Further experiments on the innervation and function of grafted supernumerary limbs in the embryo of *Eleutherodactylus martinicensis*

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WITH TWO PLATES

**Previous** studies on the transplantation of limbs in the embryo of *Eleutherodactylus martinicensis* have shown that supernumerary limbs tend to be less well innervated than are corresponding replacing grafts, and that the innervation of the former, either autografts or homografts, is progressively less extensive with increase in time after operation. The degree to which grafted supernumerary forelimbs show movement in life parallels the extent of their motor innervation when studied subsequently in serial sections. The loss of innervation and of movement is particularly drastic near the time when the animal normally hatches and the tail undergoes atrophy.

The present paper describes some further experiments made with the aim of throwing light on the causes which underly the degeneration of motor nerves within supernumerary limb grafts in this animal.

**MATERIAL AND METHODS**

The source of the embryos of *Eleutherodactylus martinicensis*, the methods of culturing them, and of limb grafting, remain the same as in other papers on this subject (Hughes, 1962, 1964a, 1964b). The same histological procedures and methods of cell counting have again been employed.

**OBSERVATIONS**

1. **Amputation of the nearby hind limb**

Supernumerary limbs derive their motor innervation in the first place from collateral branches of axons which supply the nearby normal limb. The evidence

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TEXT-FIG. 1. Diagram showing the history, the source and extent of innervation of supernumerary forelimbs grafted near hind limbs with amputation of nearby hind limb. In Groups A, B and C the amputation was performed 5, 4 and 3 days respectively after grafting. Data for the ratio of the number of lumbar ventral horn cells on operated and contralateral sides are also given. (E), femur in amputated stump with distal epiphysis; (O), complete extirpation of hind limb. X, animal of Plate 1, Fig. C; Y, of Plate 1, Figs. A, B; Z, of Text-fig. 2 and Plate 1, Fig. F.
for this statement is that the number of ventral horn cells within the cord on the
grafted and contralateral sides show no consistent differences within the limits of
error of the methods of counting employed. In discussing the phenomenon of
the regression of innervation in supernumerary limbs, one suggestion earlier put
forward (Hughes, 1964a) was that a motor neurone in late embryonic life may be
unable to maintain all the branches thrown out at earlier stages, when the
territory innervated was much smaller in volume. The obvious way of testing this
hypothesis is to amputate the nearby normal limb at some time after the graft has
become innervated, and to see what effect this additional operation has on the
survival of motor nerves within the graft at later stages.

In making these experiments the same procedure was adopted as with a series
described in the previous paper (Text-fig. 1, series C, Hughes, 1964a) so that the
results could be assessed with the help of the data there recorded. Thus in the
present series a forelimb bud was autografted near a hind limb of a 5-day embryo,
an operation which results in marked activity of the graft while its innervation
persists.

The nearby hind limb was amputated at different times after grafting the super-
numerary limb in three sets of embryos, namely at 5, 4 and 3 days after the original
transplantation (Groups A, B and C respectively in Text-fig. 1). All these animals
were fixed in early juvenile life, from 1 to 4 days after atrophy of the tail had begun.
After this period some degree of movement of the graft persisted in all examples
where movement earlier had been observed.

This is in contrast with corresponding supernumerary limbs where the nearby
normal member was left undisturbed as is shown in Group C (iii) of Text-fig. 1
of Hughes (1964), where of seven such grafts only one showed any signs of activity
after the tail of the embryo began to dwindle. In order to confirm this result, a
further series of seven similar grafts was prepared, and the nearby hind limb was
left undisturbed. Each graft again showed some activity for varying periods which
ceased in every case within a day of the atrophy of the tail.

The activity of supernumerary forelimbs where the nearby limb had been
amputated varied with the interval between grafting and amputation. Where this
interval was 5 days (Text-fig. 1A), the degree of movement recorded 2 days after
atrophy of the tail was 'very slight'. In none of this group was the graft ever used
with the normal limbs in the co-ordinated activity of swimming. In Groups B
and C (Text-fig. 1) where the interval between the two operations was 4 and 3 days
respectively, two examples in each group maintained the same level of activity
continuously until fixation (Text-fig. 2). In another animal, movement of the
graft was finally co-ordinated with the swimming movements of the other limbs.
In others, although the graft remained still during general activity of the animal, a
clear reflex movement resulted on touching the adjacent amputation stump.
Others again showed spontaneous but irregular movements of hand and fingers.
In all, activity of the grafts in Groups B and C approached the levels achieved
where forelimbs were grafted in place of hind limbs, as in Group B (ii) of Text-fig.
1 in the previous paper (Hughes, 1964a). The extent to which these various grafts were innervated was generally in proportion to their activity displayed before fixation.

We can compare these results with those already described for similar supernumerary grafts without amputation of the nearby hind limb (Hughes, 1964a, Text-fig. 1). Here, in animals of Group C (ii) which were fixed 5 to 6 days after grafting, and before the atrophy of the tail, motor nerves in the grafts extended through the forearm in four examples out of thirteen, whereas of seven animals of Group C (iii) fixed in the juvenile stage, in none was the forearm innervated. Whether nerves extend to the forearm of a supernumerary graft when fixed after the tail has atrophied thus provides a test if amputation of the nearby hind limb

EXPLANATION OF PLATES

All figures are photomicrographs of sections, 9μ thick, through embryos of Eleutherodactylus bearing supernumerary forelimb grafts, stained with haematoxylin and eosin. Lines indicate the equivalents of 100 μ.

PLATE I

Figs. A, B. Amputation of nearby hind limb 3 days after grafting supernumerary forelimb. (1) through level of S9 showing two branches entering base of graft (arrows); (2) through forearm of graft, showing the same two branches of S9.

Fig. C. Amputation of nearby hind limb 5 days after grafting supernumerary forelimb, and fixed after a further 6 days. Degeneration in branch of S9 at entry to graft.

Figs. D, E. Nerves to nearby hind limb incised at time of grafting supernumerary. Fixed 5 days later at 10-day stage. S10 sends on branch to graft (to left of Fig. E) and another to tail region. These sections are 9μ apart. (D and E to same magnification.)

Fig. F. Amputation of nearby hind limb 3 days after grafting. Animal fixed after a further 8 days (see Text-fig. 2). Section at level of dorsal root ganglion of S9, which is displaced laterally.
has any effect on the nerves of the graft. Thus in Group A of the present experiments, motor nerves did not reach beyond the upper arm except in one instance where the humerus was internal; in Group B forearm muscles were innervated in three examples out of eight, and in Group C in six out of fifteen (Plate 1, Figs. A, B, F; Plate 2, Fig. G), in two of which fibres were traced to muscles of the wrist. Cutaneous fibres, however, often reached beyond the limits of motor nerves.

The source of innervation of the grafts is related to the position in which they become attached. The grafts were placed initially on the cranial side of the nearby hind limb, and as in the supernumerary forelimbs of the previous series, the majority received their nerve supply from S7 or S8, or from both together. In the normal animal, S7 supplies some of the musculature of the pelvic girdle. It is thus not severed by amputation of the limb. Therefore only where a graft is innervated by collaterals of S8 or S9 is there any possibility of some of these branches being affected by the amputation of the nearby hind limb. In Groups B and C of Text-fig. 1 of the present paper, examples of innervated forelimbs are all in grafts penetrated by S8 or S9.

However, in nearly all those grafts of the present series which were supplied by S7 some movement continued after atrophy of the tail, whereas in the corresponding grafts of Group C (iii) of the previous series (Text-fig. 1 of Hughes, 1964a), only one out of five showed any movement immediately before fixation. This comparison suggests that the conversion of collateral fibres to main axons in supernumerary limbs is not the only result of amputation of a nearby hind limb.

In the previous paper on supernumerary limbs (Hughes, 1964a) it was shown that when the innervation of these grafts breaks down towards the end of embryonic life, signs of degeneration can be seen within the nerves which supply them. In the present series of grafts where the nearby limb is amputated, histological evidence for the breakdown of their innervation is much rarer; it was seen in two of Group A which were innervated by S7 and S8 respectively (Plate 1, Fig. C); in one of Group B supplied by S8; and in one of Group C to which a branch of S6 was traced. Hence in this group where the nearby hind limb was amputated 3 days after grafting the supernumerary limb, none of the branches of normal limb nerves in these grafts showed any trace of disintegration.

It remains to enquire what effect amputation exerts on the number of ventral

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**Plate 2**

Fig. G. Same animal as in preceding figure. Branch of S9 reaches wrist of graft (arrow). One phalange to left of figure.

Figs. H, I, J. Nerves to nearby hind limb incised at time of grafting supernumerary limb (same animal as in Text-fig. 5c). Only the hand of the graft is external; it is innervated by a branch of S7. Figure H shows normal distribution of other branches of S7. Figure I shows branch entering graft, and Fig. J within hand. Compare the calibre of S9 on operated and contralateral sides (arrows, Fig. J). At this level there are very few ventral horn cells on the ipsilateral side.
horn cells within the spinal cord. To assess this feature of these experiments we must first examine the effect of amputation where no supernumerary graft is present. In Hughes (1962) it was shown that this depends very much on the

stage at which the operation is performed. At 5 days, extensive degeneration in both ventral horn neuroblasts and in those of the adjacent dorsal root ganglia is seen within a day of amputation. The number of cells in the ventral horn on the amputated side soon drops to a small fraction of the original number. When a

TEXT-FIG. 3. Ratio of numbers of lumbar ventral horn cells in embryos where one hind limb was amputated at the stages indicated. Between 3 and 6 days after amputation at 9–10 days excentric nuclei are seen in neurones on the operated side. (Rings with black sectors.)
Function and innervation of supernumerary limbs

limb is amputated at the 9-day stage or later (Text-fig. 3), the number of ventral horn cells does not begin to decrease until more than a week afterwards. First the cell bodies swell and undergo chromatolytic changes, with displacement of the nucleus to one side of the cell. The Nissl substance is largely lost. Later, this material regenerates in a coarse and untypical pattern. In Hughes (1962), counts of the number of ventral horn cells following amputation were given only for early operations. In Text-fig. 3 of the present paper the corresponding data are provided for the results of amputation at 7 to 10 days, expressed in terms of the ratio of the number of ventral horn cells on the operated and contralateral sides. Not until these counts were made was it realised that in relatively late embryos during the period following amputation the total number of cells on the operated side becomes greater than normal. At this period of development in normal embryos cell degeneration is abundant among the ventral horn cells, and it seems that one effect of the severance of the axons of these motor neurones in *Eleutherodactylus* is to halt the normal cell turnover (Hughes, 1961). No pycnotic nuclei are seen among the swollen neurones for the first few days after amputation. It seems that ventral horn cells continue to differentiate under these abnormal conditions. From the 6th to 7th day after amputation, however, at a time depending on the age of the embryo, ventral horn cells then begin to degenerate, and the total number gradually falls to a level below that of the normal ventral horn.

In Group A of the present experiments, where the limb adjacent to the supernumerary grafts was amputated 5 days after transplantation, the embryos were then at the 9-day stage, and in the period when amputation is thus followed by chromatolysis, and temporary increase in cell number. These animals were fixed before the population of ventral horn cells had fallen to their final level, and in one example of Group A the ratio of cell numbers on the two sides is still above unity. In this animal, some cells on the operated side still have excentric nuclei. In Groups B and C of Text-fig. 1, however, the nearby hind limb was amputated between 7 and 8 days of development. Text-fig. 3 shows that under these circumstances the decline in number of ventral horn cells on the operated side would be largely complete by the 3rd day after amputation. This decrease is less drastic where an innervated supernumerary limb was present. Comparison of the number of ventral horn cells remaining under these circumstances with the corresponding total in simple amputations might therefore be expected to indicate the actual number of neurones which supply the graft.

One difficulty in making this comparison is that the loss of ventral horn cells after amputation depends very much on how large an amputation stump is left, and how far this regenerates before fixation of the animal. In amputating the normal limb of an embryo bearing a supernumerary graft, the tendency was to leave a larger stump than would otherwise have remained for fear of disturbing the nearby graft, so recently established. This was particularly true for the embryos of Groups B and C of Text-fig. 1. In most of these, the amputation stump
regenerated a distal epiphysis of the femur (E in column 5 of Text-fig. 1) and the
thigh musculature is largely complete. It can be seen from column 4 in Text-fig. 1
that in those examples of Groups B and C, where no distal epiphysis had re-
generated in the amputated femur, that the ratio of cell numbers in the ventral
horns is generally lower than where the femur was complete. In one example in
Group C, where the amputation was total and the graft was innervated only at the
base, very few ventral horn neurones were present on the operated side.

In selecting examples of amputated juveniles for comparison it was thus
necessary to choose only those with similar amputation stumps. Eight examples
from Group C in which the forearm of the graft is innervated were compared with
six amputated animals in an unpaired ‘t’ test (Snedecor, 1938). The difference
between the two groups was found to be significant at approximately the 5 per
cent. level of probability. Mean values of the ratios of number of ventral horn
cells differed by 8 per cent., which corresponds to some thirty-two neurones for
an average of about 400 cells in the normal juvenile ventral horn.

II. Severance of the nerve supply to the nearby hind limb at the time of grafting a
supernumerary member

When a limb bud is ablated in a 5-day embryo of Eleutherodactylus, extensive
degeneration results within a few hours among the cells of the related dorsal root
ganglia and in the ventral horn (Hughes, 1962). Pycnotic nuclei appear in the
latter far in excess of the number of ventral root fibres present in the limb nerves
at that time, a fact which indicates that the degeneration of ventral horn neuro-
blasts may be a secondary effect consequent on that within the spinal ganglia.
If at the time of amputation a limb bud is grafted in place of the ablated member,
neurones are later found within the ventral horn greater in number than those
which remain after amputation. Under these circumstances, the cells which
differentiate are largely, if not wholly, a new set. In neuroblastic stages they are
clearly less advanced in development than are the cells of the contralateral horn
(Hughes, 1962).

The aim of this second group of experiments was to secure the innervation of
both a supernumerary limb and the adjacent hind limb by a regenerated set of
ventral horn cells, and to see whether both would continue to move in the juvenile
stage of the animal. The first attempts at grafting a forelimb simultaneously
with the replacement of the hind limb were not successful, but it was found that
the aim could be achieved if the hind limb was nearly severed at the time of opera-
tion, leaving it connected with the body at one point by a shred of ectoderm.

Two types of experiment were performed, either with a forelimb (Text-fig. 4)
or the contralateral hind limb as a supernumerary graft. There were seventeen
and twenty-eight examples respectively of each type. In a further series, a hind
limb was incised and replaced without grafting a supernumerary member. The
animals were fixed at varying times either in later embryonic stages or in early
Innervation of supernumerary limb degenerates

Supernumerary limb grafted

Supernumerary limb grafted

Ipsilateral limb incised and replaced

Degeneration of original neuroblasts

Fresh neuroblasts differentiate and innervate both Supernumerary and ipsilateral limbs

Amputation of ipsilateral limb—

and innervation of supernumerary retained

(a)

(b)

(TEXT-FIG. 4. Diagram showing general results of grafting supernumerary forelimbs. (1) with (a), or without (b) amputation of nearby hind limb, and (2) with severance of nerves to nearby hind limb at the time of operation.)
### Table 1

**Data from Grafted Animals of Group 2**

<table>
<thead>
<tr>
<th>Operation: Hind limb incised and replaced</th>
<th>Ipsilateral side</th>
<th>Contra-lateral side</th>
<th>Ventral horn cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limb</td>
<td>Articulation at hip</td>
<td>Movement</td>
<td>Innervation</td>
</tr>
<tr>
<td>1. H/L Normal</td>
<td>Kick</td>
<td>N</td>
<td>Undisturbed</td>
</tr>
<tr>
<td>2. H/L Femur divided</td>
<td>A</td>
<td>N</td>
<td>Undisturbed</td>
</tr>
<tr>
<td>3. H/L Ankylosed</td>
<td>—</td>
<td>N (S)</td>
<td>Undisturbed</td>
</tr>
<tr>
<td>4. H/L Normal</td>
<td>Kick (P)</td>
<td>N (S)</td>
<td>Amputated</td>
</tr>
<tr>
<td>5. H/L Normal</td>
<td>H</td>
<td>N (S)</td>
<td>Amputated</td>
</tr>
<tr>
<td>6. H/L Normal</td>
<td>H</td>
<td>N (S)</td>
<td>Amputated</td>
</tr>
</tbody>
</table>

**Operation: Hind limb incised and replaced; forelimb grafted nearby**

| 7. H/L Normal                            | Kick            | N                   | Undisturbed       | 405 | 404 |
| 8. H/L N (tibio-fibula divided)         | Kick            | N                   | Undisturbed       | 317 | 390 |
| 9. H/L Normal                            | Kick (late)     | N (S)               | Undisturbed       | 233 | 508 |
| 10. H/L Ankylosed                       | A               | S₈, S₉             | Undisturbed       | 172 | 446 |
| 11. H/L Femur divided                   | W               | F: S₇, S₈          | Undisturbed       | 220 | 468 |
| 12. H/L Disarticulated                  | A               | S₈, S₉             | Undisturbed       | 192 | 484 |
| 13. H/L Normal                           | —               | N (S)               | Undisturbed       | 125 | 438 |
| 14. H/L No acetabulum                   | A               | N (S)               | Undisturbed       | 183 | 394 |
| 15. H/L Femur divided                   | A               | N (S)               | Undisturbed       | 237 | 418 |

**Operation: Hind limb incised and replaced; other hind limb grafted nearby**

| 16. I/L H/L Normal                       | Kick            | N                   | Amputated         | 481 | 55  |
| Other H/L Ankylosed                     | H & K (L)       | S₈, S₉             | Amputated         | 432 | 141 |
| 17. I/L H/L Normal                       | Kick            | N                   | Amputated         | 137 | 20  |
| Other H/L Femur internal                | A (L)           | S₈                  | Amputated         | 114 | 73  |
| 18. I/L H/L Normal                       | H & K (P)       | S₁₀                 | Amputated         | 95  | 71  |
| Other H/L Femur internal                | H               | S₉, S₁₀             | Amputated         | 73  | 35  |
| 19. I/L H/L No acetabulum               | H & K (L)       | S₉, S₁₀             | Amputated         | 71  | 35  |
| Other H/L Girdle unattached             | H               | S₇, S₈             | Amputated         | 71  | 35  |

**Abbreviations**

- I/L, ipsilateral; C/L, contralateral; F/L, forelimb; H/L, hind limb.
- *Movement in graft:* A, at ankle; E, at elbow; H, at hand; K, at knee; (L), late in development; (P), partial; W, at wrist.
- *Innervation:* Cut, cutaneous only; F, forearm; T, thigh; N, normal; N (S), normal but nerves slender; S₇ etc., innervated by 7th etc. spinal nerve.
Function and innervation of supernumerary limbs

Two examples without a supernumerary limb and with a reduced number of ventral horn cells (Nos. 5 and 6) showed free movement at the hip and none whatever at the knee. In both of these, the knee joint was perfectly normal. When at juvenile life. In Table 1 are given data concerning twenty such animals which included representatives of each type of operation.

In each group were examples (Nos. 1, 2, 7, 16, 17) in which the number of lumbar ventral horn cells was within the normal range; if the contralateral limb was present, the two lumbar horns were similar in size (Nos. 1, 2, 7). In these cases it is probable that the incision at operation was not deep enough to cut the nerves to the limb bud. Only in these examples was a full and normal action of the ipsilateral leg developed. In one example (No. 2) where the number of ventral horn cells was normal was the action of the limb imperfect, and here the femur was divided.

Where a supernumerary forelimb was present, and the lumbar nerves were not affected by the operation (No. 7, Table 1); the result is the same as in series C (iii) of Text-fig. 1 of Hughes (1964a) and, as before, movement of the supernumerary limb was lost near the time when the tail of the animal began to atrophy. Where, however, supernumerary limb and the ipsilateral hind limb were innervated by a regenerated set of ventral horn cells, even though less in number than those of the normal horn, both members continued to show some movement after the hatching stage was reached. This was particularly evident with supernumerary forelimbs (Nos. 8, 10–15) (Text-fig. 5), and here the primary aim of this series of experiments was largely achieved.

In a recent paper concerned with the normal development of function in the limbs of Eleutherodactylus (Hughes, 1964b), it was shown that extension at the knee is the last of the major movements of the thigh and leg to appear. At 10 days a light touch on the hind limb results in flexion followed by extension, both at the hip and the knee. This reaction may be referred to as a ‘kick’. In the experimental animals of Table 1 of the present paper, wherever the number of ventral horn cells was below normal (Nos. 3–6, 10–15, 18–20) extension at the knee was either imperfect or late in appearance (No. 9). Movement at the hip is decreased by the presence of a supernumerary graft of a hind limb in some cases (Nos. 18, 19) even where the articulation has regenerated.

In Nos. 11–12, 14 and 15, the injury to the femur at operation was not repaired, and in No. 10 the femur is ankylosed to the girdle. In these instances movement of the ipsilateral limb is largely restricted to the ankle joint. In No. 8 (Text-fig. 5a), the ipsilateral leg was disarticulated at the knee and rotated through a right angle. Yet this limb kicked repeatedly before the animal was fixed. The grafted forelimb, which moved at the wrist, was attached to the leg with humerus and a rudimentary pectoral girdle fused to the tibio-fibula. At operation, the middle segment of the limb must have been incised; this resulted in some decrease in the number of ventral horn cells, but how far fresh neuroblasts regenerated in this animal as a consequence of the degeneration caused by cutting limb nerves cannot be decided.

Two examples without a supernumerary limb and with a reduced number of ventral horn cells (Nos. 5 and 6) showed free movement at the hip and none whatever at the knee. In both of these, the knee joint was perfectly normal. When at
TEXT-FIG. 5. Legs and supernumerary forelimbs in five animals (a–e) in which nerves to the ipsilateral hind-limb were cut at the time of grafting, showing degree of movement at the time of fixation. (K) indicates extension at knee. The corresponding entries in Table 1 are as follows: (a) No. 8; (b) No. 12; (c) No. 13; (d) No. 11; (e) No. 10. Sections through (c) are shown in Plate 2, Figs. H, I, J. Equivalent scale to 1 mm. on each figure.
the hatching stage these animals attempted to swim, the leg rotated as a whole from the hip, while the knee joint remained fully flexed. In No. 6 (Text-fig. 6), the innervation of the extensor muscles of the thigh was more sparse than normal, and in No. 3 was absent altogether. Where there was no extension at the knee, the toes also remained permanently flexed.

In six examples a supernumerary forelimb moved freely at the wrist before fixation (Nos. 8, 10–14), and in No. 14 at the elbow as well. None showed any movement at the shoulder, the absence of which was correlated with the poor development of the pectoral girdle. In these animals, simultaneous action of the supernumerary forelimb at the wrist and of the ipsilateral leg at the ankle provided examples of the 'homologous response' of Weiss (1923), though the absence of movement at shoulder and hip was due to the special circumstances of these grafted limbs. In these animals, the earliest movements of supernumerary forelimb and of ipsilateral hind limb were not the same as those seen at later stages. The first reaction of the latter was the usual flexion on light touch, though first apparent some time later than in the contralateral member. The early activity of the graft was usually vague and ill-defined, and was only gradually concentrated into clear movements (Text-fig. 7).

The nerves within these supernumerary forelimbs were compared in two sets of animals fixed at different periods, namely six which were fixed 5–6 days after operation at about the 9-day stage, and seven which were allowed to survive for 15 days after operation into early juvenile life (Nos. 9–15 in Table 1). In both sets...
the proportion of individuals in which nerve fibres had penetrated to the forearm or beyond was over half. There was no evidence of regression in the extent of innervation in the older group, but in them the nerves traced into the supernumerary forelimbs were often very slender.

With supernumerary hind limbs innervated from an original set of ventral horn cells (Nos. 16 and 17), movement of the graft ceased before fixation. Where, however, the two hind limbs were innervated from a regenerated group of neurones, some movement continued in both members in two examples out of three (Nos. 18 and 20) and in three others which are not included in Table 1. In no example of this type of experiment, however, was a full action developed in both grafted and ipsilateral hind limbs, even though the degree of movement seen was much greater than has hitherto been observed in supernumerary hind limbs. Where two such large members were present on the same side of an animal, they impeded each other's motion, to an even greater extent than with supernumerary forelimbs.

In some examples in Table 1 of the present paper (Nos. 10–11, 18–20) the supernumerary graft and the ipsilateral hind limb are innervated by different spinal nerves, though anastomoses can usually be traced between them. In other animals
of Group II, however, there are clear instances of a lumbar nerve sending branches to both a supernumerary graft and the ipsilateral member (Plate 1, Figs. D, E). Thus no general difference can be traced in the pattern of nerve supply to supernumerary limbs when innervated by original or by regenerated ventral horn cells.

In the previous paper on supernumerary limb grafts in *Eleutherodactylus* (Hughes, 1964), it was reported that if embryos with hind limbs grafted near forelimbs were kept at sub-normal temperatures (about 26°C), the grafts were found to be better innervated than in similar grafts maintained at room temperature and fixed at the same stage of development, and to show some signs of movement. Accordingly, a batch of embryos of the present series bearing supernumerary hind limbs were kept at 26°C. The operation of grafting the supernumerary limb and of incising and replacing the ipsilateral hind limb was performed at 5 days of development. The animals were fixed 14 days later, by which time the tail had largely atrophied in all. Again the results fell short of the aim of full movement in both members, but were somewhat better than were seen in similar batches of grafts kept at room temperatures (29–30°C). Thus, of the cool series, there was some motion of the supernumerary limb before fixation in five out of six animals, with movement at foot and ankle in three, in each of which failure to regenerate a functional articulation precluded any further movement of the graft. In the ipsilateral limb, a full kick was seen in two examples, and a considerable degree of extension in two others. Such a result indicates that the failure of grafted hind limbs to achieve a greater degree of freedom of motion is due not only to mechanical obstruction, but also to an inadequate innervation, which as before, is somewhat improved in animals kept at a sub-normal temperature.

**DISCUSSION**

The hypothesis that supernumerary grafted limbs in *Eleutherodactylus* lose their motor innervation towards the end of embryonic life because of the withdrawal of side branches from motor axons is supported by the results of Group I of the present experiments which demonstrate that by amputation of the nearby normal limb, the innervation of a supernumerary member may be maintained. There is reason to think, however, that the effect may not be due solely to a decrease in the extent of axonal branching, for detailed study of the innervation of the normal limbs in the embryo of *Eleutherodactylus*, to be presented in a further paper, has shown that considerable changes occur in the composition of the limb nerves during development.

In Group 1 of the present experiments, the fact that the motor innervation of supernumerary limb grafts persists after amputation of the nearby normal limb may be due in part to the diversion into the grafted limb of axons which enter the lumbar nerves at relatively late stages, though this explanation does not necessarily supersede that which has already been advanced. This second
hypothesis, however, does suggest how the innervation of the supernumerary limb might persist even when supplied by collateral of S₇, the main branches of which are not served by amputation.

In the ontogeny of the normal limb, the number of fibres in the nerves to some muscles rises to a maximum in late stages of development, and then decreases against just before the hatching period. The most striking example is the triceps femoris, which is mainly responsible for the extension of the knee joint. The fibres in the ramus profundus anterior which supply the triceps begin to increase in number shortly before the muscle becomes functional, and the leg begins to kick. In Group II of the present experiments, limbs are innervated by regenerated ventral horn cells less in number than the normal set. Under these conditions, movement at the knee joint is more severely impaired than at the hip, and may fail entirely. Thus the triceps femoris which normally acquires an effective innervation at a relatively late period in development may fail to receive sufficient motor axons to become functional if the total motor supply to the limb is reduced (Table 1, Nos. 5, 6).

Supernumerary forelimbs, however, when innervated by regenerated lumbar ventral horn cells, even though supplied by extremely slender bundles of fibres, not only move with considerable freedom, but their mobility persists into the juvenile stage. The paradox remains unexplained that under these circumstances the innervation of a supernumerary limb from a reduced number of ventral horn cells is more stable than when derived from a normal complement of motor neurones.

**SUMMARY**

1. The innervation of supernumerary limb grafts in *Eleutherodactylus* breaks down near the end of embryonic life, but this process may be checked by amputation of the nearby normal limb.

2. Under the conditions of these experiments, supernumerary limb grafts appear to be innervated by collaterals of axons which have already reached the nearby limb. If the latter is amputated, some collateral branches may become main axons. Moreover, motor fibres which enter the limb nerves relatively late in development may thereby be diverted from their normal course and into the supernumerary graft.

3. When a limb is amputated at the 5-day stage, degeneration occurs both of the innervating fibres and of their cell bodies, in dorsal root ganglia and in the ventral horn. If in place of the ablated limb another is at once grafted, its muscles become innervated by a regenerated set of ventral horn cells.

4. If two limbs are grafted in place of an ablated limb, then both become innervated by regenerated ventral horn cells, and the innervation persists into juvenile stages.

5. Such limbs show continued function, and exhibit the ‘homologous response’ of Weiss, though with some limitations on their movements.
Nouvelles expériences sur l'innervation et le fonctionnement de membres sur-
uméraires greffés chez l'embryon d'Eleutherodactylus martinicensis

1. L'innervation de membres surnuméraires greffés chez Eleutherodactylus se
dégrade vers le fin de la vie embryonnaire, mais ce processus peut être freiné par
l'amputation du membre normal proche.

2. On montre que dans les conditions de ces expériences, les membres sur-
uméraires greffés sont innervés par des collatéraux d'axones qui ont déjà atteint
le membre voisin, et que, si ce dernier est amputé, quelques branches collatérales
deviennent alors des axones principaux.

3. Quand un bourgeon de membre est amputé au stade de 5 jours, les deux
types de fibres innervatrices dégénèrent, ainsi que leurs corps cellulaires dans les
ganglions des racines dorsales et dans la corne ventrale. Si on greffe immédiatement
un autre membre à la place de celui qu'on a amputé, ses muscles deviennent
innervés par un lot régénéré de cellules de la corne ventrale.

4. Si on greffe deux membres à la place d'un membre amputé, tous deux
deviennent alors innervés par des cellules de la corne ventrale, et l'innervation
persiste aux stades juvéniles.

5. De tels membres montrent un fonctionnement continu et présentent la
'reaction homologue' de Weiss, quoique leurs mouvements soient quelque peu
limités.

6. On discute de différences possibles dans l'innervation des membres sur-
uméraires innervés par les cellules des cornes ventrales originelles et régénérées.

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