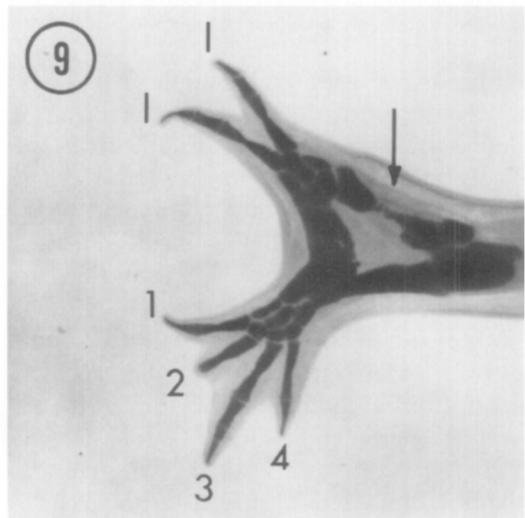
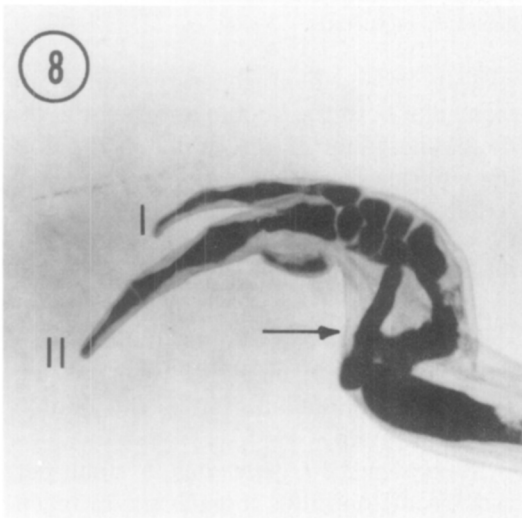
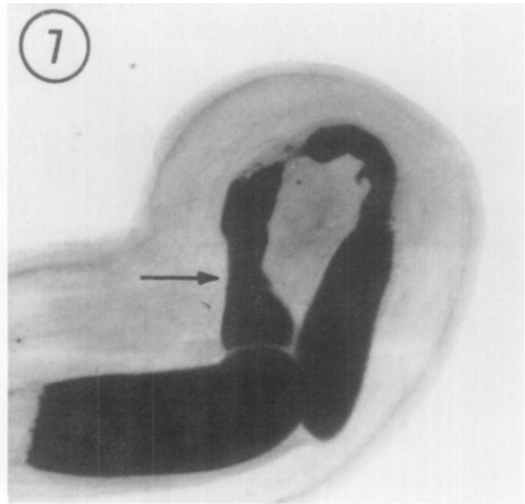
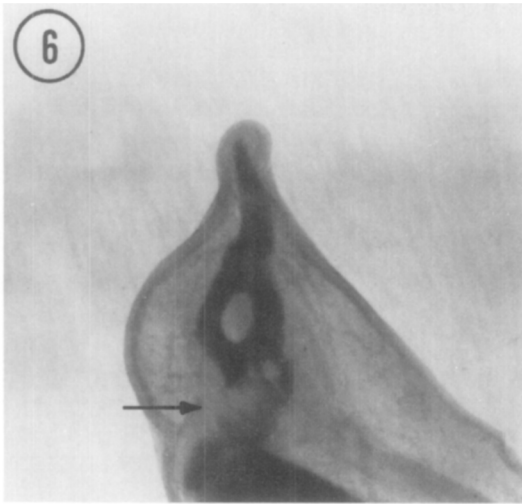
midline structures. For example, in a regenerate with three digits which arose from a double-posterior limb (Fig. 5), the digits are easily identified as digits IV, III, and IV, with the plane of symmetry appearing to pass through digit III. This result indicates that during regeneration of a limb that began with digits IV, III, III, and IV, parts of both of the digits III have been lost. Similarly, a regenerate with two digits which arose from a double-anterior limb with an original digital complement of I, II, II, and I consisted of digits I and I (Fig. 4). Both digits II have failed to form in this regenerate. When only one digit forms as illustrated in Fig. 3, this digit, in most cases, can be identified with certainty as being a digit from the periphery rather than from the center of the original pattern. The regenerate, a single digit IV, was derived from a limb possessing digits IV, III, III, and IV. It is evident from Table 1 that even further reduction in the pattern of the regenerated limb sometimes occurs, with the symmetrical limb terminating at the level of the wrist (Fig. 6). In the most extreme cases, limbs failed to form any new separate skeletal structures distal to the amputation plane (Fig. 7). Examination of Table 1 shows that while various types of symmet-

rical limbs and nonregenerating limbs are formed in both the double-anterior and the double-posterior groups, limbs in the double-anterior group regenerate on average a larger number of skeletal elements and a larger number of digits than do limbs in the double-posterior group.

The results of amputation at different times showed no clear trends in the number of digits or skeletal elements regenerated in either the double-anterior or the double-posterior group.

Asymmetrical regenerates. A small percentage of the limbs in each group regenerated asymmetrical structures. In the double-anterior group, this amounted to 5%; in the double-posterior group, it was 23%. These asymmetrical regenerates are classified in Table 1 as either normal or partial regenerates. Of the total of 13 asymmetrical regenerates found in both groups, 9 were normal limbs. More normal limbs regenerated in the double-posterior group than in the double-anterior group. Only four limbs were classified as partial regenerates, and all had different skeletal patterns. One of these is shown in Fig. 8.

Supernumerary regenerates. Supernumerary limbs often developed during regeneration of both double-anterior and double-



posterior forearms and at all of the different healing times. Supernumerary limbs developed in association with all types of regenerates and nonregenerates, with one notable exception. In the nine limbs classified as normal regenerates, supernumerary limbs did not form. Of the remaining limbs, 50 of 94 (53%) formed supernumerary limbs. All of these were formed on the grafted side of the double-anterior or double-posterior limb. In all cases in which it was possible to determine handedness, the supernumerary limbs had the same handedness as the stump (Fig. 10). The degree of separation between the regenerate arising from the distal amputation plane and the supernumerary regenerate varied from case to case. In Fig. 9, the supernumerary limb is clearly separated from the distal symmetrical regenerate. In some cases, as illustrated in Figs. 10 and 11, the supernumerary outgrowth and the symmetrical outgrowth from the plane of amputation are poorly separated. A higher percentage of supernumerary limbs developed in association with double-posterior regenerates (67%) than formed in association with double-anterior regenerates (45%).

DISCUSSION

The experiments described in this paper were performed to compare the capacity for distal transformation of surgically con-

structed symmetrical forearms of axolotls with the previously described behavior of similarly constructed upper arms and legs of both salamanders and newts (Tank, 1978a; Tank and Holder, 1978; Stocum, 1978; Bryant, 1976; Bryant and Baca, 1978). We have found that double-half forearms form symmetrical regenerates with fewer digits than the original limb. Although these results may appear to be unlike those obtained with surgically constructed double-half upper arms, we believe that the underlying cellular behavior is the same in both cases and that our results can be understood in terms of the model for distal transformation proposed by Bryant (1978) and Bryant and Baca (1978). This model suggests that distal transformation is accomplished by intercalation between circumferential positional values beginning at the plane of amputation and that each newly intercalated circumferential value assumes a progressively more distal value than the adjacent identical circumferential value.

This model makes several predictions about regeneration from surgically constructed symmetrical limbs:

(1) *Symmetrical limbs will show incomplete distal transformation.* In symmetrical limbs, interactions between like positional values on either side of the midline will cause progressive loss of circumferential po-

FIG. 6. A regenerate terminating in the wrist from a double-anterior forearm. Arrow indicates plane of amputation. 14 \times .

FIG. 7. A nonregenerate from a double-posterior forearm. Arrow indicates plane of amputation. 16 \times .

FIG. 8. A partial regenerate from a double-anterior forearm. Digits I and II arise on the host side of the limb. Arrow indicates plane of amputation. 9 \times .

FIG. 9. A clearly separated supernumerary limb (with left handedness) on a two-digit symmetrical regenerate from a double-anterior (left) forearm. The digits on the supernumerary limb are numbered from the anterior border with Arabic numerals. Arrow indicates plane of amputation. 5 \times .

FIG. 10. A supernumerary limb (with left handedness) on a three-digit symmetrical regenerate from a double-anterior (left) forearm. While the separation between the supernumerary limb and the distal symmetrical regenerate is not as pronounced as that of the specimen shown in Fig. 9, it can be seen that the carpals and digits are separated. Arrow indicates plane of amputation. 6 \times .

FIG. 11. A supernumerary limb (with right handedness) on a three-digit symmetrical regenerate from a double-posterior (right) forearm. There is no separation of the two hands. The supernumerary hand (Arabic numerals) and the symmetrical regenerate (Roman numerals) share the fourth digit. Arrow indicates plane of amputation. 7 \times .

sitional values on the line of symmetry as each new radial value is generated (Fig. 12). The degree of this loss will depend upon the extent of interactions occurring across the midline. Any interactions between po-

sitional values within each of the halves will not lead to pattern contraction. Incomplete distal transformation occurs after amputation of both surgically constructed upper arms (Bryant, 1976; Bryant and Baca,

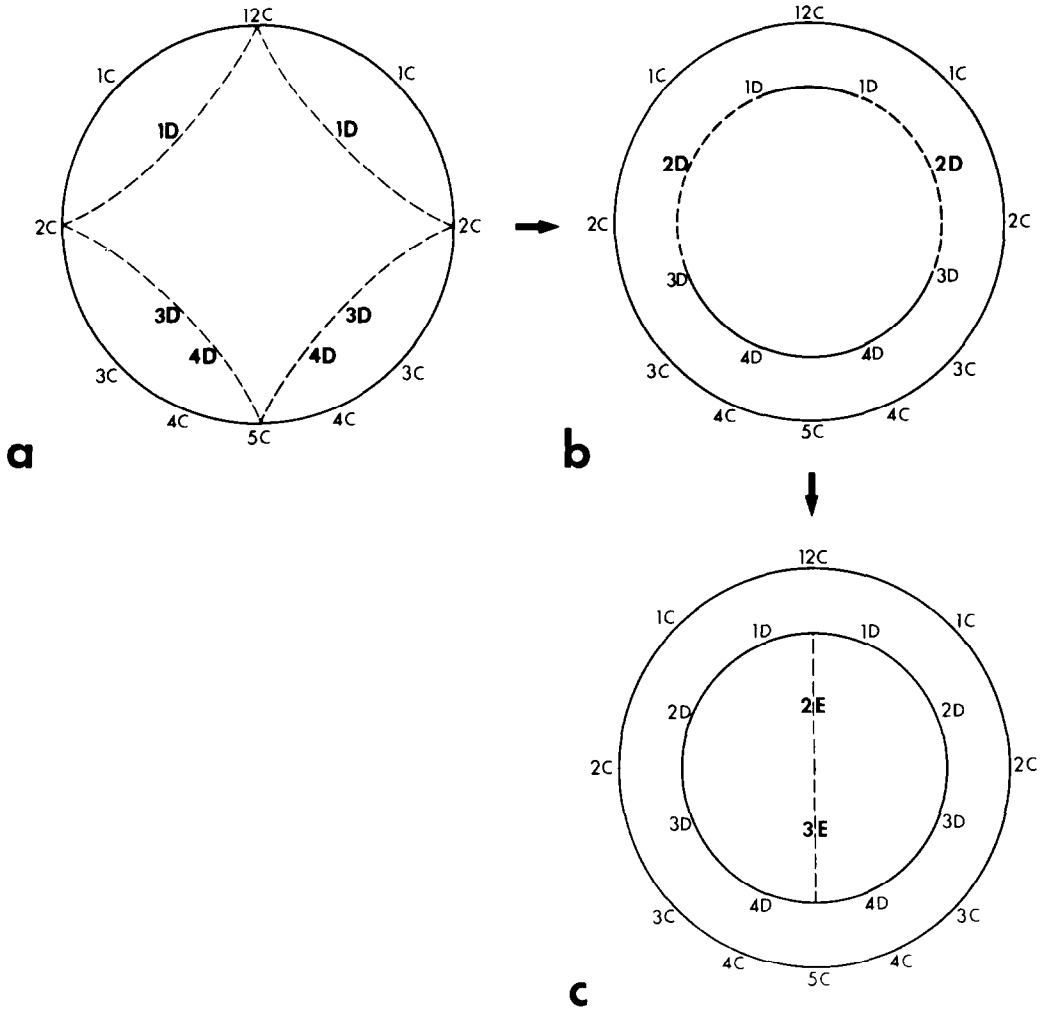


FIG. 12. A representation of the interactions between cells with positional values across the midline of a symmetrical forearm (based on an illustration from Bryant (1978) and Bryant and Baca (1978)). The midforearm amputation plane is level C. The distal tip of the limb is level E. The newly intercalated distal levels are indicated with dashed lines and boldtype. The unequal spacing of positional values may represent the spacing *in vivo* of a double-posterior forearm, as suggested in the text. (A full circumference is represented by positional values 1–12.) For simplicity, only cells of circumferential positional values 12, 2, and 5 are shown interacting. (a) Normally nonadjacent cells of level C come into contact with each other during formation of the blastema. This interaction causes intercalation of the intervening positional values which assume the next most radial value (D) to the adjacent identical circumferential values. (b) Intercalation between cells of level D produce the intervening values at the same level to complete this circumference. Note that midline values have been lost in the formation of level D. (c) Intercalation between cells of level D will generate level E, the distal level of the pattern. Note that the number of circumferential positional values at level E is considerably less than that at the starting level, level C.

1978; Tank, 1978a) and in the experiments reported here on lower arms.

(2) *Regenerates from surgically constructed symmetrical limb stumps will be symmetrical.* As mentioned above, the vast majority of the double-anterior and double-posterior forearm regenerates was symmetrical. A small minority of the regenerates was classified as partial regenerates, and these were asymmetrical. The elements which were regenerated were always on the host side of the limb. Similar partial limbs have been shown to develop in other experiments on double-half limbs (Tank and Holder, 1978; Holder *et al.*, 1979). It may be that the grafted tissue failed to participate in the outgrowth, while the host tissue developed autonomously, but the presence of the graft prevented the host tissue from replacing the entire limb. The regeneration of a small number of normal, asymmetrical limbs in the present study can be attributed either to loss of the graft followed by regeneration of a normal limb from the remaining half limb stump (Bryant and Baca, 1978) or to regression of the limb stump after amputation proximal to the level of the graft (i.e., back to a level at which only normal tissues contributed to the blastema). This explanation is supported by the observation that these normal asymmetrical regenerates are never accompanied by the supernumerary limb expected if the graft is present (see below). The predominant occurrence of normal regenerates in the double-posterior group correlates well with the difficulty of making this particular graft combination and the obvious loss of grafts in this group immediately after surgery.

(3) *Symmetrical regenerates from more distal planes of amputation will be more likely to contain the most distal elements of the pattern.* Given the same starting number of circumferential positional values which permit a given number of rounds of intercalation before distal transformation ceases, amputation through a symmetrical limb at a distal level is more likely to lead

to regeneration of the distal tip prior to termination of intercalation than amputation at a more proximal level. This is due to the systematic loss of positional values in symmetrical stumps as each round of intercalation proceeds. Therefore, many more double-half limbs in these experiments regenerated terminal elements (i.e., one or more digits) than did double-half limbs amputated at a more proximal level (Bryant and Baca, 1978; Tank, 1978a).

(4) *Regenerates from symmetrical stumps will show progressive loss of midline pattern elements.* The majority of symmetrical regenerates in these experiments does taper toward the tip, and some pattern loss always occurs on the midline. Variation in the extent to which midline loss occurs during distal transformation ranges from the minimum loss, leading to regeneration of three rather than four digits, to the maximum loss, leading to regeneration of less than one digit, or no regeneration.

(5) *The extent of distal transformation will depend upon the number of circumferential positional values present at the plane of amputation.* In other words, the larger the starting number of positional values at the level of amputation, the more rounds of intercalation will be possible before all positional disparities are resolved and distal transformation ceases. In the present experiments, double-anterior forearms produced on the average a greater number of new skeletal elements than did double-posterior forearms, thus implying that the anterior half of the forearm contains more circumferential positional values than the posterior half. This is in contrast to the finding by Tank (personal communication) that in the upper arm, positional values appear to be clustered in the posterior half. Tank and Holder (1979) have recently suggested that the apparent clustering of positional values may correlate simply to the amount of tissue in each half of the limb. Thus in the upper arm, more cells are present in the posterior half than

in the anterior half. It is possible that the apparent clustering of positional values in the anterior of the lower arm may correlate with a larger number of cells in this half.

The one result of the present study which appears to be inconsistent with what is known about the behavior of double-half upper arms in axolotls concerns the effect of variation in graft healing time prior to amputation. Experiments by Tank and Holder (1978) and Holder *et al.* (1979) have shown that the extent of distal transformation from symmetrical upper arms is inversely correlated with the amount of graft healing time before amputation. Similar results have been reported by Stocum (1978) in experiments on upper hindlimbs of salamanders. This correlation has been attributed by Holder *et al.* (1979) to a possible lack of interactions across the graft junction (i.e., across the line of symmetry) at shorter healing times, permitting each half of the double-half limb to regenerate autonomously. At longer healing times, when graft and host tissues are more intimately healed together, cells on either side of the junction are presumed to be free to interact across the midline during blastema formation, leading to a loss of midline positional values and hence to a loss of structures during distal transformation. In the most extreme cases, little or no regeneration results. Our results do not show any effect of graft healing time, and similar experiments by Stocum (1978) on the shank of the hindlimb of the salamander also fail to show any effect. Furthermore, in our experiments, the number of cases showing the extreme condition of little or no regeneration is small. We can only speculate that factors such as the details of how the wound heals and the topology of the amputation surface strongly influence where and which cells will be more likely to confront each other. In the lower arm, the amount of healing along the graft-host junction may have less influence on the pattern of cellular interactions than some of these other fac-

tors. The converse may be true in the upper arm, as argued by Holder *et al.* (1979).

The results presented here show some similarities to the recent results of Slack and Savage (1978a, b). These authors amputated symmetrical double-posterior axolotl limbs through the lower arm (wrist region). These symmetrical limbs, in contrast to those described here, were produced following surgical manipulations in the embryo prior to limb bud outgrowth. Nevertheless, as in the case of surgically constructed symmetrical lower arms, all of the regenerates produced from this level of amputation showed a reduction in the width of the pattern due to loss of midline structures. Subsequent reamputations of these regenerates sometimes resulted in the further loss of, or occasionally in a gain of, midline structures.

Finally, as in previous experiments on double-half limbs (Bryant, 1976; Bryant and Baca, 1978), a supernumerary regenerate was frequently formed at the junction between the proximal edge of the graft and the host. In these experiments, such supernumerary regenerates had the position, orientation, and handedness (where this could be determined) predicted by the polar coordinate model (see Bryant, 1976). The degree of separation between the supernumerary regenerate and the symmetrical terminal regenerate varied from case to case. It is likely that this variation reflects the length of graft tissue remaining after dedifferentiation at both the proximal and the distal ends of the graft. In the well-separated cases, the length of remaining graft tissue would be the longest. In the least separate cases, the length of remaining graft tissue would be the shortest. In some cases, fusion occurred between the supernumerary limb and the regenerate, resulting in a loss of structures lying on the midline between the two outgrowths (Fig. 11). In these examples, it is possible that the entire graft dedifferentiated. Similar fusions between a supernumerary limb and a

graft of opposite handedness have been observed previously (Iten and Bryant, 1975; Tank, 1978b). It is likely that the mechanisms we have proposed to account for loss of midline structures in symmetrical regenerates can also account for the loss of structures when a supernumerary regenerate and a distal regenerate grow out side by side.

In summary, we feel that the results obtained in this study are entirely consistent with the model for distal transformation and with the previous results obtained with surgically constructed symmetrical upper arms.

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