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Combination of Two Limb-rudiments in Urodele *Triturus*, with Special Reference to Their Symmetry Relations

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With 41 Figures

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Introduction

When two limbs are existing in close proximity, enantiomorphism is frequently found between them. The simplest and most probable interpretation for the phenomenon is to assume that mutual interaction is operating between them, as WILHELMI (1922) has postulated, between paired limbs such as right and left, or orthotopic and heterotopic ones, as of course between reduplicants of a single rudiment. With regard to this problem, I have made on two previous occasions (1934, 1936) preliminary accounts of my experiments which were carried on from 1933 to 1935 on a Japanese Urodele under the direction of Prof. Yô K. OKADA. The present paper is a full description of the result of the experiments.

Before going further I wish to express my hearty thanks to Prof. OKADA for suggesting this interesting subject and for his kind guidance and encouragement during the whole course of the work.

Material and Methods

Embryos of *Triturus pyrrhogaster* (BOIE) were used, the stages of which varied from stage 25 to 38 according to OYAMA's normal plate; namely, the embryos were in the tail bud stages. In most cases, operations were done on the left side of the embryo. Combinations of limb rudiments were made in two different ways, that

is, in one case by adding another rudiment to the normal one (additional transplantation), and in the other case by transplanting two rudiments at the same time in the heterotopic position. In the first case a circular disc of three somites ranging from the 3rd to the 5th somite was removed below the pronephros, and this was grafted to another embryo in a suitable position; reception of the graft was made in one of 4 positions anterior, posterior, dorsal, and ventral with respect to the limb disc of the host (see fig. 1). For each transplantation a circular wound was cut out in one of these positions encroaching slightly to the normal limb disc, but care was taken particularly to remove the mesodermal material of this area as little as possible at the time of operation. The operated embryos were reared separately in glass bowls and developmental records were taken when necessary. In most cases the grafted limb was clearly contrasted to the normal one by its aberrant situation, but an attempt was sometimes made to stain the graft previously with Nile blue sulphate.

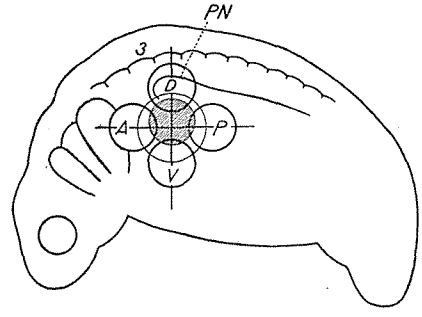


Fig. 1. *Triturus* embryo in st. 26, showing large circle in light line normal limb disc, shaded area within area used for experiment and small circles in thick line around grafting positions; A, anterior; P, posterior; D, dorsal; V, ventral; 3, the third myotome; PN, pronephros.

Results

I. *Additional Transplantation*

Grafting of an extra rudiment near the normal one of the host was not always followed by the production of two limbs, but on the other hand developmental arrest frequently occurred in one of the rudiments. The subsequent description is concerned with this problem.

In all four positions mentioned above, the graft invariably started its development and, simultaneously with the host, produced one limb bud, standing side by side with a limb bud produced by the host. But a few days later one of them ceased to develop and began to shrink. In the case of the dorsal transplantation such reduction took place mostly in the bud developed from the host rudiment.

Out of 14 cases where the single limb was produced, there were as many as 11 cases in which the grafted rudiment developed, whereas only in 3 cases the host rudiment grew. In other cases where two limbs were produced complete fusion of two buds was frequent, whereas independent development was rare (see tables 3, 6), and if such occurred at all, it was limited to those cases where the graft was always shifted postero-dorsally to the normal limb. Therefore, it seems very difficult or impossible for two limbs to develop simultaneously in the combination position of grafting under consideration. In other positions reduction took place generally in the grafted limb, the process being particularly marked in the anterior transplantation when the graft was united with the normal limb at the base. Reduction of the graft is found to be invariably brought about where the transplantation was made in relatively earlier stages of development. In the ventral transplantation and when the graft was shifted to the ventral side of the embryo, reduction also took place, but not so completely as in the positions so far described, some of the graft remaining as a slight nodule for over 20 days after the reduction began. In the posterior transplantation a simultaneous development of normal and transplanted rudiments was obtained.

Transplantations of the limb rudiment in varied orientations were tried in each of the four positions mentioned with, however, almost no variation from the general tendency of development as above mentioned (see tables 1-4, 6).

(a) *Posterior transplantations*

The orientation of the graft can be given in four different directions according to the *ap* and *dv* axes of the rudiment, as per diagram. In the posterior position *hom aa dd*, *het aa dv*, *hom ap dv* and *het ap dd* transplantations were made. The relations of the *ap* axis between two rudiments were, as shown in fig. 2, in the same direction in the first two cases and in the opposite direction in the latter two cases of the named combination.

In the *hom aa dd* transplantation, two limb buds were generally produced pointing postero-dorsally (fig. 3a). But in some cases the anterior limb bud showed slight deviation from this direction of development which, however, soon returned to the proper direction, and had nothing to do with the change of asymmetry. In most cases two buds found in such close approximation united with each other in the proximal portion. A complete fusion sometimes took place,

with the resulting production of a single normal limb on that side of the body. Partial fusions of the limbs entailed, as a rule, irregularity of the structure in one of them, including reversal of the prospective asymmetry. The change was always found in the anterior member, which was developed from the regular rudiment of the host, and enantiomorphism appeared in 8 cases out of 13 limb-combinations. Naturally, the posterior member, which underwent

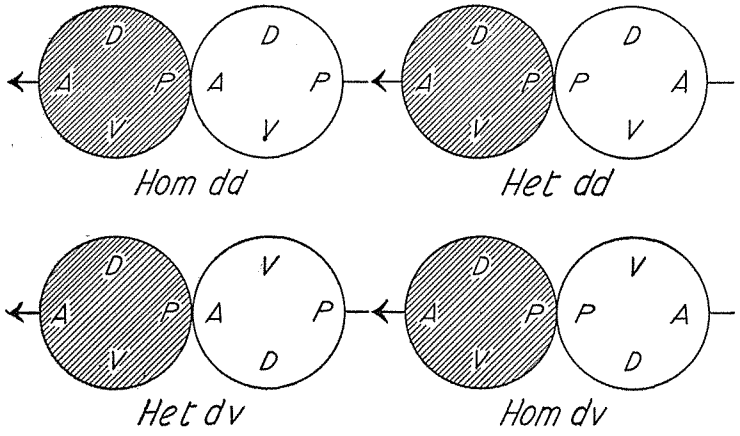
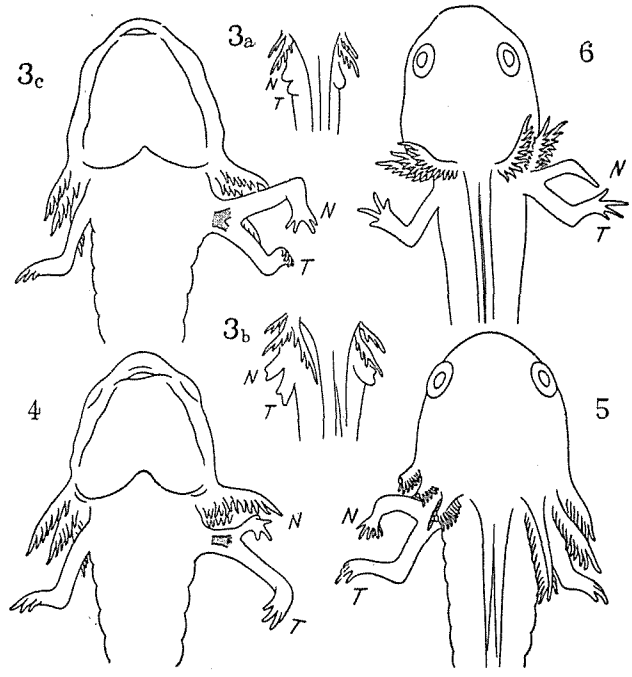


Fig. 2. Diagrammatic representation of axial relation in limb combinations in posterior position: Shaded circle indicates host regular rudiment, and arrow points to anterior end of embryo.
Hom dd, homopleural transplantation in dorso-dorsal orientation;
Het dv, heteropleural transplantation in dorso-ventral orientation;
Het dd, heteropleural transplantation in dorso-dorsal orientation;
Hom dv, homopleural transplantation in dorso-ventral orientation.

no change of direction, developed into a normal limb. As a consequence, a radial mirror image was brought about in these cases (see figs. 3–6). A close inspection of their developmental record shows us that reversal of the asymmetry in the anterior member sets in at a comparatively later period of development, when the 3rd digit begins to appear, about 10 to 15 days after the operation. The change of developmental direction was brought about by the rotation of the whole limb structure; the process took place very slowly and required several days to complete it. In consequence of this rotation, the extensor-flexsor surface of the limb was reversed, the dorso-ventrality became generally indistinct, and, moreover, irregularities ensued in the autopodium which was mostly deficient in the number



Some specimens of the posterior transplantation in hom aa dd orientation.
Figs. 3-6. Partial fusion of limbs with asymmetry reversal in anterior host limb, radial mirror image being resulted. Fig. 3a, 6 days; b, 12 days; c, 42 days after operation of the same specimen; fig. 5, 42 days; fig. 6, 32 days of different embryos respectively. In figs. 3 and 4 fusion takes place in humerus. N indicates host limb and T transplanted limb.

of digits, being provided with only three digits, one long in median and one smaller at each side in symmetrical arrangement (fig. 4). In some specimens, the limb became filiform because of lacking the autopodium (fig. 6). On the contrary, there were cases where excess digits were produced (fig. 5). At any rate, a perfect asymmetrical limb with four digits was found only as a rare occurrence (fig. 3). Such irregularities in the structure of the anterior limb were recognized even when no reversal was brought about by the fusion (figs. 7-8).

Besides the partial fusion there was also an extensive fusion which occurred almost throughout the length of the limbs, two types of fusion being distinguished: In one group the process was so complete as to be regarded as a simple structure excepting the distal

Table 1

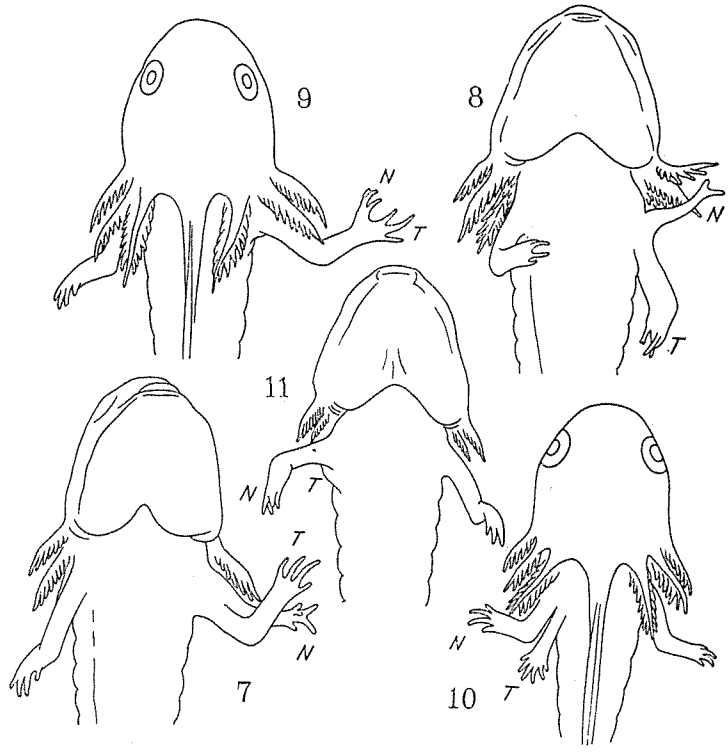
Summary of posterior transplantations

Operation	Total	Available	Single limbs			Compound limbs			Separated limbs		
			host	graft	fused	divided in autopodium	divided in zeugopodium	divided in stylopodium	both host and graft single	host alone double	graft alone double
Hom aa dd	56	53	1	1	1	7	9	18	9	3	4
Het aa dv	23	14	—	—	1	2	3	6	1	—	1
Het ap dd	26	17	1	1	1	2	2	3	1	—	6
Hom ap dv	28	15	3	—	—	3	2	1	1	—	5
Total	133	99	5	2	3	14	16	28	12	3	16

portion (fig. 9) and in another group fusion was superficial, extending only to the soft parts, with the resulting production of a thick irregular structure having two sets of skeletons. In the first group bifurcated parts of the limb were found to be mirrored in the radial plane in 2 cases, one in the autopodium (fig. 9) and the rest in the distal portion of the zeugopodium. In these cases reversal of the asymmetry was found to have taken place in the anterior member, without rotation of the distal portion. In the second group reversal not having occurred in either member of the fusion, either two parallel limbs were produced with the same asymmetry, or reduplication was found in one of them.

Separate development of limbs occurred also in other cases of this series of transplantations, without reversal of the asymmetry either in the host or in the grafted member. But instead of being parallel, the two limbs were disarranged in most cases by frequent occurence of reduplication in the posterior grafted limb (fig. 10). The reduplication can be attributed to the heterotopic situation of the graft being influenced by the surrounding tissues and has no direct connection with the presence of host limbs in the anterior position.

There was one case in which the grafted rudiment failed to develop, remaining as a slight nodule at the base of the host limb (fig. 11). Nevertheless, the host limb underwent rotation in the course of development and became disharmonic to the body side. Therefore, the presence of the undeveloped graft can be, in this case at least,



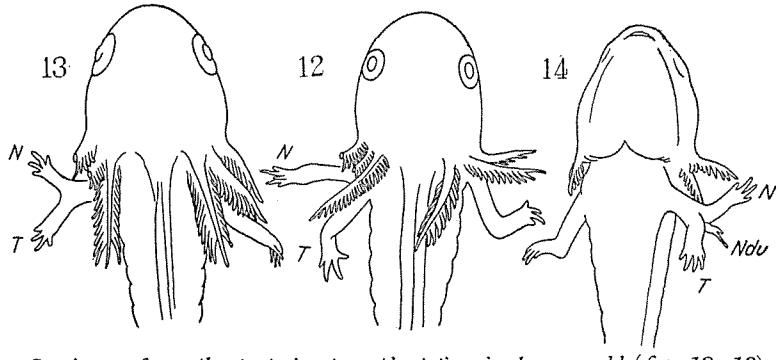
Some specimens resulted from the posterior transplantation in hom aa dd orientation.

Figs. 7, 8. Partial fusion of limbs without asymmetry reversal, host anterior limb being irregular in development. Fig. 7, 37 days after operation; fig. 8, 28 days. Fig. 9. Radial mirroring in an extensive fusion of limbs. 54 days. Fig. 10. Reduplication of grafted limb in separated development. 20 days. Fig. 11. Asymmetry reversal in host limb in spite of undevelopment of graft. 24 days.

referred to as a capital factor for the reversal of the asymmetry.

In all cases so far enumerated, combination was accomplished between two rudiments nearly in the same stage of development. However three different stages were used; 26-28, 31-32, and 35-36. No discrepancy in the result was brought about by the difference of the developmental stages of the embryo employed. A combination was further tried between rudiments of different stages, the host in stages 32-38 and the graft in stages 26-28, or reciprocally the graft being chosen in stages 32-35 and the host in stages 25-26. In both cases development took place similarly in two rudiments of limb and the results were quite comparable to those obtained in the preceding

experiment. Rotation occurred also in the anterior member of the united limb, resulting in a production of the radial mirroring. In one case where a younger graft was used, the anterior host limb was rotated incompletely and an imperfect mirroring as shown in fig. 12 was produced. Exceptionally in one case where the graft was in an advanced stage, an ulnar mirror image was produced (fig. 13). In this case reversal of the asymmetry took place in the posterior grafted limb. Since, however, there was no similar case throughout the whole series of experiments, a faulty orientation of the graft at the time of transplantation can be assumed and we may safely take this case out of our consideration.



Specimens from the posterior transplantation in hom aa dd (figs. 12, 13) and in het aa dv orientation (fig. 14).

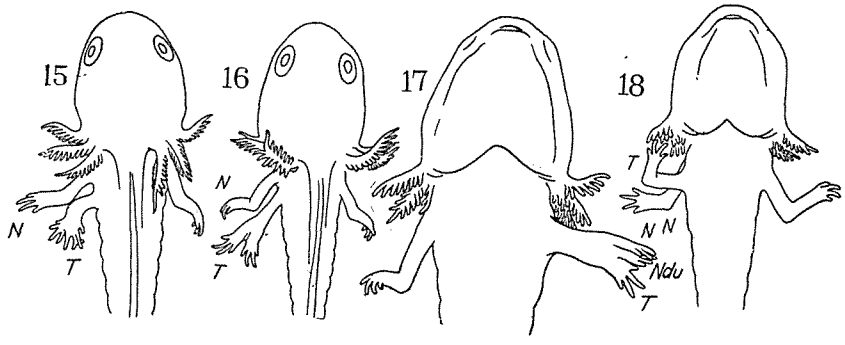
Fig. 12. Grafting of younger rudiment to older embryo with the result of incomplete reversal of asymmetry in host limb. 27 days after operation. Fig. 13. Ulnar mirroring. 21 days. Fig. 14. Reduplication in host limb by fusion of graft, inverted direction of *dv* axis being maintained in the latter. 25 days.

Excluding this doubtful case we arrive finally at the conclusion that the host limb, being affected by the fusion of the graft in the posterior position, reverses its asymmetry, while in the separated development the grafted limb generally undergoes reduplication and the host limb remains intact.

In the *het aa dv* transplantation (see diagram in fig. 2) two rudiments were combined so as to point their *ap* axis in the same direction, but the *dv* axis in the opposite direction. The experiments were carried out in two groups: In one group, embryos in stages 25–26 were used and in the other group, embryos in stage 35. The inverted direction of the *dv* axis of the graft was maintained during development and produced a limb with the palm side turning upward

in 14 cases out of 23. Such grafts frequently fused with the host limb, and in most cases superficially united along the whole length of the stylopodium (fig. 14). However, no change of direction was brought about by the fusion in any member, even when reduplication occurred in the anterior host limb. In the remaining cases in which limb-fusion took place, the *dv* axis of the graft was reversed so that it was harmonic to the body side, and as a result the same condition as in the *hom aa dd* transplantation was brought about between two limbs. However, there was no reversal of the asymmetry in either member and, therefore, no establishment of mirroring, except production of irregularities in the structure of the anterior member.

A combination of limb rudiments with the *ap* axis in an opposite direction was made by *hom ap dv* and *het ap dd* transplantations. The graft developed together with the host limb in most cases, as in the preceding experiment. In this case an ulnar mirroring, irrespective of the fusion of limbs, was expected because the direction of their *ap* axis was inverted beforehand. On the contrary, however, a mirroring pair of limbs was found in no case of these two series of transplantations even when the grafted rudiment established an



Some results of the posterior transplantation in het ap dd (figs. 15, 16) and in hom ap dv orientation (figs. 17, 18).

Fig. 15, showing embryo in 27 days, fig. 16, 19 days, and fig. 17, 29 days after operation respectively. Fig. 18. Grafted limb twisted forward without symmetrical arrangement to host limb. 25 days.

independent limb. Reduplication frequently occurred in the grafted limb, whereas no change was brought about in the host limb (figs. 15-16). If the grafted limb remained single and separated, its palm surface, because of strong pronation, always pointed upward when in repose, and the limb assumed an appearance of having the same

asymmetry as the host limb (fig. 18). If fusion took place with the host limb, the process proceeded, as a rule, in the surface of the whole length of both limbs, with the resulting production of a thick irregular structure as shown in fig. 17. In such cases, the anterior member, i. e., the host limb was frequently reduplicated, and the posterior member (the grafted limb) remained single. In one case, in which fusion was more complete, a single limb was produced. But this single limb was subsequently divided in the course of a further development and a radial mirroring ensued in the divided portions.

In conclusion, when we consider the limb-combination in these two series, the result is generally uniform in spite of the antagonistic direction of the *dv* axis given to the grafted rudiment. The results of the posterior transplantations as a whole may, therefore, be summarized as follows: A limb rudiment transplanted immediately posterior to the normal one develops normally, together with the latter, irrespective of its orientation of transplantation. Fusion frequently occurs between them, and the most marked effect of the process is a reversal of the asymmetry of the anterior member produced from the host rudiment. However, the reversal of asymmetry in the anterior member is found only in such combinations as when two rudiments are all in the same direction with respect to both *ap* and *dv* axes. Therefore, the mirroring produced in this case is radial. In the other combinations, in which either *ap* or *dv* axis is inverted, no mirroring is produced, even when two limbs are combined in an antagonistic direction with respect to their prospective axes. Reduplication is frequent on the side of the grafted limb when developed separately from the host, whereas the latter undergoes reduplication if fusion takes place in both limbs.

(b) *Anterior transplantations*

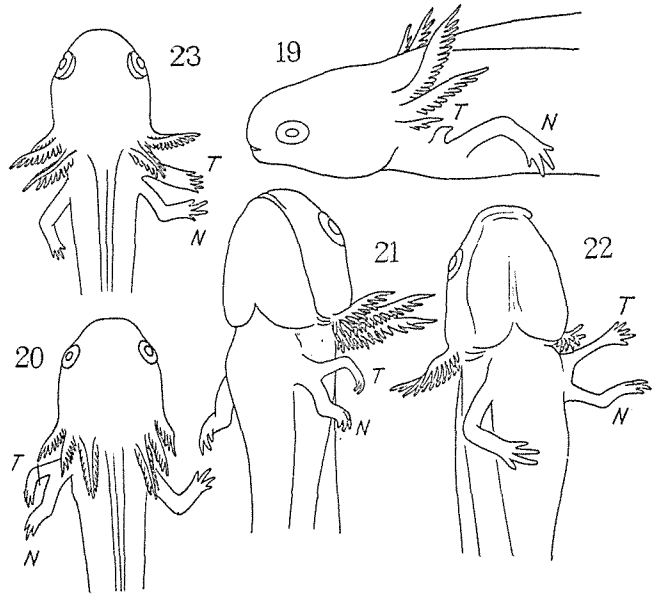
As mentioned above, shrinkage took place quite frequently in the grafted limb, especially when the latter was taken from those embryos in a comparatively younger stage, i. e., in stages 26–28. The graft was, therefore, taken mostly from older embryos in stages 32–38, in which the limb bud appeared merely as a slight elevation from the body side. In this case the result was much improved, and yet the grafted limb was likely to be deficient in the distal portion, becoming filiform in most cases as shown in fig. 19. On the other hand, the regular limb of the host underwent no change and grew up into a single normal limb in all cases. At any rate,

Table 2

Summary of anterior transplantations

Operation	Total	Available	Single limbs		Compound limbs			Separated limbs
			host	fused	divided in autopodium	divided in zeugopodium	divided in stylopodium	graft alone double
Hom aa dd	18	17	11	1	—	—	4	1
Het aa dv	8	8	1	—	—	—	6	1
Het ap dd	6	6	—	—	—	—	6	—
Hom ap dv	8	8	3	—	2	1	2	—
Total	40	39	15	1	2	1	18	2

fusion was prevalent between two limbs, but it was limited always to the base and an extensive fusion along the whole length was found only in rare cases (see table 2).



Anterior transplantation in hom aa dd (figs. 19, 20, 23), in hom ap dv (fig. 21) and in het ap dd orientation (fig. 22).

Fig. 19. Showing underdevelopment of grafted limb. 25 days after operation. Figs. 20, 21. Asymmetry reversal of grafted limb with mirroring to host limb in radial plane; fig. 20, 14 days and fig. 21, 26 days respectively. Fig. 22. Grafted limb without elbow stretched out to straight symmetrical structure. 22 days. Fig. 23. Reduplication of graft in separate development. 29 days.

In the *hom aa dd* transplantation, i. e., the grafted limb rudiment being placed in the normal orientation, the axial relation of two rudiments was the same as in the corresponding transplantation in the posterior position of the host. The result was also comparable to that of the latter transplantation, and if fusion took place between two limbs the anterior member, which was developed from the grafted rudiment, reversed its asymmetry, with the resulting production of a radial mirroring. But in this case the mirroring structure was somewhat imperfect on account of the prevalent deficiency in the anterior grafted member (fig. 20). On the other hand, a supernumerary of the autopodium was found in one case in the grafted limb, the elbow joint of which pointed in a disharmonic direction with respect to the body side, and mirrored the host limb in the radial plane. If the *dv* axis was reversed during development in a *het aa dv* transplantation, the radial mirror image was also developed between two limbs. But when the inverted direction of *dv* axis was maintained in the grafted limb, no reversal of the asymmetry occurred, and of course no mirroring arrangement was developed. Usually, however, the grafted limb became irregular in the structure.

When the *ap* axis of two limb rudiments was combined so as to occur opposite each other, i. e., in the *het ap dd* and the *hom ap dv* transplantations, no change of asymmetry took place in either member, although the production of the normal asymmetry was quite rare in the grafted limb. Only in one case in each series of these transplantations was the grafted limb normal with respect to its asymmetry. Naturally in other cases the grafts developed more or less anomalously. However, there was one characteristic which appeared in several cases in common: This was an apparent lack of the elbow joint, and the limb stretched straight without bending in any direction. In such limbs, a symmetrical structure was produced as shown in fig. 22. A separate development was found only in one case where the graft was placed in the normal orientation. Irregular reduplication was brought about in this case on the transplanted member, while the host remained unchanged (fig. 23).

From the results so far enumerated a limb rudiment transplanted just anterior to the host, does not have any influence upon the latter even when fusion takes place between them. The grafted limb, on the other hand, is always influenced by the presence of the host limb behind and the graft in some cases even reverses its asymmetry. From these facts, together with those obtained in the posterior

transplantations, we know that in the antero-posterior combination of two limb rudiments, it is always the anterior member which is affected and becomes irregular or even reverses the prospective asymmetry.

(c) *Dorsal transplantations*

Two kinds of transplantation, i. e. *hom aa dd* and *hom ap dv*, were examined in this position. The rudiment-combination in this position resulted always in a single limb which represented one of

Table 3

Summary of dorsal transplantations

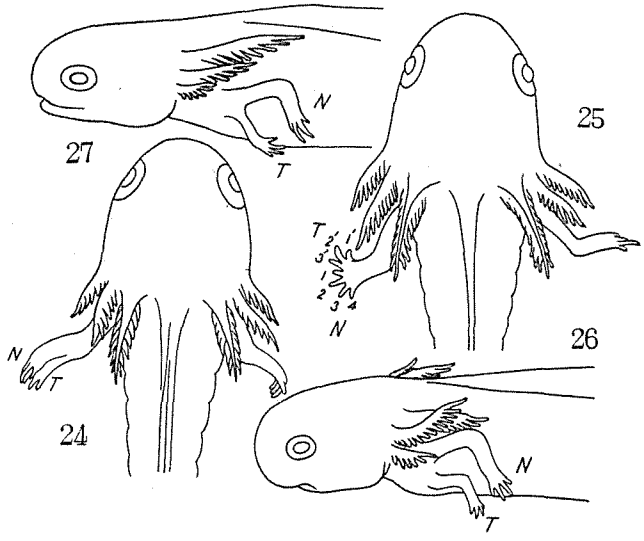
Operation	Total	Available	Single limbs			Compound limbs		Separated limbs	
			host	graft	fused	divided in zeugopodium	divided in stylopodium	both host and graft single	graft alone double
Hom aa dd	20	19	3	9	5	1	—	1	—
Hom ap dv	16	14	—	2	8	—	2	1	1
Total	36	33	3	11	13	1	2	2	1

two rudiments or both in fusion, a complete fusion being especially frequent in operations upon embryos about in stage 30. In younger embryos (about in stage 26) the grafted rudiment developed in most cases and the host limb remained undeveloped. Therefore, neither reversal of the asymmetry nor mirroring of limbs were found in any case of dorsal transplantations.

(d) *Ventral transplantations*

The graft in this position was frequently detached from the host limb and was placed on the ventral side of the embryo. This was particularly marked both in the *hom ap dv* and *het aa dv* transplantations, where the *dv* axis of the graft was inverted. Further the absorption of the graft was a usual occurence in these transplantations, especially when the graft was taken from embryos in a comparatively younger stage of development.

In the *hom aa dd* transplantation, if two limbs developed simultaneously, they generally fused. The process was in most cases so extensive and complete as to extend over half the length of the limbs, forming a simple structure (see fig. 24). The host limb, which



Ventral transplantation in hom aa dd (figs. 24, 25, 26) and in het aa dv orientation (fig. 27).

Fig. 24. Dorso-ventrality of grafted limb is reversed to be mirrored host limb in palmar plane. 37 days. Fig. 25. Production of radial mirroring as the result of limb fusion. 23 days. Fig. 26. Parallel limbs in separate development. 23 days. Fig. 27. Production of radial mirroring by asymmetry reversal of grafted limb. 23 days.

represented the dorsal member of these compound limbs, was sometimes bifurcated and the ventral grafted limb was deficient. Normal development took place also in several cases when both rudiments were combined, which resulted in a production of parallel limbs with the same asymmetry (fig. 26). In one of these, however, the reversal of the asymmetry was brought about on the side of the grafted limb, in which, differing from both posterior and anterior transplantations, the direction of the *dv* axis was reversed with the resulting production of a palmar mirror image (fig. 24). A radial mirroring was also produced in one case where the graft was shifted from its initial position and became attached to the radial border of the host limb (fig. 25). However, the grafted rudiment started its development normally in the radio-ulnar direction as did the host limb, and only in the subsequent development did change take place in the mirroring of the host limb. In other cases, separate development of limbs was found without changes in the parallel direction given to them.

In the *het aa dv* and the *het ap dd* transplantations the grafted

Table 4

Summary of ventral trasplantations

Operation	Total	Available	Single limbs		Compound limbs			Separated limbs
			host	fused	divided in autopodium	divided in zeugopodium	divided in stylopodium	both host and graft single
Hom aa dd	21	18	4	3	2	3	3	3
Het aa dv	4	4	1	—	—	—	3	—
Het ap dd	6	6	2	—	—	1	3	—
Hom ap dv	5	5	5	—	—	—	—	—
Total	36	33	12	3	2	4	9	3

limb was found generally at the antero-ventral border of the normal limb and was more or less deficient. Moreover, in one case of the former transplantation, reversal of asymmetry appeared in the grafted limb, the result of which was the production of a radial mirror image. Inasmuch as the fusion took place at the antero-ventral border of the host limb (fig. 27), this case can be compared with those obtained in the corresponding transplantations in the anterior position. The *hom ap dv* transplantation of limb rudiment was tried in several cases without success; the grafted rudiment was always absorbed, and there was no case where two limbs were produced.

From the results of these ventral transplantations, together with those in the dorsal position, we can conclude that in the dorso-ventral combination of two limb rudiments, the predominance is always on the side of the dorsal member, the influence of which renders the development of the ventral member difficult, and even occasionally causes the reversal of the asymmetry.

II. *Combination of two limb rudiments in heterotopic position*

In the present series of experiments, two rudiments were grafted at the same time in a heterotopic position, and thus afforded similar conditions of development, for the purpose of trying to analyze the effect of the position and the orientation of rudiments with respect to the body side. The method of transplantation used was to make a wound of about five somites in diameter, in which two rudiments were inserted in close contact with one another. Particular care was taken to cut the rudiments the same size, which was uniformly three somites in diameter in all cases. In the present experiments, however,

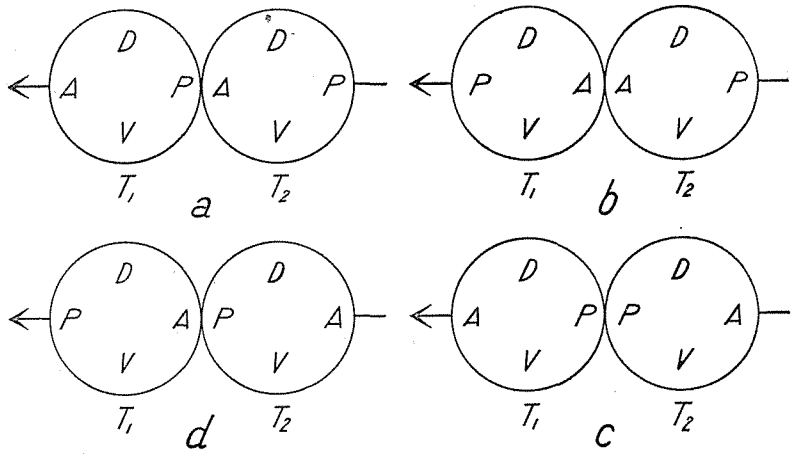


Fig. 28. Diagrammatic representation of axial relation in two combined rudiments in heterotopic position. Arrow points to anterior end of animal.

Table 5

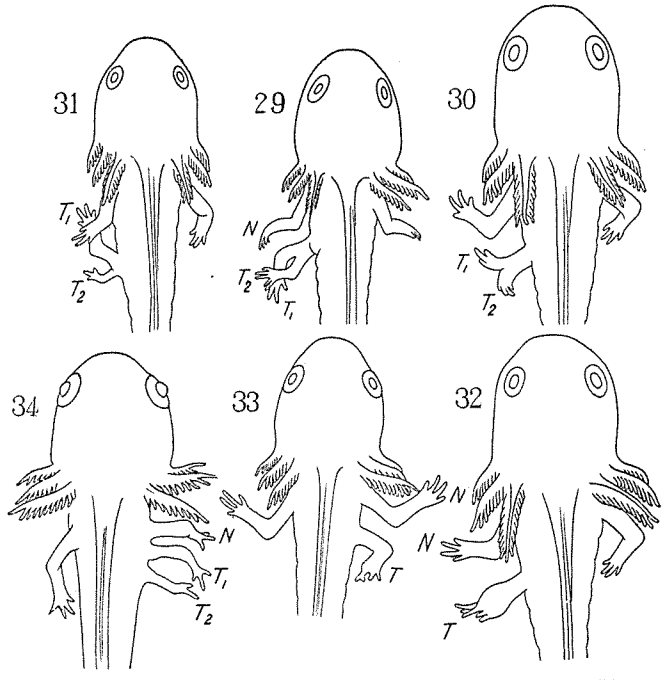
Summary of heterotopic transplantations of two rudiments

Operation	Total	Available	Single limbs			Compound limbs			Separated limbs			Absorbed
			anterior graft	posterior graft	fused	divided in autopodium	divided in zeugopodium	divided in stylopodium	both anterior and posterior single	anterior alone double	posterior alone double	
HTa	24	22	1	—	4	1	2	3	1	5	2	3
HTc	21	17	3	3	1	2	3	3	1	—	—	1
HTd	14	4	—	—	1	—	—	1	1	—	1	—
HTb	31	29	1	—	3	1	6	8	1	5	1	3
Total	90	72	5	3	9	4	11	15	4	10	4	7

antero-posterior combinations of two rudiments only were examined, uniting them in different orientation as shown in fig. 28: i. e., in *a*) both rudiments were placed in the *hom aa dd* orientation with respect to the *ap* axis of the host embryo (series *HTa*); in *b*), the anterior rudiment was in the *het ap dd*, and the posterior rudiment in the *hom aa dd* orientation (series *HTc*); in *c*) the anterior rudiment was in the *hom aa dd* and the posterior rudiment in the *het ap dd* orientation (series *HTd*); and in *d*) both were placed in the

het ap dd orientation (series *HTb*). In all cases development took place in each rudiment irrespective of its orientation, and in most cases two limbs resulted. Only in rare cases where the posterior member was shifted to the caudal portion of the embryo, did absorption take place in that member. The results of each series of transplantations are summarized in table 5.

In the transplantations of series *HTa*, in which both rudiments were in the same orientation and harmonic to the body side, the results were quite similar to those obtained in the corresponding transplantations in the orthotopic position. The limbs fused mostly in proximal portions so completely as to form a single structure. The phenomenon was accompanied in some cases also by the reversal of asymmetry in the anterior member, and as a result, a radial or



Representatives of the heterotopic transplantation in series HTa.
Figs. 29, 30. Asymmetry of anterior member (T_1) is reversed to be mirrored in radial plane; fig. 29, 17 days and fig. 30, 28 days after operation respectively. Fig. 31. Parallel arrangement of limb without change of initial direction. 22 days. Figs. 32, 33. Complete fusion of two limbs, both being 28 days. Fig. 34. Serial arrangement of three limbs, most anterior being host regular one; asymmetry of anterior two limbs (N, T_1) is reversed. 25 days.

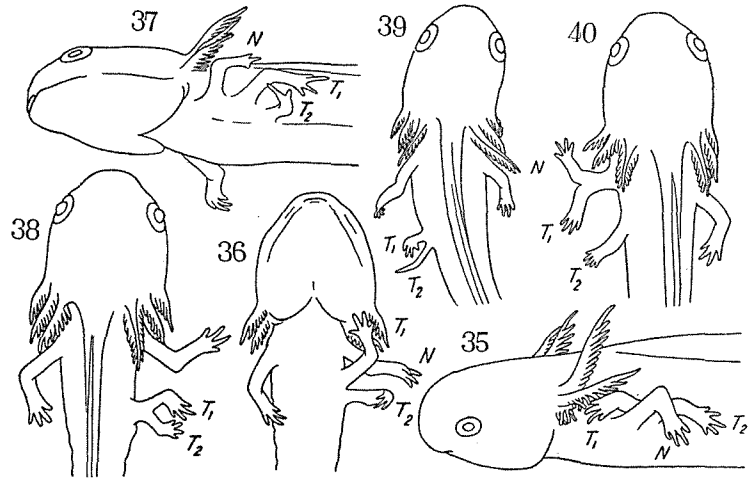
radio-dorsal mirroring was produced. In 3 such cases, division was found respectively in stylo-, zeugo- and autopodium (figs. 29, 30). In other cases, fusion of limbs caused reduplication of the anterior member or other deficiencies were observed. However, there was one case where both members of the fused limb remained single and a parallel arrangement was clearly recognized between them (fig. 31). If fusion was complete, the limb was always anomalous with an indefinite asymmetry (fig. 32), or even disharmonic to the body side (fig. 33).

On the other hand, if the combined limbs developed separately, usually bifurcation took place in one member, especially in that found in the anterior position, and there was no case where two normal limbs were observed in the harmonic direction. In one case there was a development of three limbs in close approximation, being arranged antero-posteriorly in a row (fig. 34). Of these, two posterior ones belonged to the grafted rudiments, while the most anterior one developed from the regular rudiment of the host. Uniformly they were somewhat deficient in the autopodium. However, their asymmetry was distinct; it was reversed in the anterior pair in a disharmonic direction, and only the hindermost one was retained in the original harmonic direction.

In the combination of rudiments, the radial side of which faced each other (series *HTc*), production of the radial mirroring was expected as a natural consequence, but the symmetrical arrangement of limbs in this plane was found only in one case where two limbs were fused at the base (fig. 35), while in most cases reduplication occurred either in one or both of the limbs, and failed to establish a mirroring relation between them. In one case where limbs developed separately, the anterior member was twisted forward by remarkable pronation and became seemingly parallel with the posterior member which retained the given direction (see fig. 36). A complete fusion of limbs with slight irregularities in structure was found in one case, the resulting limb being disharmonic to the body side. There was also one case where reversal of asymmetry was brought about on the regular limb of the host (fig. 37). In this case the anterior graft was situated close to the posterior border of the host limb. The reversal, therefore, may be duly inferred to be the result of the influence of the graft as in the *hom aa dd* transplantation already considered (ref. p. 356), but no mirroring was established between this limb and the host limb.

A simultaneous transplantation of two limb rudiments with their ulnar side facing each other (series *HTd*), was tried in 14 cases. But mortality of the operated embryos was so high as to give results in only 4 cases (ref. table 5). In 3 cases, limbs developed, without producing, however, mirroring in the given direction, and in 2 of these cases, reduplication took place in the posterior member. In the remaining case, although both limbs were normal in structure, the anterior member was situated near the ventral side of the embryo and it was difficult to define its symmetry in relation to the posterior member.

At any rate, the results of the heterotopic transplantations so far obtained are quite similar to those already found in the orthotopic position. This fact indicates that there is almost no influence of position upon the mirroring relationship of two grafted limbs. The next problem is, therefore, to show how the axial relation between two limbs is affected by the *ap* axis of the host embryo. For this purpose two rudiments were grafted in a heterotopic position, so that their *ap*



Representatives of the heterotopic transplantation in series HTc (figs. 35-37) and in series HTb (figs. 38-40).

Fig. 35 Symmetrical arrangement of grafted limbs in radial plane, original asymmetry being retained in both members. 27 days after operation. Fig. 36. Same as before, but irregular posture taken by anterior member (T_1) disturbs enantiomorphic arrangement in given direction. 26 days. Fig. 37. Asymmetry reversal induced in host regular limb by grafting. 27 days. Figs. 38, 39. Production of radial mirroring by asymmetry reversal in posterior member (T_2) of grafts; fig. 38, 23 days and fig. 39, 28 days respectively. Fig. 40. Ulnar mirroring as the result of fusion between host limb and anterior member of grafts (T_1). 29 days.

axes were inverted with respect to that of the host embryo (series *HTb*). In this combination, having examined the combination in its normal orientation, a radial mirror image was observed. There were 3 such cases out of 8 where fusion took place at the base of the limbs (figs. 38, 39). The production of the radial mirror image was brought about by the reversal of asymmetry in the limb situated posterior to the other. In cases where two rudiments were grafted with their *ap* axis in the normal orientation, reversal of asymmetry took place always on the side of the anterior member. Therefore, the limb which is reversed, is in the opposite relation to the body. However, if we consider the direction of the *ap* axis of the limb rudiment alone, without taking the body axis into our consideration, we can reconcile this discrepancy to a certain extent. The limb which underwent the reversal in this case, corresponds to the anterior limb in the other case with respect to the direction of the *ap* axis of the graft, although it is caudal in its position with respect to the body axis of the host embryo. Thus we find finally in the serial combination of two limb rudiments with the *ap* axis in the same direction, reversal of the asymmetry takes place always on the side of the anterior member irrespective of the direction of the grafts against the body axis. No influence of position and orientation is perceptible upon the symmetrical relation between two grafted limbs.

Separate development as well as complete fusion of limbs was also found in this series of transplantations, without any marked difference in the results as compared with the corresponding combinations in the normal orientation. Namely, in separate development, reduplication was brought about in most cases in the anterior member and in complete fusion, irregularity ensued. In a few cases of separated limbs, fusion took place between the anterior member and the regular limb of the host along the whole length of the stylopodium, and resulted in the ulnar mirroring (fig. 40). In this case reversal of asymmetry took place in none of them, because the ulnar side of the graft was placed from the beginning to the ulnar side of the host limb. By this chance occurence, however, we obtained examples of enantiomorphism in the ulnar plane which was lacking in prior experiments.

III. *Regeneration of the compound limb*

Amputation of one member of a compound limb, having reversed asymmetry, was tried to ascertain if the same asymmetry as before

was reproduced after the operation. But before proceeding with this experiment, amputation was performed in one member of a pair of reduplicated limbs derived from a single rudiment. In this case, a rapid regeneration followed in the amputated member which always repeated the same structure as before, if the cut was well in the distal portion of the branched point. This always held true whether the limbs were situated either in the orthotopic or in the heterotopic positions. On the other hand, in the case of compound limbs produced by the fusion of two rudiments, the amputated member, whether it belonged to the regular host or to the grafted rudiment, showed no regeneration beyond wound healing, so far as correcting its asymmetry was concerned. The position of amputation apparently did not affect this phenomenon. Whereas, in those cases where one of the members was not completely reversed in its asymmetry as well as in those cases where both members were normal without changes in their asymmetry, regeneration took place as usual and always reproduced the missing part with the same asymmetry. Therefore, the hope to solve whether or not reversal of asymmetry is accompanied by reversal of the limb axis is unfulfilled by the amputation experiment.

IV. *Internal structure of the compound limb*

Fusion of two parallel limbs was sometimes followed by the reversal of asymmetry in one member, and in some cases fusion was found even in the humeri, which were visible in the living specimen in a ventral view (figs. 3, 4). In other cases where fusion was superficial, accompanying no reversal of asymmetry in either member as in the separate development, the internal structure naturally remained double. These facts suggest the existence of a certain correlation between the internal structure and the limb asymmetry. However, the closer investigation in sections as well as in anatomy of the available specimens indicated that this was not necessarily the case. In compound limbs in which mirroring was evident, fusion of the humeri generally took place. But the fusion of the bone was also found in a few cases where no mirroring occurred. Moreover, there were other cases where the limb asymmetry was reversed and yet the internal structures were separated. Naturally, where two limbs were separated the skeletons were double. Hence, we fail to find a general rule which can be applied to this condition for discriminating the two groups of limbs studied by a mere comparison

of the internal structures. Practically, however, when two limbs fused closely fusion of the internal structures, especially of the humeri, was quite frequent and brought about the reversal of asymmetry in one member of the compound limb, whereas the superficial fusion of limbs in the soft parts was never accompanied by the reversal of asymmetry in either member.

Discussion

(1)

Two limb rudiments closely combined generally develop separately, and yet a complete fusion of them into a single limb sometimes takes place. Such limb-fusion has been observed to occur as early as that stage of development when the limb bud becomes visible (NICHOLAS (1924), BRANDT (1925), SWETT (1926), DETWILER (1934), etc.). The fusion is also possible in more advanced stages than this as proved by several cases under observation during the present experiment. As shown in table 6, the frequency of fusion varies greatly according to the position of transplantation round the limb disc of the host. It is more frequent in the dorsal position, where the grafted rudiment is placed very close to the host as compared with other positions. However, different combinations of the grafted rudiment with respect to the axial direction of the host seem to have nothing to do with the phenomenon, since there exists a similar frequency among the cases observed in the combinations (ref. table 6). From these facts, the narrowness of the space between two limb rudiments combined seems to be a capital factor for the complete fusion of them. On the other hand, the limb axis has significance in connection with the asymmetry of the fused limb in such a way that the fusion in the same axial direction results in a production of the normal asymmetry, while in the antagonistic direction the fused product is generally anomalous.

As has been shown by DETWILER (1934) in the transplantation experiments of an extra limb rudiment into the position just posterior to the normal one in the same orientation, difference of growth is always noticed between two limbs combined. In the antero-posterior combination retardation or even arrest of development takes place on the anterior member, whether this belongs to the grafted rudiment or to the regular one of the host, and in the dorso-ventral combination, it is the ventral member which is affected. This rule of the

Table 6

Summary of additional transplantations of single rudiment

Operation \ Position of transplantation	Complete fusion					Partial fusion					Separated development				
	A	P	D	V	Total	A	P	D	V	Total	A	P	D	V	Total
Hom aa dd	1 (17)	1 (53)	5 (19)	3 (18)	10 (107)	4 (17)	34 (53)	1 (19)	8 (18)	47 (107)	1 (17)	16 (53)	1 (19)	3 (18)	21 (107)
Het aa dv	— (8)	1 (14)	— (—)	— (4)	1 (26)	6 (8)	11 (14)	— (—)	3 (4)	20 (26)	1 (8)	2 (14)	— (—)	— (4)	3 (26)
Het ap dd	— (6)	1 (18)	— (—)	— (6)	1 (30)	6 (6)	7 (18)	— (—)	4 (6)	17 (30)	— (6)	7 (18)	— (—)	— (6)	7 (30)
Hom ap dv	— (8)	— (15)	8 (14)	— (5)	8 (42)	5 (8)	6 (15)	2 (14)	— (5)	13 (42)	— (8)	6 (15)	2 (14)	— (5)	8 (42)
Total	1 (39)	3 (100)	13 (33)	3 (33)	20 (205)	21 (39)	58 (100)	3 (33)	15 (33)	97 (205)	2 (39)	31 (100)	3 (33)	3 (33)	39 (205)

( )=available cases; A, anterior; P, posterior; D, dorsal; V, ventral.

limb growth, so far as the present experiments reveal, is further applicable not only to the combination of two limb rudiments in the same direction of axis, but also to that in the antagonistic direction (ref. p. 355). As to the cause of the phenomenon, an incompatibility of the combined rudiments is probably the reason. Because the retardation or shrinkage of one limb takes place always when they are fused together. If they are separated, development occurs in both members even when transplantation is accomplished into the anterior position of the host limb where the shrinkage is most marked. On the other hand, the limb rudiment develops in any position of the body excluding such portions as the gill area and the abdomen, where absorption is generally found. Within the limit of the body side, therefore, so marked difference in the development of the grafted limb as found in the present experiment, cannot simply be attributed to the influence of the position in which the transplantation is done. Accordingly fusion plays some important rôle in the reduction of one of two limbs in combination.

In the splitting experiment of a limb disc, SWETT (1926) who finds also reduction in one of two limbs thus resulted supposes that this reduction is because the limb is in disadvantageous location with reference to the blood stream. This supposition is, to a certain extent, corroborated by the experiment of SCHMALHAUSEN (1925) which demonstrates the importance of nutrition, temperature etc. in the regeneration of limbs. On the other hand, BLOUNT (1934) states from his anatomical observation on the reduplicated limbs that reduplication depends solely upon the mesodermal material to be used and the weak supply of blood vessels does not necessarily follow the absorption of a reduplicant. In our present case, it is preferable to adopt the latter interpretation, because transplantation of the limb rudiment is made slightly encroaching on the limb disc of the host, a certain portion of which becomes common to both rudiments, and is employed by both of them when they begin to develop simultaneously. The equipotentiality, as has been demonstrated by HARRISON (1918, '21, '25) and HOLLINSHEAD (1934), is gradually reduced as development proceeds, and at the time of limb formation the mesoderm of the limb disc behaves, according to SWETT (1923), differently in proportion to its four quadrants; the dorsal half is important for the limb formation putting, however, the anterior half in second importance. From these facts of limb formation, the portion common to

both rudiments seems to have a significance for the incompatibility which exists between them.

(2)

Two kinds of mirroring are produced by the reversal of asymmetry in one member of the combined limb, one being radial and the other palmar. The latter is brought about in the ventral transplantation by the reversal of the dorso-ventrality in the grafted limb (see p. 366). This seems to be in turn caused by the reversal of the *dv* axis of the rudiment. The period for the irrevocable determination of this axis varies in each species tested, and it is not yet worked out in the present specimen. However, the determination of the axis was supposed to be in a labile condition in the present case when the experiment was made, and because of the *dv* axis was actually found to be reversed in some cases (see p. 361). The reversal of the dorso-ventrality in the grafted limb can be regarded as the result of the reversal of the *dv* axis of the rudiment. As a result of the phenomenon, the limb becomes disharmonic to the body side instead of the harmonic direction previously given to it. This is quite contrary to the case of a single rudiment, since in the latter the harmony of the limb asymmetry with the body side is established by the same process. Therefore, the reversal of the dorso-ventrality in question cannot be caused by the influence of the surroundings as in the case of the single limb, and an interaction between normal and grafted limbs by their fusion is considered the salient factor.

The radial mirroring is always found in the antero-posterior combination of limb rudiments with the *ap* axis in the same direction, reversal taking place in the anterior member with respect to this axis. This fact is confirmed in the heterotopic as well as in the orthotopic position and further in those cases where the *ap* axis of both rudiments is inverted. Therefore, the production of mirroring between two limbs is apparently not at all related directly to the position and orientation in which the rudiments are transplanted into the body side. Also in this case the interaction of combined limbs is regarded as the capital agent.

According to HARRISON and his associates, the reversal of the limb asymmetry is brought about in one of the following ways; 1) by the reversal of the *ap* axis of the rudiment, or 2) by the reduction of the primary bud after reduplication, or 3) by the rotation of the limb by regulation. As already described, the reversal is brought

about in my tests mostly by the rotation of the limb structure as a whole, and therefore, it belongs to the last category. As to the procedure of the rotation, it begins, as pointed out by NICHOLAS (1924) in the single limb, in a relatively later stage of development and proceeds gradually. The same process in the compound limb is described by NICHOLAS (1924), SWETT (1926) and OKA (1934). NICHOLAS states that the rotation of one member influences the other so as to rotate in the opposite direction. But in my tests rotation takes place so remarkably in one member as to reverse its asymmetry, while no change is perceptible in the other member in any case observed.

The so-called interaction between two limbs here under consideration can be recognized only in the production of enantiomorphism by the reversal of the asymmetry in one member of the compound limbs. There is no clue to detect it where no change is found in the direction of either member. In the combination of limb rudiments with the *ap* axis in the opposite direction, mirroring is sometimes produced but only in rare cases, while in most cases frequent occurence of reduplication and anomaly in one or both members hindered the establishment of mirroring previously given to the combined rudiments. Therefore, the combination of two rudiments in a direction to produce symmetry does not necessarily follow an appearance of mirroring between them.

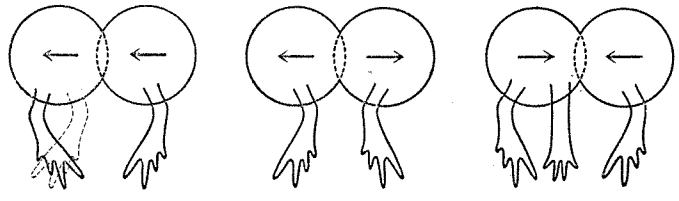


Fig. 41 Diagrammatic representation of results in antero-posterior combination of two limb rudiments; circles indicating limb discs, radial side of which is pointed by arrow. Limbs in heavy line represents those actually produced and that in broken line the one, if developed, in the given direction.

Fig. 44 is a diagrammatic representation of the symmetry relation of the limbs obtained. In the radio-ulnar combination (fig. 41, a), the radial mirroring is produced in about 30% (23 out of 76), while in the radial combination (fig. 41, c), the radial mirroring appears in about 22% (6 out of 28) of the available cases. The ulnar mir-

roring (fig. 41, c) is found in few cases but it occurred only by chance (ref. p. 372). From this result alone, however, it is very difficult to draw out a general rule for the enantiomorphic development of the combined limbs. Of course, we cannot fail to recognize an interaction which exists between them; this effect is only presented where two limbs fuse together, and when they are separated, no influence is perceptible, at least, concerning the asymmetry of limbs. For this reason, the "Symmetriefaktor" of WILHELMI cannot be accepted as it is, so far as the present experiment is concerned. Here we are of the same opinion as MANGOLD (1929) who holds the view that "der Einfluss zweier Anlagen nur dann zur symmetrischen Ausbildung führt, wenn der Wirkungsbereich der beiden Organisationsfelder sich teilweise überdeckt und diese ein gemeinsames Baumaterial besitzen. Dabei könnte auch die Quantität des Baumaterials von Bedeutung sein.....". Also in our experiments, besides the quantity, reference should be made to the quality of the mesoderm which is common to both rudiments, because the interaction is not presented always in the same manner, and the action is always one-sided and not reciprocal in all combinations examined. In the overlapping experiment of two limb rudiments of *Amblystoma punctatum*, HOLLINSHEAD (1934) has demonstrated that the independent development of the limb disc with reduction of the equipotency, begins in stages 36–37, in which the limb is a slight protuberance from the body side. In a limb bud of a little more advanced stage, the effect of the interaction becomes manifested in one member of the combined limbs. Therefore, it seems reasonable to take the qualitative difference of the combined limb discs into our consideration as being responsible for their interaction. If so, the so-called interaction between two limb rudiments can be defined as sectional conflict of the fused portion, and its influence then need not be extended to the whole limb disc in connection with the consideration of the limb asymmetry, except for such combinations as have the *ap* axis in the same direction.

(3)

The radial mirror image is predominant in the reduplication of a single limb rudiment. This is also true in the present case of the combined limbs, though mirroring is sometimes incomplete as shown in figs. 3–6. Regeneration always takes place in a reduplicant of the single limb, but it does not occur in the member of the combined limb, with the asymmetry reversed. As a reason for such differences,

the fact that two rudiments of the compound limb are brought together experimentally after determination has proceeded to a certain extent, is taken into consideration; whereas in the reduplication of a single rudiment, a connection more intimate is imagined to exist between branches, even in view of the symmetry hypothesis, which assumes in one rudiment the latent potency of two limbs with opposite asymmetry. As to the origin of reduplicants, we may follow to the centre hypothesis of HARRISON, who claims division of the limb-forming centre. But as pointed out by MANGOLD (1929), the division of the growth centre alone is not enough to solve the reason for the production of enantiomorphism. Based on his transplantation experiments, SWETT (1926) has made the following proposal: Polarization of the *ap* axis of a secondary limb bud takes place in the direction opposite to that of the primary, probably when the secondary growth centre is formed. This interpretation permits implicitly the belief in interaction between two centres of limb growth. Nevertheless, the result of his splitting experiment of a limb disc has brought about two parallel limbs retaining the original asymmetry and in no case is mirroring established between them. Viewed from our experiments, this result seems, however, to be quite natural, since a piece of indifferent tissue which is inserted into a limb disc in order to bisect, would prevent a close connection of two limbs developed on both sides of the split, and this makes it difficult for one limb to interact upon another. As may be easily supposed, the interaction is more effective between two members of a single limb origin, because two limb-forming centres are located more closely than in the case of combined limb discs and the secondary centre is generally developed later than the primary one. The secondary centre is subjected from the beginning to the influence of the primary one and comes under control of the latter. Therefore, we may speak of this influence as overwhelming rather than mutual.

Summary

To investigate the mutual action between two limbs establishing an enantiomorphism, two limb rudiments were combined in the embryo of *Triturus pyrrhogaster* (BOIE). Combination was made 1) by transplanting an additional rudiment in one of these positions, anterior, posterior, dorsal, and ventral in close contact with the normal limb disc, or 2) by grafting two rudiments at the same time in a heterotopic position, the axial relation being varied in each position

with respect to the *ap* and *dv* axes. Thus in the first case the axial relation between two limbs, and in the second the relation of the combined limbs to the body axis were examined.

1) There occurred generally incompatibility in the growth between two combined rudiments. In the antero-posterior combination the posterior member, whether it belonged to the host or to the graft, always proceeded the development of the anterior member. In the dorso-ventral combination, it was the ventral member which generally checked the development.

2) Even when two limbs developed, fusion was very frequent between them and in some cases resulted in a reversal of the prospective asymmetry in one member. The reversal of asymmetry was found to be brought about generally in the direction from harmonic to disharmonic with respect to the body side, with the resulting production of an enantiomorphism. This phenomenon was actually found in the following series of operations:

a) In the *hom aa dd* transplantation in the posterior position, reversal of asymmetry took place on the side of the regular limb of the host, resulting in the production of a radial mirror image (figs. 3-6).

b) In the *hom aa dd* and *het aa dv* transplantations in the anterior position, the asymmetry of the transplanted limb was reversed and mirroring in the radial plane occurred (figs. 20, 21).

c) In the *hom aa dd* transplantation in the ventral position, the palmar mirror image was produced by reversing the dorso-ventrality of the grafted limb (fig. 24).

3) In the heterotopic transplantation, two rudiments combined in the same axial direction as in (a) and (b) of the second section, resulting similarly in the occurence of a radial mirror image even when their *ap* axis was inverted with regard to that of the body (fig. 28).

4) In the combination placing the *ap* axis of the rudiments in an antagonistic direction, reversal of the prospective asymmetry did not occur, irrespective of the fusion between them. Reduplication and anomaly, which were often met with in one or both members of the combined limbs, usually interrupted them to realize the given symmetrical arrangement, though in a few cases a figure like a radial or ulnar mirror image was actally produced according to the relative position of one rudiment with respect to the other (figs. 35, 40).

5) From these facts, it appears that an interaction really exists

between two combined limbs. However, the action, so far as their enantiomorphism is concerned, seems to work on one side and is not reciprocal between them. That is to say, its effect is noticed only in the particular member which varies according to the different combinations. This phenomenon should be attributed to the qualitative difference within the limb disc, and the action is, therefore, to be defined as a sectional conflict and not necessarily extended to the whole limb disc.

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