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Transplantation of Limb-Discs of Varying Size

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With 15 figures in the text

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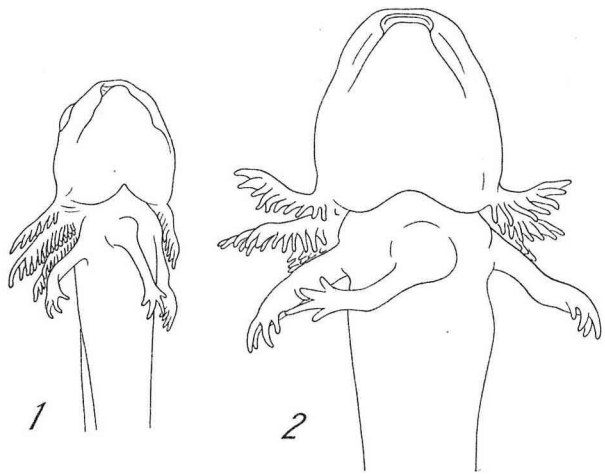
Introduction

The limb-forming area, according to HARRISON (1918), consists of a circular disc on the side of the body, measuring in the embryo of *Amblystoma punctatum*, a diameter of three and a half somites just below the pronephros. In these experiments, however, discs larger than the size named were employed in order to learn the siginificance of size as well as of the presence of the surrounding tissue for development of the grafted limbs. The experiments were carried out in 1936 and 1937 under the supervision of Prof. Yô K. OKADA, using embryos of *Triturus pyrrhogaster* as material. It is a pleasure to take this opportunity of thanking him for his valuable suggestions and criticisms.

Experiment I

The mid-ventral position of the urodelan embryo is found by WILHELMI (1922), NICHOLAS (1924) and others to be unfavorable for the development of grafted limbs. Nevertheless, this position was chosen in the present experiment to receive the graft, because it was hoped that in this position the condition for limb-growth only can be analyzed, without interference from other factors otherwise involved in the body side. Four series of experiments were performed, in each of which the graft was of varying size, i. e. 3, 4, 5 or 6 somites in diameter respectively, and operation was made only on the left side of the body. The results are summarized in the accompanying table (tab. 1).

In the case of small grafts absorption was of frequent occurrence. Even when they grew, the limbs were generally abortive, becoming filiform on account of marked deficiency of the autopodium. In rare cases limbs did develop. But these were, as a rule, stretched straight out apparently because of lack of the elbow joint and presented the so-called "Fächer-

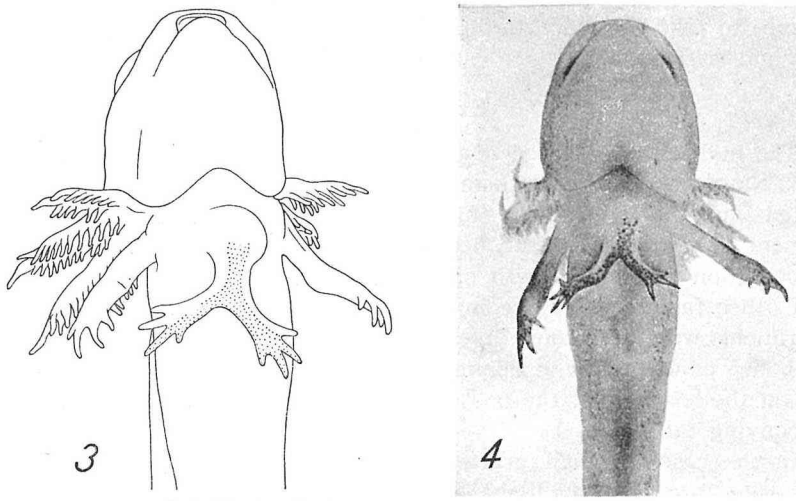


Two examples of the ventral transplantation.

Fig. 1. Irregular limb of so-called "Fächerbildung" produced in graft 4 somites in width. 32 days after operation.

Fig. 2. Normal limb with the original asymmetry developed in graft 4 somites in width. 50 days.

bildung", being unable to acquire proper asymmetrical structures (see fig. 1). Anomalous limbs of this type were also sometimes met with in the same kind of transplantation with a larger graft. Among the transplantations of 3 and 4 somites in width, perfect development of the limbs with proper asymmetry was found only in one case (fig. 2). In the grafts of 5 somites, absorption of the limbs rarely occurred (2 out of 8), although half of the limbs produced were more or less irregular in structure. When the grafts were as wide as 6 somites, limbs were produced in all cases so far as the operations were successful. In 3 of these 7 cases, limbs were normal in all respects. In one other case



Reduplicated limbs of the ventral transplantation.

Figs. 3, 4. Members of reduplicated limbs are mirrored in the radial plane. Extensor surface of limbs is toward the reader. Fig. 3, 48 days after operation; Fig. 4, 50 days.

the limb was anomalous as is shown in fig. 1. In the remaining 3 cases reduplication occurred with resulting production of ulnar mirroring (figs. 3, 4). The reduplicated limbs were found to be bifid in auto-, zeugo- and stylopodium respectively. In no case were separated limbs produced such as were reported by NICHOLAS (l. c.) in a case of ventral transplantation. In the position of transplantation in question the limb, whether it was single, double or irregular, tended to turn the palmar surface toward

Table 1 Summary of transplantations in the mid-ventral position

a) Addition of the surrounding tissue

Size	Operated	Survived	Limb absorbed	Limb developed		
				abortive or irregular	normal	reduplicated
3 somites	8	8	5	3	—	—
4 somites	8	8	4	3	1	—
5 somites	8	8	2	3	3	—
6 somites	7	7	—	1	3	3
b) Addition of portions of the surrounding tissue						
N	16	16	8	8	—	—
DV	16	15	1	6	6	2*
AP	17	16	6	7	1	2*
D	9	7	2	3	2	—

Abbreviations: N, transplantation of normal limb-disc; DV, addition of dorsal and ventral portions; AP, addition of anterior and posterior portions; D, addition of dorsal portion alone. *, reduplication in the autopodium.

the body. This contrasts with the case of NICHOLAS, in which the palmar surface faces in the ventral direction. At any rate, the above results of experiments indicate that the presumptive area for limb-development when grafted into the ventral site, fails to yield a perfect limb, and that the latter to be perfect should be accompanied by a certain portion of the surrounding tissue.

An experiment was next made to find out what part of the surrounding tissue bears the most significance for the limb-growth. For this purpose discs were cut out together with 1) dorsal and ventral portions of the surroundings (series DV) on the one hand, and 2) with anterior and posterior portions (series AP) on the other. The shape and size of grafts are diagrammatically represented in fig. 5. Transplantation was done as before in the mid-ventral portion of the body in antero-anterior and antero-posterior orientations. In either case, irrespective of the orientation, limb-growth was much better than when limb disc proper alone was grafted. Especially in those grafts to which dorsal and ventral portions of the surrounding tissue were attached, development of the limbs was most perfect (ref. tab. 1 b). On the other hand, limbs developed from the grafts to which anterior and posterior portions of the surrounding tissue were attached, were generally abortive and

irregular. They were often symmetrical in structure, stretching out straight without bending in any direction (cf. fig. 1). Reduplication occurred in 2 cases of each of the above-mentioned combinations of transplantation, always with production of ulnar mirror image. In these cases, the limbs were bifid at the autopodium; the zeugopodium and in some cases the stylopodium were thick, apparently manifesting doubleness of the internal structures.

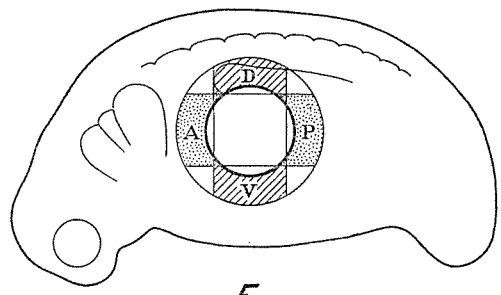


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Fig. 5. Schema, showing the size and shape of grafts in the ventral transplantation where parts of the surrounding tissue are attached to the limb-disc. Circle of thick line represents normal limb-disc. Both dotted and striated areas are portions grafted together with the rudiment.

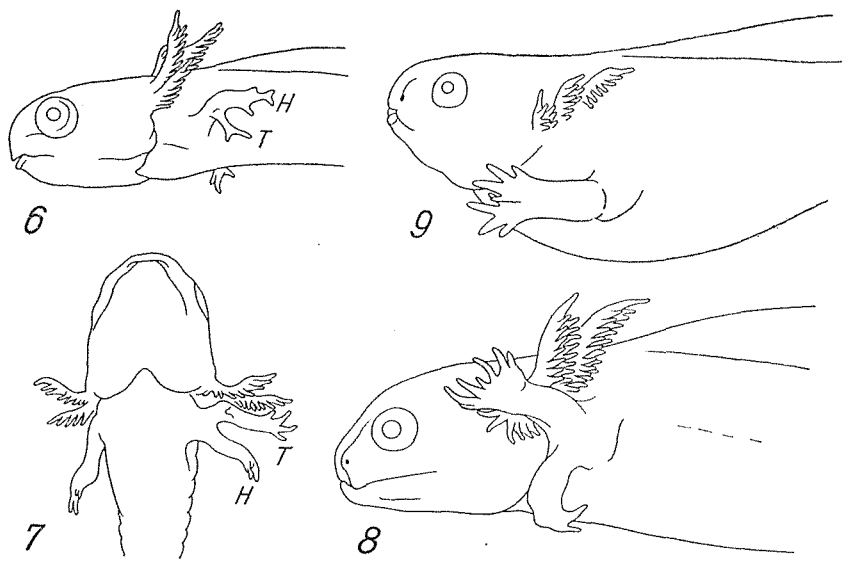
Thus it appears from the results of these experiments, that surrounding tissue from the dorsal and ventral portions has greater influence than that from the anterior and posterior portions. Furthermore, of the dorsal and ventral portions, the former may be supposed to be more powerful than the latter from the following facts noted in the experiments: Of 7 cases in which the limb-disc was grafted together with only dorsal portion of the surrounding tissue (series D), limbs were produced in 5 cases (3 were somewhat deficient in structure). Evidently the percentage of limb-formation is higher than in the case where anterior and posterior surroundings were attached to the disc (ref. tab. 1 b). In one case in which operation was made on an embryo of advanced stage of development (st. 31), limb-formation was more perfect than in the others, and moreover, there was produced a small bud-like nodule at the ulnar base of the limb. The age of the graft may have some bearing upon the limb-growth.

A few words may be added on the pigmentation of the grafted limbs. When limb-rudiments were grafted together with the dorsal portion of the surrounding tissue, pigment appeared in the majority of cases generally, as shown in figs. 3, 4, limited to the extensor surface of the limb. Only rarely the whole surface of the limb was densely covered with pigment cells. On the other hand, when anterior and posterior portions of the surrounding tissue were grafted together, pigmentation appeared in less than half of the limbs resulting. Appearance of pigment is, however, in no case to be regarded as directly related to the growth of limbs, because pigment appeared in the grafted tissues irrespective of the presence or absence of limbs. But the fact that the dorsal portion of the surrounding tissue is more effective in producing pigment than other portions, may support DUSHANE's finding (1935) that the melanophores of *Amblystoma* migrate at the tail-bud stage from the neural crest toward the flank.

Experiment II

From the foregoing experiments it is evident that reduplication was brought about only when large grafts consisting of the limb-disc and surrounding tissue were used. Presumably the size of grafts has some relation to the production of twin limbs. In order to verify this assumption, transplantation of limb-discs of varying size was performed in the flank of the embryo. Homopleural transplantation was made both in ortho- and heterotopic positions in stages from 27 to 31, i. e. in the tail-bud stage of the embryo.

Orthotopic transplantation in hom dv ap orientation: In the first series of experiments only the central portion of the limb-disc, 2 somites wide, was grafted. This resulted in 3 single and 4 reduplicated limbs in the distal portion (tab. 2). Fig. 6 is an example of the latter in which one of the members is small, deficient, and has no symmetrical relation to the other. Such independent development of limbs may be brought about by



Some representatives of the orthotopic transplantation.

Fig. 6. Reduplicated limb developed in graft 2 somites in width. The member (*T*) derived from grafted parts is small, deficient, and attached to the other one (*H*) which originated in the host tissue. 23 days after operation.

Fig. 7. Reduplicated limb developed in graft 3 somites in width. The grafted member (*T*) is perfect and mirrors the host limb (*H*) in the radial plane. 44 days.

Fig. 8. Irregular reduplication of limb produced in graft 4 somites in width. 51 days.

Fig. 9. Reduplicated limb produced in graft 5 somites in width, showing apparent doubleness of the internal structure. 44 days.

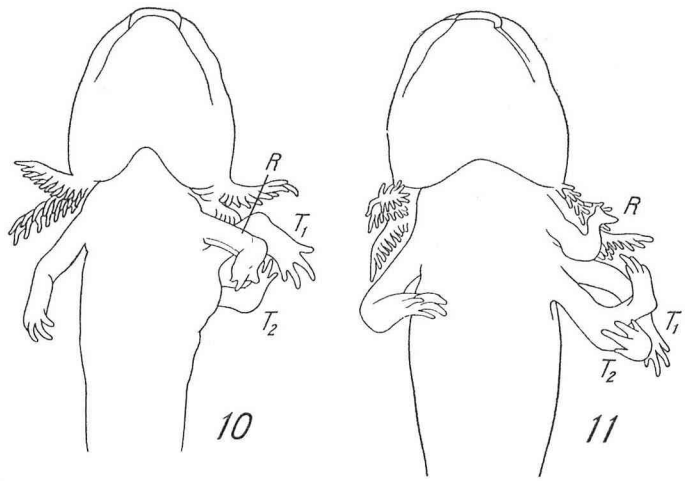
the fact that the grafted central portion behaves in development independently of the peripheral portion which belongs to the host limb-disc, the former being in reverse orientation with respect to the latter. Generally speaking, the limb developed from the host periphery is normal in all respects, whereas the limb resulting from the grafted centre is more or less deficient and under-developed, often attaching to the distal portion of the other, or in some cases it even failed to develop. Also in the transplantation of 3-somites grafts, similar limb-reduplication was frequently observed. In one case, however, the graft showed full growth and produced a perfect limb at the radial border of the limb derived from the host. In this case the limb developed from the graft was disharmonic to the body side and established a radial mirror image of the host (fig. 7). On the other hand, a single limb was produced in 2 cases. In the beginning of development the limb was disharmonic, but later it became harmonic to the body side through rotation.

When grafts were larger than the proper width of limb-disc, being 4 somites in diameter, limb-reduplication was still prevalent. In this case, however, two limb-buds were actually produced, but one of these was gradually resorbed in the course of development. 3 such cases were obtained, 2 of them were harmonic and the other disharmonic to the body side. In the reduplicated limbs, division occurred in the proximal portion (ref. tab. 2).

Table 2. Summary of transplantations in the flank

Size	Operated	Survived	Single Limb		Reduplicated Limb				Per cent of reduplicated limbs
			harmonic	disharmonic	in autopodium	in zeugopodium	in stylopodium	separated	
a. Orthotopic *hom ap dv*									
2 somites	8	7	3	—	2	2	—	—	57%
3 somites	8	8	2	—	2	3	1	—	75%
4 somites	12	12	2	1	—	3	6	—	75%
5 somites	22	10	—	3	2	3	1	1	70%
6 somites	11	11	—	2	—	—	2	7	82%
b. Heterotopic *hom aa dd*									
4 somites	8	3	—	1	2	—	—	—	69%
5 somites	8	7	2	—	1	1	3	—	71%
6 somites	8	6	—	—	1	1	1	3	100%
c. Heterotopic *hom ap dv*									
4 somites	8	7	—	2	3	1	—	1	71%
5 somites	8	6	—	1	1	1	3	—	83%
6 somites	8	5	—	1	—	—	1	3	80%

Generally the structures were anomalous (fig. 8), and no case was found where mirroring was clearly established between two members. Even in the transplantation of 5-somites grafts, the results were generally the same as in the preceding case. The only peculiarity to be mentioned here is that division took place usually in the distal portion of the limbs (see tab. 2). But internally the latter was apparently of double structure through the whole length (fig. 9). Beside these, there were also 3 cases in which a single



Two examples of the orthotopic transplantation of 6-somites grafts, showing production of the pair of limbs (T_1, T_2). In both cases one more timb (R) is produced from the host.

Fig. 10. 40 days after operation; Fig. 11, 57 days.

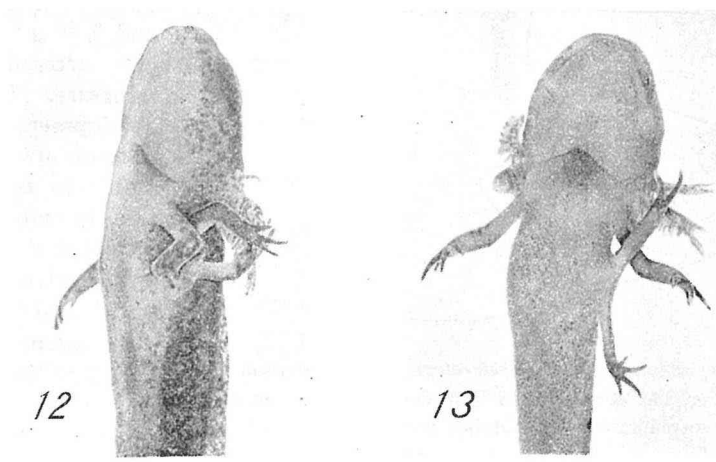
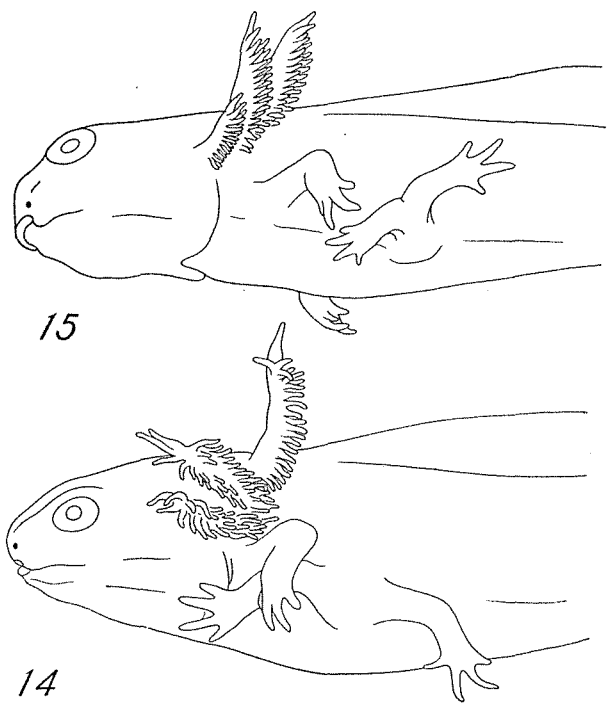


Fig. 12. Photographic representation of the same specimen as in fig. 10.
Fig. 13. The same as in fig. 14.

limb was produced. All these limbs commenced their development in the disharmonic direction, retaining the original polarity of the limb-axis. But 2 of them gradually changed their posture to become harmonic to the body side. Nevertheless, it was uncertain whether or not this was brought about by regulatory rotation of the limb as a whole.

In one case of 5-somites grafts, reduplication of limbs occurred, two members being completely separated one from the other. This was, moreover, common when grafts were made as large as 6 somites in width (ref. tab. 2). Figs. 10-12 represent two examples of the results of this number of somites. Not only in these but also in other cases, the two members of reduplicated limbs mirrored each other in the radial plane. Sometimes one of them anomalous, being deficient or a further reduplication taking place (fig. 11). Moreover, the limbs were often shifted more or less from the regular limb-position to the caudal side (figs. 10-12), probably on account of technical difficulty in cutting out a circular disc so wide as 6 somites in diameter with the proper limb-forming area precisely at the centre of it. Also perhaps for the same reason, one more limb appeared in a position a little anteriorly to the anterior member of the two limbs, sometimes in a level corresponding to the contra-lateral one of the host (fig. 10). As a rule, appearance of the third limb in question was retarded 2 or 3 days over that of the others, and it was more or less irregular in structure. But the developmental direction was always harmonic to the body side, in spite of the fact that it was derived from the tissue transplanted in the disharmonic direction (figs. 10-12). In one of the reduplicated limbs, in which limbs were fused proximally,



Representatives of the heterotopic transplantation.

Fig. 14. Reduplicated limb developed in *hom dv ap* transplantation of 6-somites graft. 52 days after operation.

Fig. 15. Pair of limbs produced in *hom dd aa* transplantation of 6-somites graft. 54 days.

the ulnar mirror image was found. In this case the limbs were shifted to the ventral side toward the abdomen and turned the flexor surface upward; the same condition was always found in the ventral transplantation of limb-discs (ref. p. 322-323).

As a conclusion so far as the experiments described are concerned, the size of grafts has significance for the mode of limb-development in the orthotopic position, all varieties of reduplication produced depending upon it.

Transplantation in heterotopic position: A larger area than the proper limb-disc was grafted in *hom dd aa* and *hom dv ap* orientations into the body side. Irrespective of the differing orientations, the results were the same, and they are compared with those in the orthotopic position (ref. tab. 2). Also, in the heterotopic position reduplication of limbs was more intense as the size of the grafts increased. When the latter was as large as 6 somites in width, the two members of reduplicated limbs were generally separated from each other (figs. 13, 14). Mirroring was radial, as a rule, but in rare cases ulnar mirroring was found in disharmonic transplantation. In this case the dorso-ventrality of the limb was inverted with the flexor surface turning upward (fig. 15). Single limbs occurred in both orientations irrespective of the size of grafts. As to the asymmetry of the limbs, among 3 cases of *hom dd aa* orientation 2 retained the original direction and one, which was derived from the 4-somites graft, reversed and became harmonic to the body side. In *hom dv ap* orientation, production of single limbs was somewhat more frequent than in *hom dd aa* orientation, and all the limbs which were produced retained the original direction of polarity, with the resulting disharmonicity to the body side.

Discussion

Problems of absorption: Absorption of the grafted limb-rudiment in the ventral position, the fact of which has been mentioned by WILHELMI (1922) and NICHOLAS (1924), is also shown in the present experiments. Our results, however, went a step further and demonstrated that absorption took place when grafts were small, but in larger grafts comprising the surrounding tissue, limbs were generally produced. At the same time, each portion of the surrounding tissue seems to have different significance for their growth. However, the dorsal and ventral portions or even the dorsal portion alone was sufficient for complete development of limbs. Hence, it may be assumed that addition to the graft of the dorsal portion of the surrounding tissue bears a significance for the limb-development in the ventral location, important beyond simple augmentation of the size of the graft. Within the limb-forming area the cells of the dorsal half, especially those situated below the pronephros, according to SWETT (1923, pp. 215-216), play the most important part in limb-formation. So the absorption of smaller grafts above mentioned may have been caused by the deficiency of this important portion of the surrounding tissue.

Absorption of the grafted limb is further reported in other positions beside the abdomen, e. g. gill area, head (DETWILER 1922, 1930), mid-dorsal (NICHOLAS 1924), and flank of the body (RUUD 1931, HOLLINSHEAD 1936). In these positions except the last, the cause of absorption would be the same as in the ventral position, and insufficiency of the material for limb-growth can be assumed. In the flank position RUUD (l. c.) pointed out regulatory reversal of *dv* axis which would be the cause of the developmental check of the grafted limb-rudiment. But in the author's figures of the specimens operated on (cf. figs. 12-16) limbs are indicated much shifted toward the ventral side of the body. In such a position, as is evident in the results of the present experiments, the limbs must suffer from the lack of material to complete their full growth. So it may be regarded that RUUD's case is also due to the deficiency of the material for limb-formation.

Problems of reduplication: Production of paired limbs from a single rudiment was recorded by NICHOLAS (l. c.) in both mid-dorsal and mid-ventral positions of the body. In looking for the reason for this characteristic development of grafted rudiments he resorted to HARRISON's rules of minor symmetry, and claimed the presence of a potency to make them divide into two in these median positions of the body. In my study of ventral transplantation, reduplication of limbs did really occur in several cases, but in none of them were two reduplicants separated from each other. Production of two distinct limbs as mentioned by NICHOLAS might appear if operation were made on more specimens. It occurred frequently in the transplantation into the flank of the body. Especially in the latter position, whenever the grafts were as large as 6 somites in width, production of two limbs was rather common in both orthotopic and heterotopic positions. In the heterotopic transplantation, moreover, the orientation of the grafts had nothing to do with the phenomenon. On the other hand, the size of the grafts bore always an important significance for producing the phenomenon. Actually the experiments with smaller grafts, less than 4 somites in width, consistently failed to produce two limbs in the flank as well as in the abdominal position.

As a general rule, reduplication of limbs is brought about as a result of changed surroundings, especially when the tissue immediately surrounding the rudiment is changed (ref. TAKAYA 1938). So according to this rule, the transplanted limb-rudiment together with the surrounding tissue should develop into a single structure having the original asymmetry. However, contrary to expectation, grafts larger than the normal limb-disc still yielded reduplicated limbs in more than half of the specimens operated on. As is evident in tab. 2, reduplication appears in nearly the same frequency throughout the whole series of experiments, with only a slight increase in proportion to the increase of the size of the grafts. So far as the flank region is concerned, limb-reduplication seems not to depend for its appearance on the position and orientation of the grafts. On the other hand, in the transplantation of the normal size of limb-discs it is well established since HARRISON (1921) that production of reduplication is most frequent in the orthotopic

position in the disharmonic orientation and in the heterotopic position in the harmonic orientation. To reconcile our results with this rule, we are forced to assume that the tissue surrounding the limb-disc and the disc itself forms, when grafted together, a unit which affects the development of a limb, although in the normal course of limb-development the surrounding portion has its own separate function.

Problems of symmetry: Generally mirroring relation was established in reduplicated limbs. However, two groups are distinguishable in the present case: first, mirroring was radial when reduplication occurred in the flank region; second, mirroring was ulnar when reduplication appeared in the abdominal region. Also NICHOLAS (l. c.) has shown two types of mirroring between two limbs produced in the mid-ventral and in the mid-dorsal positions of the body, the former being ulnar and the latter radial (cf. NICHOLAS' figs. 1. 2). On the other hand, RUUD (l. c. p. 559) obtained an ulnar mirror image in as many as 5 out of 8 reduplicated limbs produced in the heterotopic dorso-ventral transplantation. Unfortunately no precise location of the grafts is recorded, but from her figure (fig. 16) we may suppose that they were in the abdomen rather than in the flank of the body. That different mirrorings of limbs are produced according to different positions of the body, is also pointed out by OKA (1934) who finds that the mirroring near the fore-limb region is radial, while near the hind-limb region it is ulnar (p. 461). There is some discrepancy between this statement and our results, but we may venture to reconcile them by taking the following fact into consideration, that the material used by OKA belongs to the larva of advanced development (cf. OKA's fig. 2).

Finally as regards the influence of position of the grafts, one more fact may be added. When reduplication occurs in the limbs grafted in the abdomen, the arrangement of each member is always at right angles to the main axis of the body, whereas in the flank the two members generally stand in parallel.

Résumé

1. In the embryo of *Triturus pyrrhogaster* transplantation experiments of limb-discs were made in varying sizes, grafts being placed in the flank region in the orthotopic and heterotopic positions. In the abdominal region generally larger grafts were used.

2. In the mid-ventral position of the body, grafts smaller than 4 somites are generally absorbed. If developed however, they are abortive being deficient of the distal portion. When grafts become as large as 5 to 6 somites wide, perfect development of limbs is realized, generally single, normal and retaining original asymmetry. Reduplication sometimes occurs, and ulnar mirroring is established between the two members.

3. Addition of parts of the surrounding tissue always gives better results for limb-growth. Especially important is it to add the dorsal and ventral portion or the dorsal portion at least; when this is done normal limbs are most frequently produced.

4. In the disharmonic orthotopic transplantation, grafts smaller than proper limb-disc (2 or 3 somites) show deficient development, sometimes being checked by regeneration from the host. Larger grafts develop but mostly into reduplicated limbs. Two members are generally separated when grafts are as large as 6 somites in width.

5. In the heterotopic transplantation also, separated limbs are a common occurrence in larger grafts 6 somites in width, without regard to the orientation whether *hom dd aa* or *hom dv ap*. Smaller than this, reduplicated limbs result in either orientation.

6. In the flank region, mirroring of the limbs is radial as a rule, whereas ulnar mirroring is produced in the abdomen. Both separated and reduplicated limbs on the body side are always parallel to the body axis, while on the abdomen they are at right angles.

7. The description of the present research may be summarized as follows: 1) the condition for the development of limbs in the ventral location depends not on the size of the grafts, but on the presence of the dorsal portion of the surrounding tissue; 2) but production of two distinct limbs from a single rudiment depends on the size of the graft. The mid-ventral position of the body does not possess any significance for the phenomenon beside the peculiarity of arranging the direction of resulting limbs, namely vertical to the body axis and establishing the mirroring in the ulnar plane.

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