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Supernumerary Limbs in Amphibians: Experimental Production in *Notophthalmus viridescens* and a New Interpretation of Their Formation

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The formation of supernumerary limbs was studied in the adult newt, *Notophthalmus viridescens*. Forelimb blastemas at the stages of medium bud and early digits were either transplanted to the contralateral forelimb with their dorsal-ventral axis opposed to that of the limb stump, or removed, rotated through 180°, and replaced on the same limb stump with both dorsal-ventral and anterior-posterior axes opposed to those of the stump, or as a control, removed, and replaced in normal orientation. Supernumerary limbs were produced in both experimental series, but not in the controls.

Following contralateral transplantation, supernumerary limbs arose close to the graft junction at the two positions where dorsal limb tissue was in contact with ventral limb tissue. Both dorsal and ventral supernumerary limbs were of the same handedness as the limb stump and they were mirror-images of the regenerate developing directly from the transplanted blastema. Following 180° rotation, supernumerary limbs arose close to the graft junction at those positions where anterior-ventral and posterior-dorsal limb tissues were in contact. The supernumerary limb which arose in the posterior-dorsal position with respect to the limb stump was a mirror-image of the transplant, and was therefore of opposite handedness to both transplant and stump. The supernumerary limb which arose in the anterior-ventral position was of the same handedness as both transplant and stump. A new model of pattern regulation in epimorphic fields which can account for these results and which has retrospective value in the interpretation of earlier experiments on developing limbs is discussed.

INTRODUCTION

Early approaches to the problem of pattern formation in amphibian embryos involved the transplantation of developing organs either orthotopically or heterotopically in various orientations. In many of the experiments performed on developing limb buds and regeneration blastemas it was noted that supernumerary limbs would form in addition to the transplanted limb, particularly in those cases in which transplantation was to a limb site or to the flank (see Harrison, 1921; Swett, 1926). The reasons for the development of supernumerary limbs were not understood, and often they were regarded as complicating factors in the experiments. In this paper, we will show that it is possible to understand the formation of supernumerary limbs following certain transplantation

procedures if it is assumed that their formation is a consequence of the regulative properties of the epimorphic limb field.

It has been shown that throughout the development of the limb regenerate of *Notophthalmus viridescens* interactions occur between the blastema and the adjacent limb stump (Iten and Bryant, 1975). These interactions were demonstrated by combining blastemas and stumps such that their anterior-posterior axes were opposed. At an early stage of regeneration, when the anterior-posterior axis of the blastema had been established but was not stable, contact with a stump of opposite polarity resulted in the formation of a limb in which the anterior-posterior organization was either intermediate between that of the original location of the blastema and that of the graft site, or com-

pletely like that of the graft site. However, at later stages of regeneration when the anterior-posterior axis of the transplanted blastema was stable, contact with a stump of opposite polarity led to the formation of separate supernumerary limbs at either the anterior or the posterior or at both surfaces of the limb stump. On the basis of these results, it was proposed that at the positions of maximum axial misalignment, supernumerary limbs were produced by intercalary regeneration between the adjacent anterior and posterior surfaces of the stump and the transplant.

In order to more clearly define the type of axial misalignment which can lead to supernumerary limb formation, the interaction between the blastema and the stump leading to the formation of supernumerary limbs has been further investigated under conditions in which the dorsal-ventral axes alone, or both the dorsal-ventral and anterior-posterior axes have been opposed. The results of these experiments, in particular the occurrence, number, location, orientation and handedness of supernumerary limbs, are explained in terms of a new model for pattern regulation in epimorphic fields (French, Bryant, and Bryant, 1976). Furthermore, the results of classical experiments dating back to the time of R. G. Harrison are discussed in relation to this model.

MATERIALS AND METHODS

Male and female adult newts (*Notophthalmus viridescens*) were maintained at a constant temperature of 25°C and on a 12-hour light cycle. They were kept in individual finger bowls and fed with Tubifex three times each week. The spring water in the bowls was changed daily. Chloretone (Parke, Davis and Co.) was used as an anesthetic when amputations and operations were performed.

A total of 118 newts were used, and of these 73 were analyzed at the end of the experiment. The remainder either died

too early to be analyzed or had unsuccessful grafts. Both forelimbs of each animal were amputated through the proximal half of the humerus, and the tissues at the amputation site were trimmed to give a flat wound surface. Developing regenerates were staged according to the criteria described by Iten and Bryant (1973), and transplantations of regenerates were performed at the stages of medium bud (MB) and early digits (ED). When regenerates were transplanted, care was taken not to include any stump tissue with the transplant. At both stages used, the original site of amputation was distinct due to pigmentary differences between the regenerate and the stump. Both mesodermal and epidermal tissues of the regenerate were transplanted, and all transplants were made to the forelimbs of the same animal to preclude the possibility of immunological reactions (Cohen, 1971). In most cases, a carbon mark was placed on the mid-dorsal position of the blastema to ensure that the transplant was properly situated on the recipient limb stump. Following transplantation, the animals were placed on damp cotton in such a way as to reduce the likelihood that the transplanted tissue would be dislodged. In the case of transplants at ED, small rectangles of sterile lens paper were placed over the graft junction to hold the transplanted regenerate in place during wound-healing. All operated animals were kept at 10–12°C for the first 24 h to reduce their mobility. For the remainder of the experiment they were placed in spring water and kept at 25°C.

The following types of transplantation operations were performed (Fig. 1).

Control transplantations. A right limb blastema was removed and replaced without rotation on the same limb stump. Both the dorsal-ventral and anterior-posterior axes of the blastema and stump were aligned normally.

Opposition of the dorsal-ventral axis alone. A left limb blastema was trans-

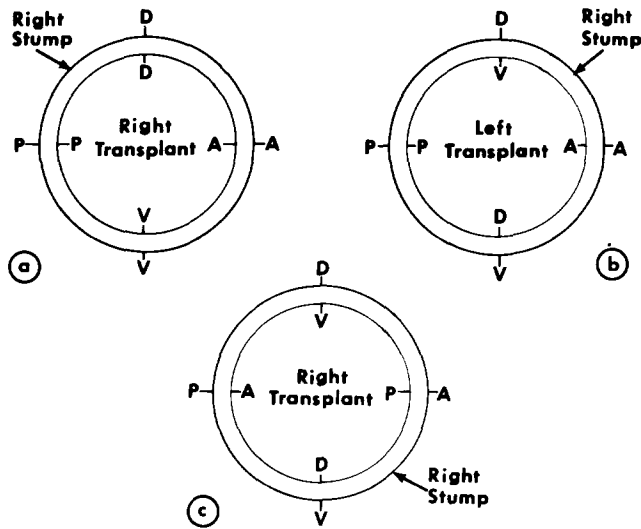


FIG. 1. Diagram of operations. Outer circles represent limb stumps, inner circles represent grafts. (a) Control transplantation in which a right limb blastema was removed and replaced in normal orientation; (b) contralateral transplantation of a left limb blastema to a right limb stump with anterior-posterior axes aligned and dorsal-ventral axes opposed; (c) 180° rotation of a right limb blastema on its limb stump. A, anterior; P, posterior; D, dorsal; V, ventral.

planted to a right limb stump from which a blastema of a similar stage had been removed. The anterior-posterior axes of the blastema and stump were aligned, and the dorsal-ventral axes were opposed.

Opposition of both dorsal-ventral and anterior-posterior axes. A right limb blastema was removed, rotated around the proximal-distal axis through 180° and replaced on the same limb stump, such that both the dorsal-ventral and anterior-posterior axes of the blastema and stump were opposed.

All limbs bearing transplanted regenerates were observed daily during the first week following operation, and three times each week during the subsequent weeks. Any experimental limbs showing signs of resorption of the transplanted tissue were discarded (see Iten and Bryant, 1975). Development of the transplanted regenerates was briefly arrested; however, they became revascularized between 3 and 9 days after grafting. Occasional regression of one or more of the digits was sometimes observed when the transplant was a regenerate at ED.

When the transplanted regenerates reached the stage of late digits and no further changes were apparent in the experimental limbs, they were amputated at the shoulder, placed in Bouin's fixative, and stained as whole mounts with Victoria Blue (Bryant and Iten, 1974) in order to examine the skeletal elements. Details of the arrangement of the skeletal elements of regenerated forelimbs have been described (Iten and Bryant, 1975). All experimental and control limbs were analyzed to determine their orientation and handedness and these interpretations relied on the following criteria. The dorsal surface of the regenerate can be distinguished from the ventral surface because it is usually more darkly pigmented. In addition, the digits usually curve towards the ventral surface. However, neither of these characteristics is sufficiently reliable to be used alone, and more reliable markers are the cartilago pre-pollicis carpal which lies in a more ventral position than the other carpals, and the crista ventralis humeri, a muscle attachment site located in an

anterior-ventral position on the proximal region of the humerus. The anterior surface can be distinguished from the posterior surface on the basis of the digital formula, the fact that digits one and two articulate with the same basal carpal, the clear difference between the small anterior carpals (the radiale and cartilago pre-pollicis) and the large posterior carpal (the fused pars ulnaris and pars intermedia), and the fact that the radius lies in the anterior position and the ulna in the posterior position.

RESULTS

Control Transplantations

Twenty control transplantations (11 at MB; 9 at ED) were performed in which regenerates were removed from their stumps and replaced in normal orientation. All of the transplants formed normal limbs and none had any additional skeletal elements.

Opposition of Dorsal-Ventral Axis Alone

Regenerating left limbs developing from an amputation site in the proximal half of the stylopodium were transplanted at MB or ED to the contralateral right limb stump from which a regenerate of a similar stage and level of amputation had been removed. The transplanted regenerates were positioned such that their dorsal-ventral axis was opposed to that of the stump and the anterior-posterior axes of transplant and stump were aligned.

This type of transplantation operation was followed in many instances by the production of supernumerary limbs (Table 1). The percentage of operations leading to the development of supernumerary limbs was larger at ED (80%) than at MB (57%). In most instances, supernumerary limbs which formed after transplantation of ED regenerates were more complete in the proximal-distal axis and more clearly separated from the transplant than were those which formed following operations at MB. In all but two of the cases where supernumerary limbs formed, the transplant was well developed and clearly retained its original handedness. The remaining four cases in which the transplant's handedness was indeterminate did not form supernumerary limbs, although two formed extra skeletal elements, usually in dorsal or ventral locations. These extra elements could not be identified during their development as separate supernumerary structures since their development was temporally coincident with that of the transplant. A further three specimens, one of which also formed a clear supernumerary regenerate, formed additional skeletal elements.

The supernumerary limbs arose in the region of the graft junction, in either a dorsal position (Figs. 2 and 3), a ventral position, or in both positions (Figs. 4 and 5). The frequency of occurrence of limbs in each of these locations is shown in Table

TABLE 1
RESULTS OF TRANSPLANTING LIMB BLASTEMAS WITH THEIR DORSAL-VENTRAL AXIS
OPPOSED TO THAT OF THE STUMP

Stage of transplant ^a	Total number of cases	Handedness of transplant		Occurrence of supernumerary limbs			
		Same as site of origin	Indeterminate	Number forming super. limbs	Number forming dorsal super. limb only	Number forming ventral super. limb only	Number forming both dorsal and ventral super. limbs
MB	14	8 (57%)	6 (43%)	8 (57%)	1 (7%)	3 (21%)	4 (29%)
ED	15	15 (100%)	0 (0%)	12 (80%)	1 (7%)	5 (33%)	6 (40%)
Total	29	23 (79%)	6 (21%)	20 (69%)	2 (7%)	8 (28%)	10 (34%)

^a MB, medium bud; ED, early digits.

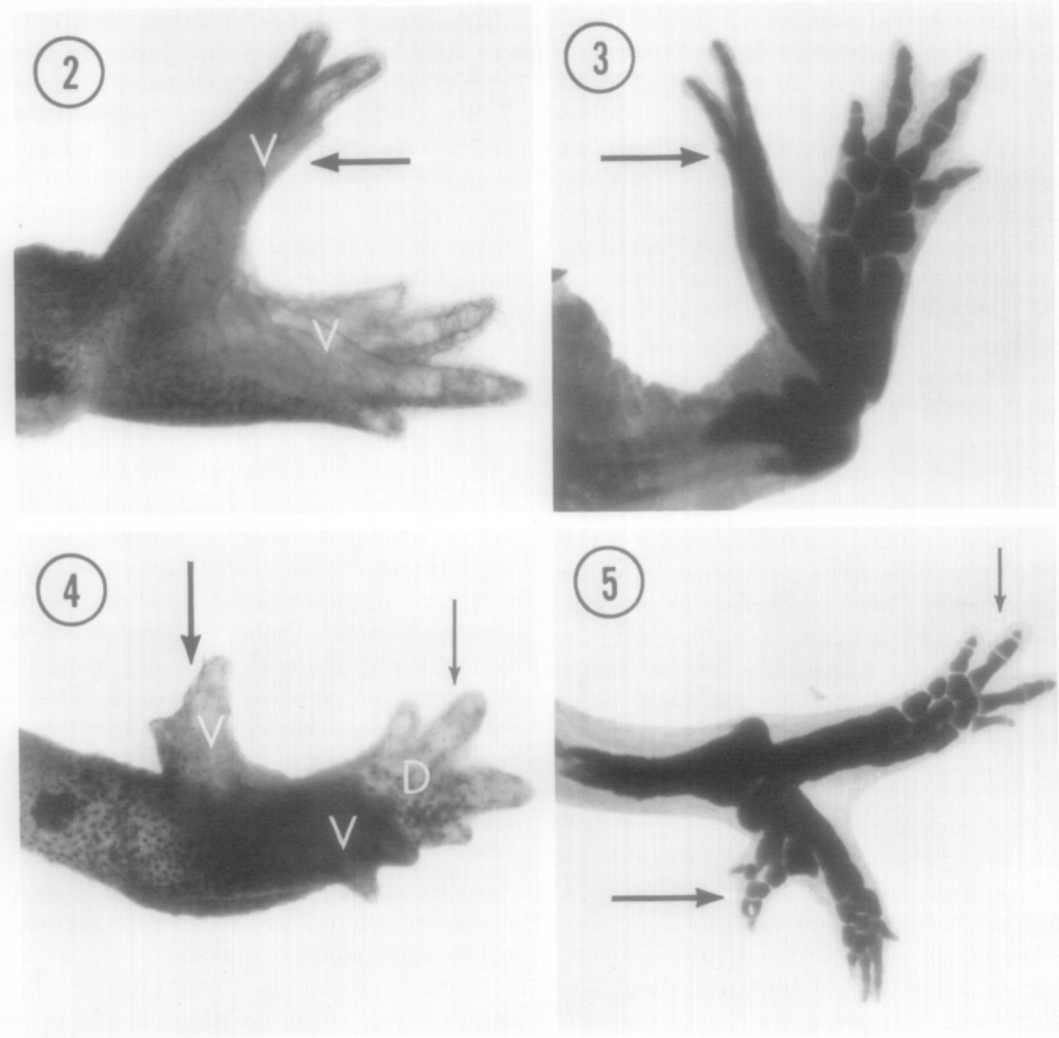


FIG. 2. Limb with a single dorsal supernumerary limb (arrow) which formed after contralateral transplantation of a regenerate at the stage of early digits from the left to the right limb stump with dorsal-ventral axes opposed. The pale, ventral surface (V) of the transplant (lower hand) is uppermost and faces the ventral surface of the supernumerary limb. $\times 30$.

FIG. 3. Skeletal preparation of limb shown in Fig. 2. The ventral surface of the transplant is seen. The dorsal supernumerary limb is indicated by the arrow. $\times 25$.

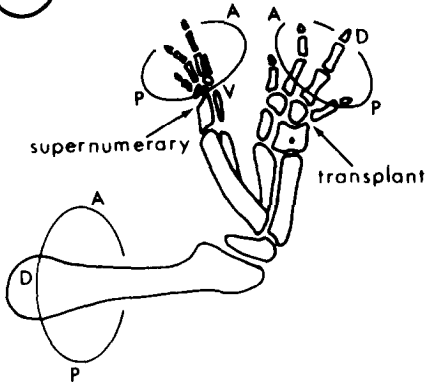
FIG. 4. Limb with both dorsal and ventral supernumerary limbs which formed after contralateral transplantation of a regenerate at the stage of early digits. The ventral supernumerary limb (thin arrow) is better developed than the dorsal one (thick arrow). The dorsal surface (D) of the ventral supernumerary limb and the ventral surfaces (V) of the transplanted regenerate and the dorsal supernumerary limb are seen in this figure. $\times 15$.

FIG. 5. Skeletal preparation of limb shown in Fig. 4. The upper hand in the figure is the ventral supernumerary limb (thin arrow) seen from the dorsal side. The middle hand is the hand developed from the transplant, also seen from the dorsal side. The lower, least well developed supernumerary limb (thick arrow), is in fact dorsal in location and its dorsal surface is seen here. $\times 11$.

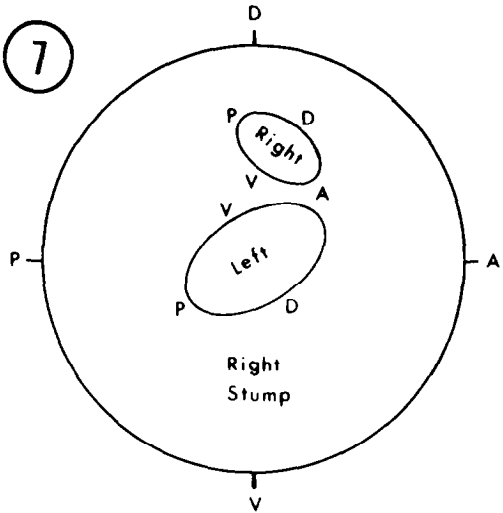
1. Ventral supernumerary limbs were first seen, on the average, 18 days after transplantation, whereas dorsal supernumerary limbs did not arise until slightly later (an average of 20 days after transplantation).

An analysis of each supernumerary limb was made to determine its handed-

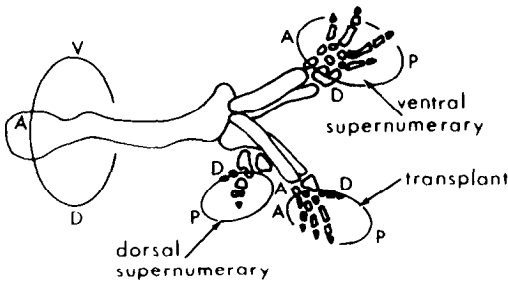
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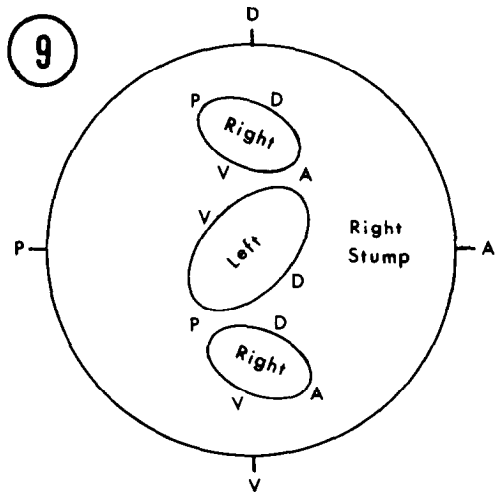


FIG. 6. Diagram of limb shown in Figs. 2 and 3, to illustrate the relationship of the supernumerary limb with respect to the limb stump and the transplant. Ellipses are shown to indicate orientation. Abbreviations as in Fig. 1.

FIG. 7. Diagram of the limb shown in Figs. 2 and 3, to illustrate the orientation and handedness of the transplant, supernumerary limb, and limb stump, as viewed from the distal end of the limb. The limb stump is shown as the outer circle; the transplant as the large ellipse and the supernumerary limb as the small ellipse. The minor axes of the ellipses correspond to the flattened dorsal-ventral axes of the distal regions of the transplant and supernumerary limb. The dorsal supernumerary limb is a mirror-image of the transplant. Abbreviations as in Fig. 1.

FIG. 8. Diagram of the limb shown in Figs. 4 and 5 to illustrate the relationship between the dorsal and ventral supernumerary limbs, the limb stump and the transplant. Ellipses are shown to indicate orientation. Abbreviations as in Fig. 1.

FIG. 9. Diagram of the limb shown in Figs. 4 and 5 to illustrate the orientation and handedness of the transplant (large ellipse), the dorsal and ventral supernumerary limbs (small ellipses), and the limb stump (outer circle), as viewed from the distal end of the limb. See legend to Fig. 7 for explanation of ellipses. Both dorsal and ventral supernumerary limbs are mirror-images of the transplant. Abbreviations as in Fig. 1.

ness and its orientation with respect to the limb stump and transplant. Twenty-two of the total of 30 supernumerary limbs

could be identified as right hands, and in each case, the orientation of this hand was normal with respect to the limb

stump. In all cases in which the supernumerary structures were developed sufficiently to allow this determination to be made the relationship between the limb stump, the transplanted regenerate, and the supernumerary regenerates was similar to that diagrammed in Figs. 6-9. As can be seen from these figures, each supernumerary limb is a mirror-image of the transplant. The remaining eight supernumerary limbs were classed as indeterminate.

Opposition of Both Dorsal-Ventral and Anterior-Posterior Axes: 180° Rotation

Regenerating right limbs, developing from an amputation site in the proximal half of the stylopodium, were removed, rotated around the proximal-distal axis through 180°, and replaced on the same limb stump, at MB or ED. By this procedure, both anterior-posterior and dorsal-ventral axes of transplant and stump were opposed.

This type of operation was followed in many instances by the production of supernumerary limbs (Table 2). Combining the results of both stages at which the transplants were performed, 79% of the cases formed supernumerary limbs of which 41% formed single supernumerary limbs, and 38% formed two supernumerary limbs. There were no cases in which more than two supernumerary limbs formed. The supernumerary limbs arise consistently in

either the posterior-dorsal position (Figs. 10 and 11) the anterior-ventral position (Figs. 12 and 13), or both positions, with respect to the limb stump, but rarely in any other location (see Footnotes to Table 2). As in the experiments in which a single axis of the regenerate was reversed with respect to the limb stump, the supernumerary limbs arose in the region of the graft junction. The anterior-ventral supernumerary limbs began to develop before the posterior-dorsal supernumerary limbs (an average of 15 and 19 days after rotation, respectively).

Rotation of regenerates at MB always led to the development of supernumerary limbs (Table 2). In general, these were much less separated from the transplant than in the case of those produced following transplantation at ED. In 5 out of the 13 operations at ED, no supernumerary limbs arose. In four of these cases, it was clear that the transplant had rotated back towards its normal orientation through 90 to 180°. An additional two specimens underwent a correctional rotation, and in these cases, the supernumerary limbs which formed were rudimentary. The occurrence of similar compensatory rotations have been noted previously in experiments on reoriented limb buds (Harrison, 1917, 1921; Nicholas, 1924b; Ruud, 1926; Swett, 1924, 1926, 1937), but the mechanisms responsible for this behavior are not known.

An analysis of each supernumerary limb

TABLE 2
RESULTS OF 180° ROTATION OF LIMB BLASTEMAS

Stage of transplant ^a	Total number of cases	Handedness of transplant		Occurrence of supernumerary limbs			
		Same as site of origin	Indeterminate	Number forming super. limbs	Number forming AV super. limb only	Number forming PD super. limb only	Number forming both AV and PD super. limbs
MB	11	7 (64%)	4 (36%)	11 (100%)	3 (27%) ^b	2 (18%) ^c	6 (55%)
ED	13	11 (85%)	2 (15%)	8 (62%)	4 (31%)	1 (8%)	3 (23%)
Total	24	18 (75%)	6 (25%)	19 (79%)	7 (29%)	3 (12%)	9 (38%)

^a MB, medium bud; ED, early digits.

^b In one of these specimens, an additional anterior supernumerary limb was also present, and in a second, an additional posterior supernumerary limb.

^c In one of these specimens, an additional anterior supernumerary limb was also present.

was made to determine its handedness and orientation with respect to the limb stump and transplant. Of the 16 supernumerary limbs which arose in an anterior-ventral location with respect to the limb stump, 8 were identified as right limbs which were oriented normally with respect to the limb stump (Figs. 16 and 17). These supernumerary limbs were therefore not mirror-images of the transplant (see Fig. 17). The remaining eight limbs were too poorly developed to be accurately classified. Of the 12 supernumerary limbs which arose in a posterior-dorsal location with respect to the limb stump, 5 were sufficiently developed to be identified as left hands, arranged in mirror symmetry with the transplant (Figs. 14 and 15), and 7 could not be assigned a definitive handedness. The diagrams in Figs. 15 and 17 illustrate schematically the relationship between the supernumerary limbs, the transplant, and the limb stump.

DISCUSSION

Supernumerary Limb Formation

In a previous paper (Iten and Bryant, 1975) we showed that despite the capacity of limb blastemas to undergo self-differentiation (de Both, 1970; Faber, 1971; Stocum, 1968; Stocum and Dearlove, 1972) a limb stump and blastema do interact when they are adjacent to each other. When a regeneration blastema was transplanted to the contralateral limb with the anterior-posterior axes of graft and stump opposed, the interaction between the two led to either a modification of the anterior-posterior organization of the transplanted blastema (at the early stages of regeneration) or to the production of separate anterior and posterior supernumerary limbs (at the later stages of regeneration).

Results presented in this paper show that a similar interaction occurs when a limb blastema and a limb stump are combined such that their dorsal-ventral axes are opposed. When limb regenerates at the stages of medium bud or early digits were

grafted to the contralateral limb stump so that their dorsal-ventral axes were opposed, supernumerary limbs developed in a dorsal and/or a ventral location with respect to the limb stump, but not in other locations. In all cases in which the supernumerary limbs were sufficiently well formed, they could be identified as being of the same handedness and orientation as the stump, and were arranged as mirror images of the transplant. Figure 9 illustrates this relationship diagrammatically. Similarly, following transplantation of a limb blastema to a contralateral stump so that the anterior-posterior axes were opposed, both supernumerary limbs were of stump handedness and orientation, and both were mirror images of the transplant (Iten and Bryant, 1975). This relationship is illustrated in Fig. 18.

Earlier experiments carried out to investigate the time of determination of the axes of the regeneration blastema in axolotls (Lodyženskaja, 1928, 1930), *Triturus cristatus* (Milojević, 1924), and *Triturus taeniatus Triturus alpestris* (Schwidefsky, 1935) provide results which are consistent with our own. Although the formation of clearly separate supernumerary limbs was rare in these experiments, various kinds of double limbs were produced. It appears that when transplants were made so that the anterior-posterior axes of the regenerate and the limb stump were opposed, supernumerary structures formed along the anterior or posterior borders of the regenerate developing from the transplant. When the dorsal-ventral axes were opposed, supernumerary limbs usually formed in a dorsal or ventral position with respect to the transplant. In those cases in which the supernumerary structures were clearly developed, they were mirror images of the transplanted regenerate. Abe-loos and Lecamp (1931) also reported the occurrence of supernumerary limbs under similar conditions, but did not comment on their handedness. From these results on regenerating limbs in which only a single

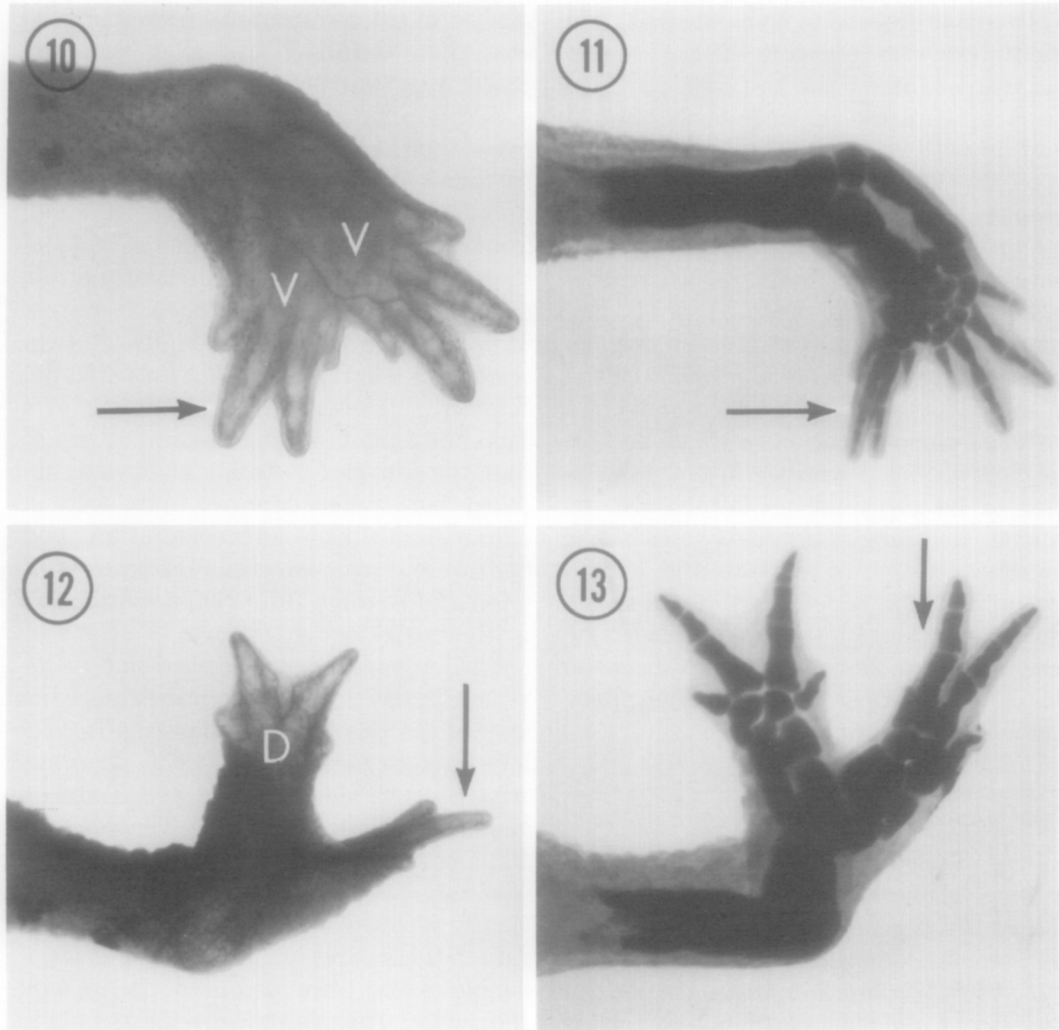


FIG. 10. Limb with a single posterior-dorsal supernumerary limb (arrow) which formed after 180° rotation of a limb regenerate at the stage of early digits. The ventral surfaces (V) of the supernumerary limb and the transplant are uppermost. $\times 16$.

FIG. 11. Skeletal preparation of the limb shown in Fig. 10. The hand to the right in the figure is the inverted limb which developed from the transplant and it is seen from its ventral side. The other hand (arrow) is the posterior-dorsal supernumerary limb. $\times 12$.

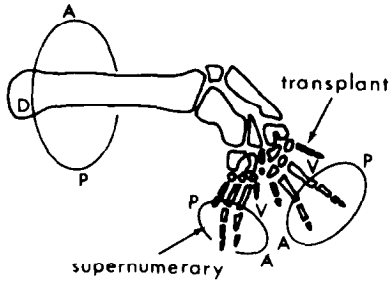
FIG. 12. Limb with a single anterior-ventral supernumerary limb (arrow) which formed after 180° rotation of a limb regenerate at the stage of early digits. The hand to the upper left is the transplant, and in this figure its dorsal surface (D) is seen. $\times 10$.

FIG. 13. Skeletal preparation of the limb shown in Fig. 12. The hand to the left in the figure is the transplant, seen from the dorsal side. The hand to the right is the anterior-ventral supernumerary limb (arrow), also seen from the dorsal side. $\times 11$.

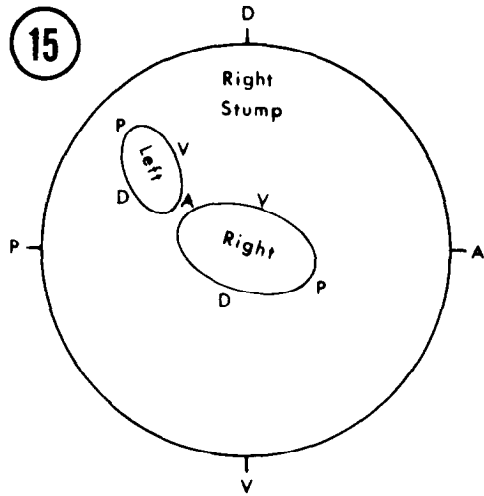
axis is misaligned during grafting, it is possible to conclude that supernumerary limbs are capable of developing wherever posterior limb tissue is confronted with anterior limb tissue, and wherever dorsal limb tissue is confronted with ventral limb tissue.

A reexamination of the results of experiments performed on the developing limb buds of *Ambystoma punctatum*, *Ambystoma tigrinum*, and *Triturus pyrrhogaster* shows that this conclusion is also valid as an explanation for the occurrence or the absence of supernumerary limbs following

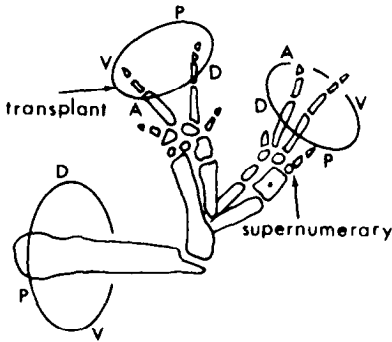
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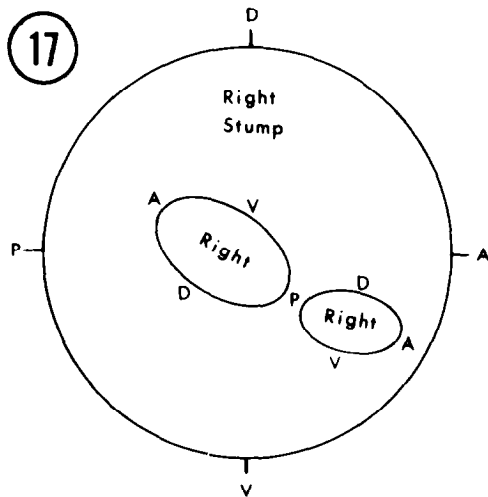


FIG. 14. Diagram of the limb shown in Figs. 10 and 11 to illustrate the relationship between the posterior-dorsal supernumerary limb, the limb stump, and the transplant. Ellipses are shown to indicate orientation. Abbreviations as in Fig. 1.

FIG. 15. Diagram of the limb shown in Figs. 10 and 11 to illustrate the orientation and handedness of the transplant (large ellipse), the limb stump (outer circle), and the posterior-dorsal supernumerary limb (small ellipse), as viewed from the distal end of the limb. See legend to Fig. 7 for explanation of ellipses. The posterior-dorsal supernumerary limb is a mirror-image of the transplant. Abbreviations as in Fig. 1.

FIG. 16. Diagram of the limb shown in Figs. 12 and 13 to illustrate the relationship between the anterior-ventral supernumerary limb, the limb stump, and the transplant. Ellipses are shown to indicate orientation. Abbreviations as in Fig. 1.

FIG. 17. Diagram of the limb shown in Figs. 12 and 13 to illustrate the orientation and handedness of the transplanted regenerate (large ellipse), the limb stump (outer circle), and the anterior-ventral supernumerary limb (small ellipse), as viewed from the distal end of the limb. See legend to Fig. 7 for explanation of ellipses. The anterior-ventral supernumerary limb is not a mirror-image of the transplant. Abbreviations as in Fig. 1.

certain transplantation operations. For example, when limb buds of developing amphibian embryos were removed and replaced on the limb site in the normal ori-

entation (orthotopic, homopleural, anterior-anterior, dorsal-dorsal, medial-medial) no tissue from the posterior of the limb was in contact with tissue from the

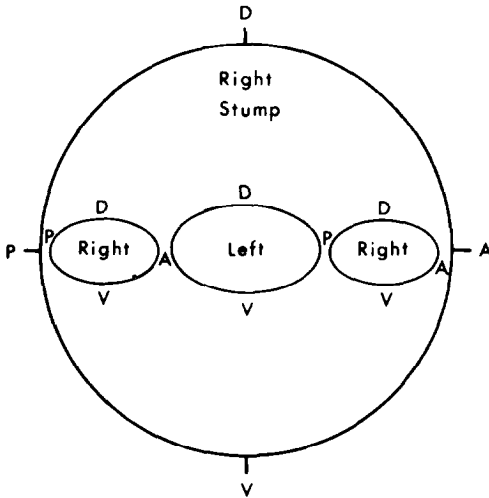


FIG. 18. Diagram to illustrate the relationship between a transplanted regenerate (large ellipse), the limb stump (outer circle), and the anterior and posterior supernumerary limbs (small ellipses) following contralateral transplantation of a limb regenerate with anterior-posterior axes opposed, as viewed from the distal end of the limb. See legend to Fig. 7 for explanation of ellipses. Both supernumerary limbs are mirror-images of the transplant. Abbreviations as in Fig. 1. (From the results of Iten and Bryant, 1975.)

anterior of the limb, and no dorsal limb tissue was in contact with ventral limb tissue. Consequently, no supernumerary limbs would be expected to form, and with rare exceptions (Stultz, 1936, with one case of a supernumerary limb), none did form (Harrison, 1917, 1921; Ruud, 1926). Likewise, when limb buds were transplanted to a contralateral limb site at a stage prior to the establishment of the dorsal-ventral axis, with the anterior-posterior axis in normal orientation (orthotopic, heteropleural, anterior-anterior, dorsal-ventral, medial-medial), supernumerary limbs would not be expected and they rarely formed (Harrison, 1917, 1921; Ruud, 1926; Stultz, 1936). On the other hand, when whole limb buds were transplanted to the contralateral limb site with the anterior-posterior axes of the transplant and limb site opposed (orthotopic, heteropleural, anterior-posterior, dorsal-dorsal, medial-medial) supernumerary limbs were fre-

quently produced (Harrison, 1917, 1921; Ruud, 1926; Stultz, 1936; Swett, 1926). In those cases in which the supernumerary limbs were adequately described, they were found to occur, as expected, at the anterior or the posterior margin of the transplant, and they were mirror images of the transplant. Similarly, when limb buds were rotated through 180° and replaced on the same limb site at a stage in development prior to the establishment of the dorsal-ventral axis, the anterior-posterior axes of the rotated limb bud and its base were opposed (orthotopic, homopleural, anterior-posterior, dorsal-ventral, medial-medial). In these cases, supernumerary limbs were formed, and where they are described, they were located on the anterior or posterior limb margin, and they were usually mirror images of the transplant (Blount, 1935; Harrison, 1917, 1921; Ruud, 1926; Swett, 1926).

Transplantations of developing amphibian limb buds to the contralateral limb site at a stage after the establishment of the dorsal-ventral axis, and with the dorsal-ventral axes of transplant and limb site opposed, are surprisingly rare in the literature. However, one series of such operations was performed by Swett (1930) (orthotopic, heteropleural, anterior-anterior, dorsal-ventral, medial-medial), who found that supernumerary limbs developed in connection with almost all of the transplants. Although these supernumerary limbs were not described in detail, it can be inferred that the juxtaposition of dorsal and ventral limb tissues was responsible for their initiation.

From the results discussed above on regenerating and developing amphibian limbs, the conclusion that supernumerary limbs tend to form whenever tissue from the anterior part of the limb is placed next to tissue from the posterior part of the limb, or when dorsal limb tissue is placed next to ventral limb tissue seems to be clearly reasonable. Similar conclusions about the formation of supernumerary

structures have been drawn from experiments in which tissues within the mature limb have been reoriented with respect to each other. When limbs with such rearranged tissues are amputated, regenerates with multiple autopodia and digits frequently form (Carlson, 1974, 1975; Droin, 1959; Glade, 1957; Lheureux, 1972, 1975; Rahmani, 1960; Settles, 1967).

It might be expected then, that when developing regenerates are rotated through 180° and replaced on their original limb stumps, thereby opposing both dorsal and ventral limb tissue and anterior and posterior limb tissue, at least four supernumerary limbs would form, corresponding to the positions at which these tissues were in contact. A larger number of supernumerary limbs would be expected if such outgrowths arise in positions of maximum disharmony between transplant and stump. However, in our experiments, a maximum of two supernumerary limbs was formed after 180° rotation of a regenerate on its limb stump. These supernumerary limbs were not randomly placed with respect to the limb stump, but occurred in anterior-ventral and posterior-dorsal locations. Furthermore, unlike the supernumerary limbs which developed following juxtaposition of anterior and posterior, or of dorsal and ventral limb tissues, only one of the two supernumerary outgrowths (the posterior-dorsal supernumerary) was a mirror-image of the transplant. Specifically, the rotated regenerate was a right limb, the posterior-dorsal supernumerary was a left limb, and the anterior-ventral supernumerary was a right limb like the transplant, but unlike the transplant it was positioned in normal orientation with respect to the right limb stump. Milojević (1924) and Lodyženskaja (1930) performed similar 180° rotations of developing regenerates in *Triturus cristatus* and in axolotls, respectively, to investigate the time of axis determination, and both authors report the occurrence of some supernumerary limbs which were not mir-

ror-images of the transplant. Cameron and Fallon (1974) report the occurrence of supernumerary limb elements in *Xenopus laevis* following 180° rotation of the distal tip of the developing limb bud.

Among the transplantation experiments performed on developing urodele limbs, similar experiments to those just described, at stages when both dorsal-ventral and anterior-posterior axes were determined have not been reported. Those experiments which were performed on stages before the establishment of the dorsal-ventral axis, (orthotopic, homopleural, anterior-posterior, dorsal-ventral, medial-medial) might be expected to give results similar to those in which the anterior-posterior axis alone had been opposed, i.e., the development of supernumerary limbs in anterior and/or posterior locations which were mirror-images of the transplants. As reported by Blount (1935), Harrison (1917, 1921), Ruud (1926), Stultz (1936), and Swett (1926), this is the result in the majority of the cases. However, all of these authors (see also Swett, 1924) describe some exceptional cases which did not conform to Bateson's (1894) rules of symmetry (as reformulated by Harrison, 1921), that is, some cases in which one supernumerary of a duplex or triplex did not form a mirror-image of the transplanted limb. These exceptions may represent instances in which the dorsal-ventral axis had already been established.

A New Interpretation of Supernumerary Limb Formation

Until the present time, no satisfactory explanation for the production of supernumerary limbs in amphibians or in other animals has been put forward, even though their formation following certain grafting procedures has been noted since the earliest experiments on amphibian limb buds (see Harrison, 1917). Those explanations which have been developed may address certain features of their production, but not all. For example, Milo-

jević (1924) was of the opinion that supernumerary limbs from the host stump arise when part of the stump is left uncovered in grafting. This explanation cannot account for the predictable location of supernumerary limbs following a particular type of transplantation, nor can it account for the fact that following 180° rotation of a limb bud or regenerate, one supernumerary appendage is a limb of opposite handedness to that of the stump. Przibram's (1926, 1931) suggestion that one supernumerary limb may be produced by distal transformation (regeneration) from the limb stump and one by distal transformation (duplication) from the transplant, would account for the handedness of supernumerary regenerates in all types of transplantation operations, but it does not account for their constant locations.

In the early years of this century, a great variety of experimental manipulations on developing amphibian limb buds were performed, not just those described above, but others in which limb buds were grafted to the flank region of the embryo. In many of these cases supernumerary limbs were found to develop. It appeared to Swett (1926) that a disturbance of nearly any kind to the limb bud before it becomes differentiated, could cause the formation of supernumerary limbs. However, experiments in which limb buds were transplanted to sites other than the limb site and the flank adjacent to the limb (Dettwiler, 1930; Nicholas, 1924a; Swett, 1945) showed that supernumerary limb formation was very rare or absent, regardless of the orientation of the graft with respect to its new surroundings. These results suggested that an influence of the flank region or of the limb site itself on the transplanted limb was necessary for supernumerary limb formation.

An understanding of the production, location, orientation and handedness of supernumerary limbs following various grafting procedures has recently become possible with the development of a new for-

mal model of pattern regulation in epimorphic fields by French, Bryant, and Bryant (1976). In using this model to describe the formation of supernumerary limbs, the assumption is made that their occurrence is a manifestation of the ability of the limb to undergo pattern regulation. This model proposes that positional information (Wolpert, 1971) in epimorphic fields is specified in terms of polar coordinates. One component of positional information is a value corresponding to position on a circle, and the second component to position on a radius (see Fig. 19). Positional values on the circles are shown on this diagram by numbers from 0 to 12, where position 12 is equal to position 0. Positional values on the radii are represented by letters from A to E. In this diagram the values in each sequence are arbitrarily shown as being equally spaced. The outer circle represents the proximal limb boundary, and the field center the distal tip of the limb. The radius joining the field center to the outermost circle represents

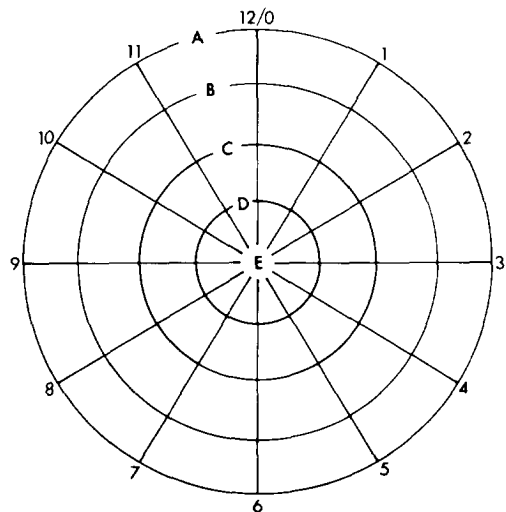


FIG. 19. Diagram to illustrate the concept of polar coordinates in an epimorphic field. Each cell in such a field is assumed to have information with respect to its position on a radius (A through E) and its position around a circle (0-12). In the amphibian limb, the field center (E) is the distal tip and the edge of the field (A) is the most proximal boundary of the limb field.

the distal-proximal axis of the limb.

French, Bryant and Bryant (1976) have proposed rules for the behavior of cells in epimorphic fields:

1. When cells with normally nonadjacent positional values in either the radial or circular sequences are placed together, growth occurs at the junction until cells with all intermediate positional values have been intercalated. In the circular sequence, the sequence is continuous. This means that there are two possible sets of intermediate positional values between any two nonadjacent positional values. The model stipulates that intercalation in the circular sequence is by the shortest of the two possible routes. This rule is known as the "shortest intercalation rule."

2. The radial sequence of positional values is also capable of distal transformation, during which cells with any radial positional value are capable, under certain conditions (see below), of dividing to produce cells with more distal positional values. In order for distal transformation to occur, a complete circular sequence of positional values must either be exposed, as in the case when an appendage is amputated, or be generated by intercalation, according to the shortest intercalation rule. This rule is known as the "complete circle rule." The evidence to support these assumptions and rules is drawn from experiments performed in three different types of epimorphic fields: cockroach legs, amphibian limbs, and *Drosophila* imaginal discs. This evidence is discussed in detail in French, Bryant, and Bryant (1976) and will not be repeated here.

Using these rules, it is possible to explain the formation of supernumerary limbs in amphibians following grafting operations. In those cases in which only a single axis is opposed in grafting, the occurrence of two supernumerary limbs is predicted by the model whether the positional values in the circular sequence are spaced evenly, as shown in Fig. 19, or whether there is a slight nonuniformity in

the spacing, as shown in Fig. 20. However, in order to account for the occurrence of only two supernumerary limbs when both axes are opposed in grafting, it is necessary to assume that the positional values are slightly nonuniformly spaced around the circle. Figure 20 shows the circular sequence of positional values (according to this model) of a right limb stump juxtaposed to the circular sequence of a regenerate from a left limb. The anterior-posterior axes are misaligned. According to the shortest intercalation rule, in those places around the graft junction where normally nonadjacent positional values are now in contact, growth will occur to intercalate those intermediate positional values which normally occur between the two values by the shortest route in terms of numbers of positional values, rather than physical distance. Therefore in Fig. 20, where position 6 is in contact with position 4, intercalation of position 5 will occur. There are two unique points around the graft junction where the direction of shortest intercalation reverses, and where complete circular sequences of positional values are created. These unique points occur where anterior tissue is in contact with posterior tissue (in Fig. 20, where position 8⁺ and 2⁺ are in contact). Intercalation in the graft junction dorsal to these points is in the opposite direction to intercalation in the junction ventral to these points. Therefore in Fig. 20, the placing of the positional values on the complete circles generated in the graft junction is dictated by the direction of intercalation in the adjacent regions of the junction. According to the complete circle rule, distal transformation will only occur in the places where complete circular sequences have been generated. Therefore, supernumerary limbs will develop in the places where anterior tissue is juxtaposed to posterior tissue. The orientation of the supernumerary outgrowths with respect to the stump and the transplant is a consequence of the shortest intercalation rule, as is their handedness. As

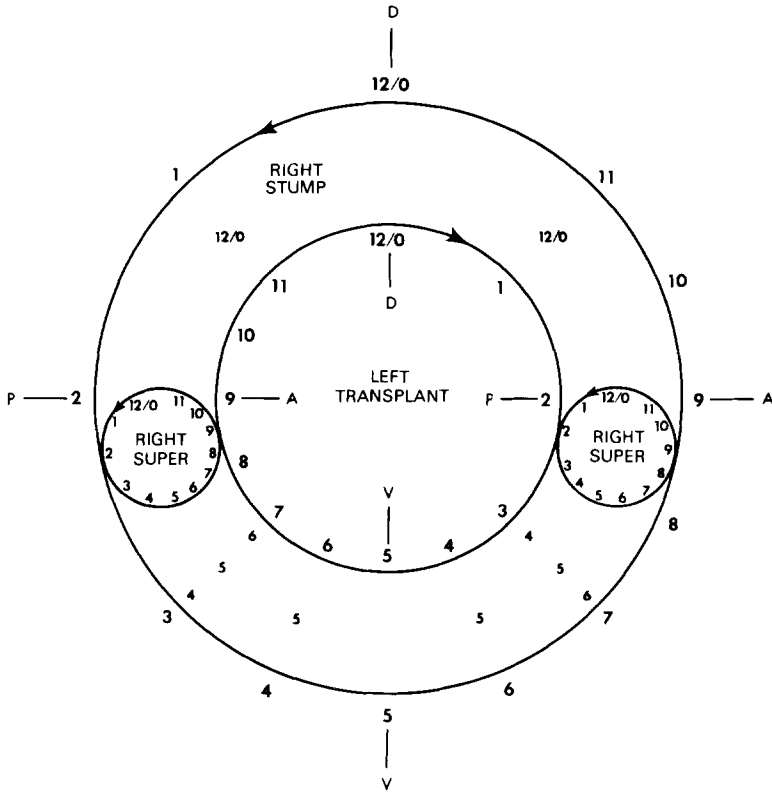


FIG. 20. Diagram to illustrate the application of the model discussed in the text to the production of supernumerary limbs following contralateral transplantation with anterior-posterior axes opposed. The outer circle represents an end-on view of the circular sequence of positional values in a right limb stump (according to the model); the inner circle represents the circular sequence of positional values of a left transplant. The diameters of graft and stump are shown to be dissimilar for convenience only. Positional value 12/0 = dorsal, 9 = anterior, 5 = ventral, and 2 = posterior. Following the shortest intercalation rule, whenever normally nonadjacent positional values lie next to each other, intercalation of intermediate positional values occurs by the shortest route. For example, where position 3 on the outer circle lies next to position 7 on the inner circle, positions 4, 5, and 6 are intercalated. There are two unique points around the graft junction where the direction of shortest intercalation reverses, and where complete circular sequences of positional values are created. These points are where anterior tissue is in contact with posterior tissue (where positions 8⁺ and 2⁺ are in contact). Intercalation in the graft junction dorsal to these points is in the opposite direction to intercalation in the junction ventral to these points. Hence, the placing of positional values on the complete circles generated in the graft junction is dictated by the direction of intercalation in the adjacent regions of the junction. Following the complete circle rule, distal transformation takes place from these complete circumferences to form separate supernumerary limbs. The arrangement of the positional values around the supernumerary limbs, which is a consequence of the shortest intercalation rule, gives the handedness and orientation of these structures. As can be seen from the figure, both supernumerary regenerates are right limbs, normally oriented with respect to the limb stump, and they are in mirror-image symmetry with the transplant. Super, supernumerary limb; other abbreviations as in Fig. 1.

can be seen from Fig. 20, both supernumerary limbs are right hands, and they stand as mirror-images of the transplanted regenerate. This result is consistent with the experimental results described by Iten and Bryant (1975). Figure 21 illustrates the application of the model to the forma-

tion of dorsal and ventral supernumerary limbs when the dorsal-ventral axes of stump and graft are misaligned. The rationale is identical to that described above for the production of anterior and posterior supernumerary limbs. The location, handedness, and orientation of the expected su-

pernumerary limbs fits the experimental results described in this paper. Figure 22 illustrates the formation of anterior-ventral and posterior-dorsal supernumerary limbs following 180° rotation of a regenerating limb on its stump, when both anterior-posterior and dorsal-ventral axes are opposed. By applying the shortest intercalation rule, one of the derived supernumerary regenerates is a mirror-image of the transplant, whereas the other is of the same handedness as the transplant and in normal orientation with respect to the stump. Again, the results presented in this paper conform to these predictions.

The many experiments performed on developing urodele amphibian limb buds can be classified by means of this model into

those graft combinations in which supernumerary limbs would be expected, and into those in which they would not. We can include heterotopic transplantations to the flank in this consideration on the assumption that in such a location, limb buds will be in a position to interact with the anterior edge of the hind limb field, the posterior edge of the forelimb field, or both of these fields, if the limb fields cover much of the flank. That this is the case is indicated by experiments by Balinsky (1933) which showed that indeed the entire flank region between the limbs is capable of forming limbs, given appropriate stimuli. Furthermore, the fact that induced limbs in the flank are "disharmonic" (Balinsky, 1933; Bovet, 1930; Ichikawa and Amano,

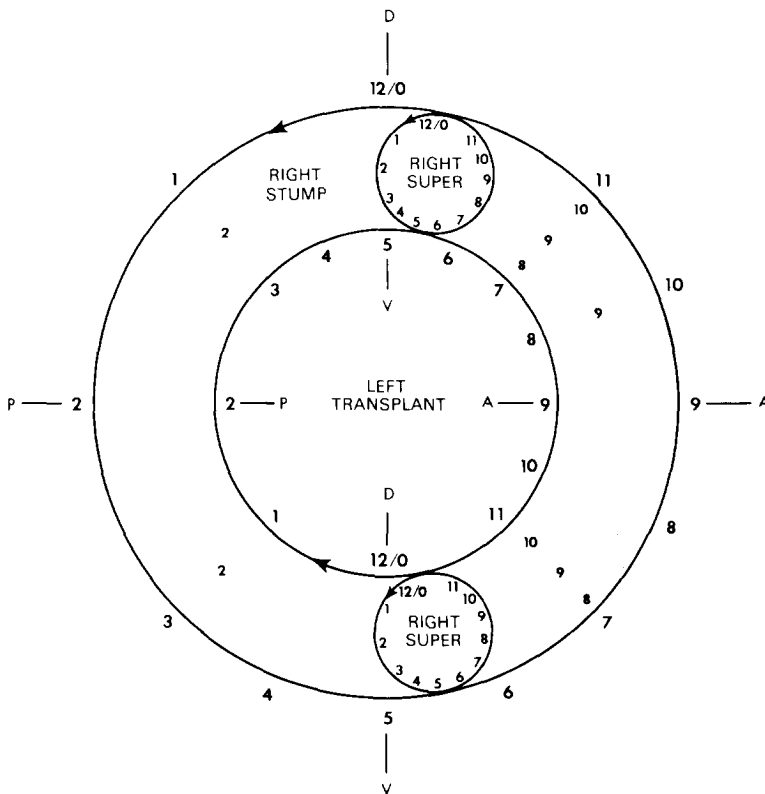


FIG. 21. Diagram to illustrate the application of the model discussed in the text to the production of supernumerary limbs following contralateral transplantation with dorsal-ventral axes opposed. Supernumerary limbs are produced as described in Fig. 20, but in dorsal and ventral locations. The arrangement of the positional values around the supernumerary limbs, which is a consequence of the shortest intercalation rule, gives the handedness and orientation of these structures, as can be seen from the figure. Both supernumerary limbs are right hands, normally oriented with respect to the limb stump, and they are in mirror-image symmetry with the transplant. Abbreviations as in Figs. 1 and 20.

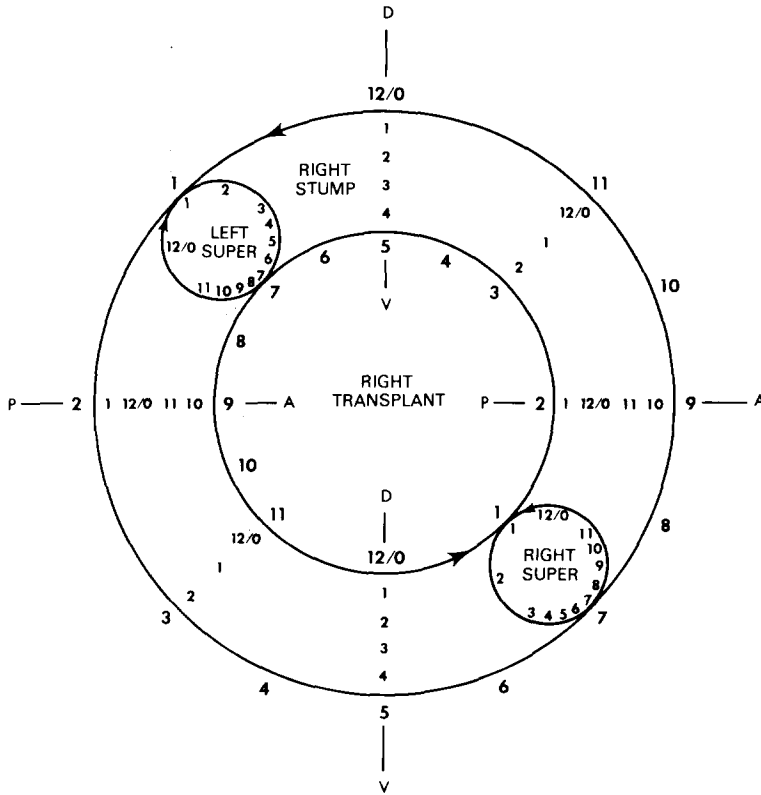


FIG. 22. Diagram to illustrate the application of the model discussed in the text to the production of supernumerary limbs following 180° rotation of a right limb regenerate on a right limb stump. Supernumerary limbs are produced as described in Fig. 20, but in posterior-dorsal and anterior-ventral locations. The arrangement of positional values around the supernumerary limbs, which is a consequence of the shortest intercalation rule, gives the handedness and orientation of these structures. As can be seen from the figure, the posterior-dorsal supernumerary is a left hand with its ventral surface uppermost, and it is a mirror-image of the transplant. The anterior-ventral supernumerary limb is a right limb which is normally oriented with respect to the limb stump, and it is not a mirror-image of the transplant. Abbreviations as in Figs. 1 and 20.

1949; Takaya, 1941), that is, of opposite handedness to their side of origin, indicates that their orientation may be controlled by the posterior border of the forelimb field and the anterior border of the hind limb field. Table 3 shows the operations in urodele embryos expected to lead to the development of supernumerary limbs. Table 4 shows those not expected to lead to such outgrowths. As can be seen from the combined results of many authors, the experiments on amphibian limb buds conform well to the predictions made about the occurrence of supernumerary limbs. A more detailed analysis of the handedness and orientation of the super-

numerary limbs in these experiments is not always possible since in several instances these features were not described. However, from those descriptions which are given, the results appear to be consistent with the model described here.

In the model developed by French, Bryant, and Bryant (1976) it is proposed that positional information is specified in only two dimensions. Since the limb is a three-dimensional organ, it might be expected that it would be necessary for positional information to be specified in three dimensions. However, it is clear from many studies that positional information within the very early limb bud is carried

TABLE 3
EXPERIMENTS ON URODELE AMPHIBIAN LIMB BUDS: TRANSPLANTATION OPERATIONS EXPECTED TO LEAD TO
THE PRODUCTION OF SUPERNUMERARY LIMBS

Type of operation ^a		Authors	Total number positive cases	Number of cases with supernumerary limbs
Orthotopic	Hom ap dv mm	Harrison (1917)	23	16
		Harrison (1921)	38	27
		Swett (1926)	146	132
		Ruud (1926)	8	2
		Blount (1935)	199	127
		Stultz (1936)	100	30
	Het ap dd mm	Harrison (1917)	33	32
		Harrison (1921)	31	25
		Swett (1926)	32	29
		Ruud (1926)	8	0
		Stultz (1936)	86	25
	Het aa dv mm (After establishment of dv axis)	Swett (1930)	9	8
Hom rotation of 135 or 225°	Nicholas (1924b)	29	17	
Transplanted to head with cuff of limb tissue: Hom ap dv mm	Swett (1945)	7	6	
Heterotopic	Hom aa dd mm	Detwiler (1918)	29	10
		Harrison (1921)	7	4
		Brandt (1924)	4	4
		Ruud (1926)	2	0
		Swett (1932)	33	16
		Carpenter (1932)	68	22
		Stultz (1936)	6	0
		Takaya (1941)	41	10
		Hom aa dv ml	Harrison (1925)	2
	Hom ap dv mm (After establishment of dv axis)	Brandt (1924)	6	3
	Het aa dv mm	Harrison (1921)	16	8
		Brandt (1924)	9	5
		Ruud (1926)	4	0
		Swett (1927)	86	71
		Swett (1930)	26	24
		Hollinshead (1936)	122	57
		Stultz (1936)	8	3
		Swett (1938)	103	65
		Takaya (1941)	91	58
	Het aa dd ml	Harrison (1925)	4	1
		Swett (1927)	40	18
		Swett (1928)	52	35
Takaya (1941)		45	16	
Hom rotation of 90, 135, 225, or 270°	Nicholas (1924b)	104	73	
Transplanted to head with cuff of flank tissue: Hom aa dd mm	Swett (1945)	18	13	
Transplanted to flank with piece of head tissue ventrally: Het aa dv mm	Swett (1945)	64	35	
Total of all experiments			1739	1027
Percentage forming supernumerary limbs				59

^a Hom, homopleural; Het, heteropleural; a, anterior; p, posterior; d, dorsal; v, ventral; m, medial; l, lateral.

TABLE 4

EXPERIMENTS ON URODELE AMPHIBIAN LIMB BUDS: TRANSPLANTATION EXPERIMENTS *NOT* EXPECTED TO LEAD TO THE PRODUCTION OF SUPERNUMERARY LIMBS

Type of operation ^a		Authors	Total number positive cases	Number of cases with supernumerary limbs
Orthotopic	Hom aa dd mm	Harrison (1917)	6	0
		Harrison (1921)	9	0
		Ruud (1926)	9	0
		Stultz (1936)	20	1
	Het aa dv mm (Before establishment of dv axis)	Harrison (1917)	18	1
		Harrison (1921)	16	1
Ruud (1926)		1	0	
Stultz (1936)		40	7	
Hom rotation of 90 or 270°		Nicholas (1924b)	108	12
Heterotopic	Hom ap dd ml	Harrison (1925)	2	0
	Hom ap dv mm (Before establishment of dv axis)	Detwiler (1918)	8	2
		Harrison (1921)	12	1
		Ruud (1926)	1	0
		Detwiler (1929)	21	2
		Detwiler (1933)	4	1
		Stultz (1936)	14	6
	Takaya (1941)	22	0	
	Het ap dd mm	Detwiler (1918)	9	1
		Harrison (1921)	10	2
		Brandt (1924)	6	1
		Ruud (1926)	4	0
		Stultz (1936)	11	0
	Het ap dv ml (Before establishment of dv axis)	Harrison (1925)	2	0
	Transplanted to head	Detwiler (1930)	307	3
		Swett (1945)	106	0
Transplanted to ventral midline	Nicholas (1924a)	8	1	
Transplanted to dorsal midline	Nicholas (1924a)	5	1	
Transplanted to flank with a ring of head tissue	Swett (1945)	31	0	
Total of all experiments			810	43
Percentage forming supernumerary limbs				5

^a Hom, homopleural; Het, heteropleural; a, anterior; p, posterior; d, dorsal; v, ventral; m, medial; l, lateral.

only in the circular disc of mesoderm (Detwiler, 1929; Harrison, 1918, 1925). It is also of interest in this connection that Carlson (1975) has shown that reorientation of muscle and dermis within the axolotl limb leads to the formation of regenerates with supernumerary elements, whereas reorientation of epidermis and bone does not. These results suggest that in the axolotl limb, positional information is carried in a hollow cylinder of mesoderm, i.e., in two dimensions rather than three.

The fact that experiments on developing amphibian limbs (Harrison, 1921, 1925; Hollinshead, 1936; Swett, 1927, 1928, 1937) as well as on other developing organs (ear: Harrison, 1969; retina: Hunt, 1975; Jacobson, 1968) indicates that there is a sequential establishment of the orthogonal axes of an organ (anterior-posterior before dorsal-ventral) need not lead one inevitably to the conclusion that positional information in these organs is specified by means of rectangular coordinate systems. Rather, the fact that polarity in the anterior-poste-

rior axis develops before polarity in the dorsal-ventral axis may indicate that positional values in the proposed circular sequence are established sequentially, with anterior and posterior values being established first, followed later by dorsal and ventral values. Similar reasoning has recently been used to argue against the concept of a rectangular coordinate system for pattern specification in the vertebrate retina (MacDonald, 1976).

It is important to recognize that the model presented here and discussed in greater detail in French, Bryant, and Bryant (1976), does not propose a specific cellular or molecular mechanism by which positional information is specified. However, it is clear that when such a mechanism is proposed, in its basic form it must be capable of accommodating the fundamental similarities in behavior of epimorphic fields in such widely separated groups of organisms as amphibians and insects. The results of leg transplantation experiments on cockroaches are essentially the same as those described here for amphibian limbs (Bohn, 1972; Bullière, 1970; French, 1976). French has independently interpreted his own results on supernumerary leg formation in insects, as well as those of other workers, in exactly the same way as described here (French, 1976; French, Bryant, and Bryant, 1976).

Finally, although the limb of a postembryonic chick cannot be considered as an epimorphic field, it is not yet clear to what extent the developing limb can be considered in this way. Many experiments on developing chick limbs point to the existence of an organizing center in the posterior mesoderm of the limb bud, the Zone of Polarizing Activity (Fallon and Crosby, 1975a; J. A. MacCabe and Parker, 1975; Saunders and Gasseling, 1968; Summerbell, 1974; Tickle *et al.*, 1975). This zone has the property of stimulating polarized outgrowths when it is transplanted to other regions of the limb bud. However, Chaube (1959) showed that the prospective

limb bud region of very early embryos already has its anterior-posterior polarity fixed, yet polarizing zone activity, as assayed in grafting experiments, does not reach a peak until considerably later in development (A. B. MacCabe *et al.*, 1973). Furthermore, A. B. MacCabe *et al.* (1973) have shown that normal limb development can occur after removal of the polarizing zone, and Fallon and Crosby (1975b) have shown that normal development of the limb occurs without regeneration of this zone. The role of the polarizing zone in pattern formation and pattern regulation in developing chick limbs is therefore unclear. Irrespective of the possible function this zone may have in normal development, experiments performed on developing chick limbs provide results which are analogous to those described here for amphibian limbs. For example, Saunders *et al.* (1958) showed that when the tips of limb buds were grafted to the contralateral limb stump with the anterior-posterior axes misaligned, (orthotopic, dorsal-dorsal, anterior-posterior) supernumerary limb elements usually developed posterior (with reference to the stump orientation) to the elements expected from the graft. Occasionally, supernumerary elements also formed anterior to the grafted limb. In both cases, the supernumerary elements were mirror-images of the elements arising from the graft. However, when similar grafts were performed, but with the dorsal-ventral axes opposed (orthotopic, dorsal-ventral, anterior-anterior), supernumerary limbs did not form, although supernumerary integumentary structures were sometimes produced (Saunders *et al.*, 1958). When the tip of the wing was reoriented on its base after 180° rotation, separate posterior and sometimes anterior supernumerary limbs also formed (Amprino, 1965, 1968; Amprino and Camosso, 1959, 1963; Camosso and Roncali, 1971; Saunders and Gaseling, 1959, 1968; Saunders *et al.*, 1958). The posterior supernumerary limb was a mirror-image of the

reoriented limb tip, whereas the anterior supernumerary, when it was present, was of the same handedness and orientation as the limb stump. Hence, with the exception of the absence of supernumerary limbs following opposition of the dorsal-ventral axes of limb tip and base, these results on developing chicks are very similar to those on amphibian embryos, and indicate that the model for pattern regulation in epimorphic fields discussed in this paper may be equally applicable to both organisms. Experiments to test this speculation are in progress.

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