

Regeneration from isolated half limbs in the upper arm of the axolotl

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SUMMARY

A technique enabling the isolation of half limb stumps using strips of skin from the head is described. Using this technique posterior, anterior, dorsal and ventral halves of the upper arms of axolotls were constructed. All halves produced regenerates and regional differences were shown in the regenerative and regulative abilities of the different halves. Posterior half stumps regenerated limbs with a mean digit number of 3.9 and had a normal dorsoventral muscle pattern. Anterior halves produced hypomorphic limbs with a mean digit number of 1.2 while dorsal and ventral halves produced an average of 3.8 and 2.6 respectively. Regenerates from dorsal half stumps had a normal dorsoventral axis but the majority of those from ventral halves were either double ventral or had little muscle on the dorsal side of the limb.

INTRODUCTION

The information for the regeneration of amphibian limbs is believed to lie in the dermis and underlying muscles (Lheureux, 1972, 1975; Carlson, 1974, 1975; Bryant, 1978; Settles, 1978; Tank, 1981*b*). This information however is not thought to be uniformly distributed within the limb. Experiments using surgically created symmetrical limbs (Stocum, 1978; Tank & Holder, 1978; Holder, Tank & Bryant, 1980; Krasner & Bryant, 1980; Burton, Holder & Jesani, 1985), irradiation (Maden, 1979) and surgical isolation of parts of the limb (Goss, 1957*a,b*) have shown differences in the regenerative ability of different regions of the limb. In particular in the upper limb the posterior side appears able to regenerate more of the normal pattern of the limb compared with the anterior side although this difference is not seen in the lower limb (Goss, 1957*a,b*; Maden, 1979; Krasner & Bryant, 1980).

In addition to differences in regeneration there are differences between the transverse axes as to whether discontinuities in the pattern of structures within the regenerate are tolerated or whether regulation occurs to restore continuity. Discontinuities seem never to be tolerated within the anteroposterior axis and surgically created discontinuities present in the stump often result in the formation of additional structures in the regenerate (Tank, 1979; Slack, 1980; Holder & Weekes, 1985). In contrast surgically created discontinuities in the dorsoventral

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axis of the stump remain in the regenerate (Maden, 1980, 1982; Maden & Mustafa, 1982; Tank, 1981a; Papageorgiou & Holder, 1983; Holder & Weekes, 1984). The presence of discrete discontinuities between dorsal and ventral muscle on the same side of the limb suggests that limb tissue can only exist as either dorsally or ventrally coded material. These two tissue codings would normally meet at a dorsoventral boundary running across the middle of the limb the exact location of which is unknown.

In the light of these results the present work was carried out to further investigate these regional differences in a direct way by examining regenerates from surgically isolated half limbs. We describe the regenerative potential of isolated half limbs from the upper limb for the first time and assess all the results in terms of pattern regulation in both transverse axes. The surgical technique used in the present study results in the complete isolation of half limb regions and avoids the presence of additional limb tissues or the effects of irradiation. In order to isolate half limb regions pieces of skin from the head were used. Autografts of this kind have the advantage that they rapidly heal into place (Tank, 1984) without inducing an immune reaction. Head skin was chosen because it contains no positional information relating to pattern formation in the limb. This has been shown by a number of previous results: complete cuffs of head skin prevent limb regeneration in the axolotl (Jeffmoff, 1931 quoted in Polezajew & Faworina, 1935), head skin unlike limb skin does not restore the regenerative capacity of irradiated limbs (Umaski, 1938; Lazard, 1967) and it does not alter the pattern of regeneration when grafted under the skin of intact limb stumps (Tank, 1981b).

Using head skin anterior, posterior, dorsal and ventral halves of the upper forelimb were created. All four halves regenerated and showed differences in their anteroposterior and dorsoventral axes. The results are discussed in terms of current theories concerned with the mechanisms of pattern regulation.

MATERIALS AND METHODS

All experiments were carried out on axolotls (*Ambystoma mexicanum*) 15–20 cm in length, spawned in the colony at King's College. Animals were kept in tap water in individual containers and fed on raw heart.

Experimental procedure

All operations were performed on animals anaesthetized in MS222 (Sigma) after which the animals were kept at 10°C in the dark for two days to facilitate healing. All operations were on the upper arm with the amputation plane lying in the middle third of the humerus. Operations were performed with the arm at right angles to the body with the elbow flexed using the axes of the body to determine the position of different halves of the limb. Experimental operations consisted of the removal of half the arm (either the dorsal, ventral, anterior or posterior half) for a distance of 3–4 mm while leaving the humerus intact. The medial wound surface thus formed was covered with a 3–4 mm wide strip of skin which was removed from the forehead and sutured into place. The limb was then amputated and trimmed to form a stump consisting of approximately 50 % of the circumference of limb skin and soft tissues together with the humerus, the remainder of the circumference being covered by head skin (Fig. 1). Controls consisted of carrying out the same procedure as described above but without grafting any head skin onto the limb.

All experimental animals were checked for retention of the graft every few days for the first few weeks after the operation. Operated animals were left for 60 days after which time the limbs were amputated, fixed in Bouin's solution, dehydrated and stained with Victoria blue (Bryant & Iten, 1974) to show the pattern of cartilages. Camera-lucida drawings were made of all regenerates. Limbs were then wax embedded and serial transverse sections were cut at 10 μ m and stained with haematoxylin and eosin. These sections were used to analyse the dorsoventral muscle pattern.

The muscle pattern was examined at two levels, the proximal metacarpals and the mid-forearm. In normal limbs at the level of the proximal metacarpals, ventral muscle is continuous across all digits while dorsal muscle occurs as discrete crescents over each metacarpal and a large vascular sinus is present only on the ventral side (Maden, 1980, 1982; Maden & Mustafa, 1982; Burton *et al.* 1985). Also at this level Leydig cells in the epidermis are relatively more abundant on the dorsal side compared with the ventral side (Holder & Weekes, 1984). At the midforearm level, limb organization along the dorsoventral axis can be determined from the overall muscle pattern and the presence of the *pronator quadratus* (*pq*) muscle which runs from a spine on the ventral side of the ulna towards the radius (Holder & Weekes, 1984; Burton *et al.* 1985).

RESULTS

Posterior half limbs

Thirteen posterior half limbs were made all of which produced a regenerate (see Table 1). Two of the regenerates had 3 digits while the remaining eleven had 4 digits and all regenerated limbs had a radius and ulna. The cartilage pattern of the eleven 4-digit limbs appeared normal (Fig. 2) with the exception of one limb that showed fusion of the two central digits in the phalangeal region and four limbs which showed loss of one or more phalanges from their anterior digits.

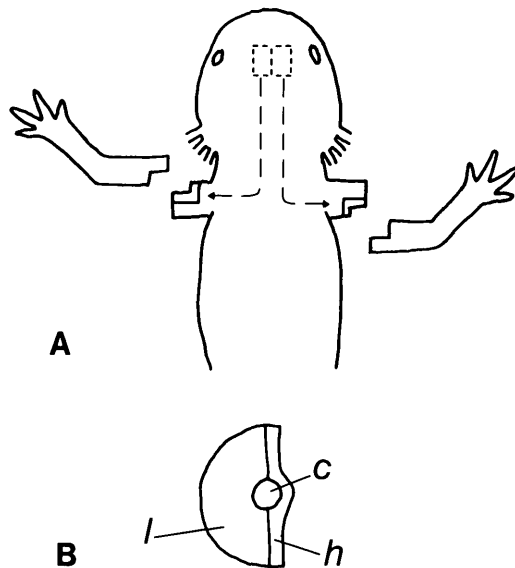


Fig. 1. Diagram of operation. Pieces of head skin were grafted onto the medial surfaces of half limb stumps. (B) End on view of stump after grafting on of head skin. The abbreviations are: c, humerus; h, head skin; l, limb tissue.

Table 1. *Analysis of the number of digits regenerating from different halves of the limb*

Stump	No. of limbs produced	No. of digits regenerating					Spike	No regeneration	Mean no. of digits
		5	4	3	2	1			
Posterior half	13		11	2					3.9
Anterior half	13	1	1		1	5	2	3	1.2
Dorsal half	9		7	2					3.8
Ventral half	11		2	5	2	2			2.6

Sections of the above limbs revealed a large fold of skin on the anterior side of the limb overlying a region of loose connective tissue (Fig. 3). This feature was continuous with the head skin graft in the upper arm and extended as far as the metacarpal region. At the interface between this fold of skin and the dorsal limb skin, frequent lateral line organs were seen (Fig. 4) and it is assumed that this fold of tissue is an outgrowth from the head skin graft. The muscle patterns of all thirteen limbs showed normal dorsoventral organization at both the metacarpal and forearm levels (see Fig. 3).

Anterior half limbs

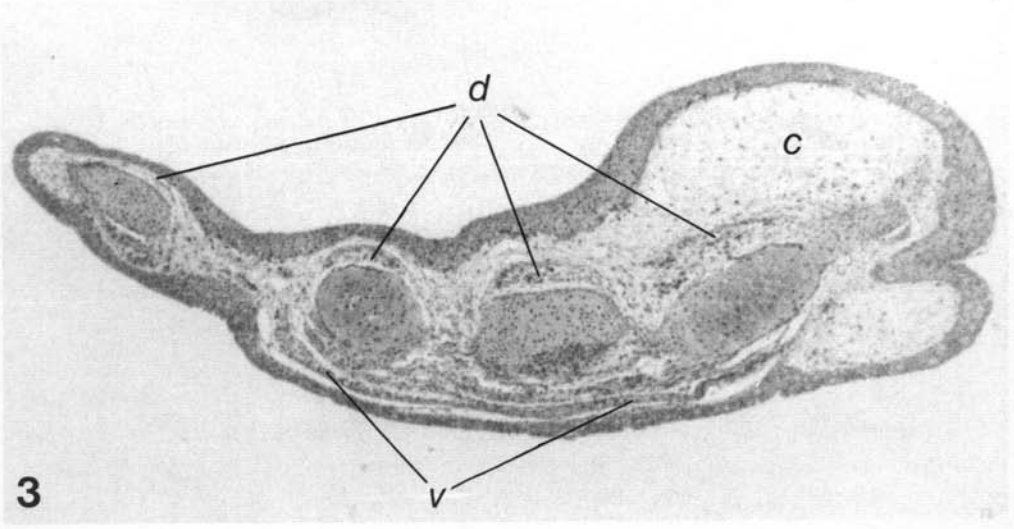
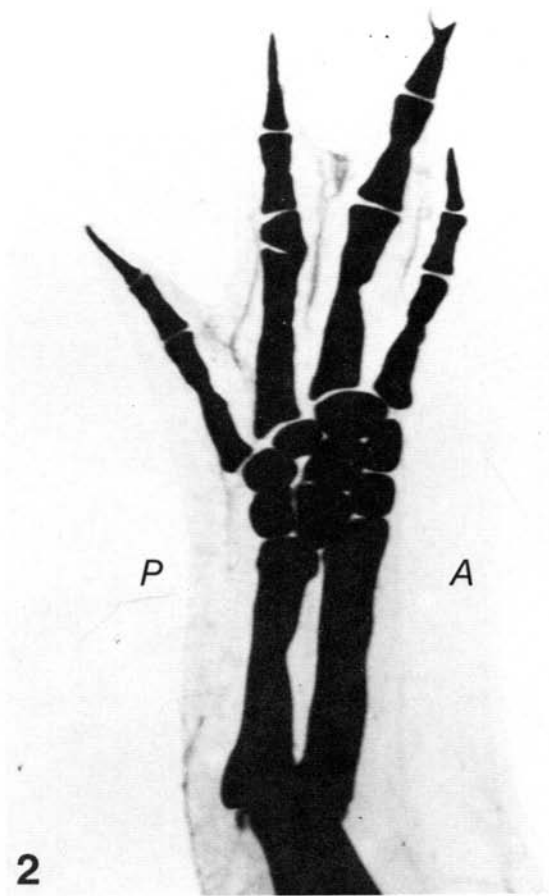
Thirteen anterior half limbs were made using the contralateral arms of the animals used to make posterior half limbs. Ten of these limbs produced a regenerate and Table 1 gives an analysis of the number of digits produced by each limb. The most commonly occurring regenerate had a single digit (five limbs) and consisted of a single forearm bone, several carpals, a metacarpal and two or three phalanges (Fig. 5). The 2-digit limb also had only a single forearm bone whilst the 4- and 5-digit regenerates had two forearm elements. Two regenerates consisted only of a short piece of cartilage classed in Table 1 as a spike and three failed to regenerate any visible structure.

Sections of these limbs showed that they too had a fold of skin and loose connective tissue which in these limbs occupied the posterior margin of the regenerate. The 5-digit limb had four digits clustered around one another with the fifth digit growing from the dorsal surface of the hand. Sections showed the midforearm region to be normal but additional carpals were present leading to a hand which was strongly curved to enclose its ventral surface (Fig. 6). The 4-digit

Fig. 2. Dorsal view of a Victoria-Blue-stained whole mount of a left limb showing the skeletal structures regenerating from a posterior half stump. The skeletal pattern is basically normal. Abbreviations: *A*, anterior side of limb; *P*, posterior side of limb. Magnification $\times 9$.

Fig. 3. Transverse section at the distal metacarpal level of a regenerate from a posterior half stump. The abbreviations are: *d*, dorsal muscle; *v*, ventral muscle; *c*, loose connective tissue on the anterior side of the limb. Magnification $\times 40$.

Fig. 4. Lateral-line organ present in the dorsal skin of a regenerate from a posterior stump. Magnification $\times 400$.



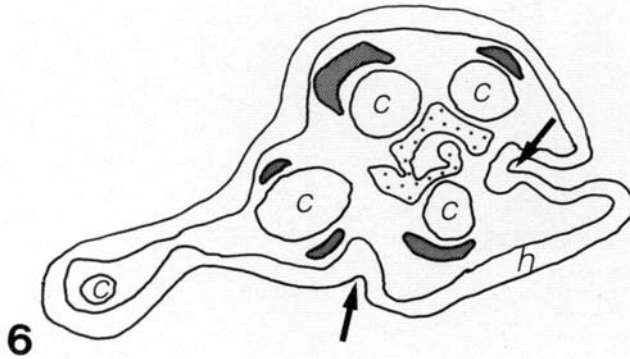
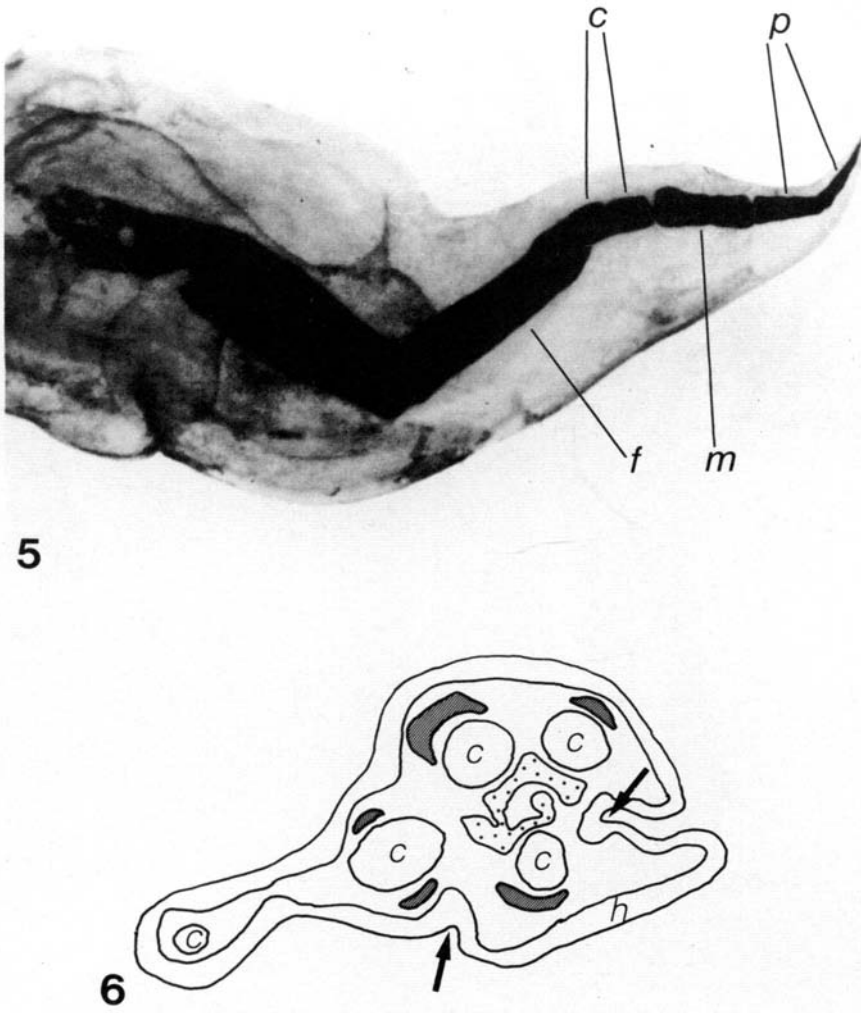


Fig. 5. Victoria-Blue-stained whole mount of a right limb showing the skeletal structures regenerating from an anterior half stump. A single forearm bone and a single digit have regenerated. Abbreviations: *f*, forearm bone; *c*, carpals; *m*, metacarpal; *p*, phalanges. Magnification $\times 9$.

Fig. 6. Camera-lucida tracing of a transverse section at the distal metacarpal level of a 5-digit regenerate from an anterior half stump. Abbreviations: *c*, cartilage; Shaded areas, dorsal muscle; Starred areas, ventral muscle. *h* marks the location of head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.

limb had double ventral muscle at the metacarpal level with a single vascular sinus on the ventral side and few Leydig cells on either side of the limb. In the forearm region the muscles were abnormal with little muscle on the dorsal side of the radius and ulna. Little muscle pattern existed in the single digit limbs although in some

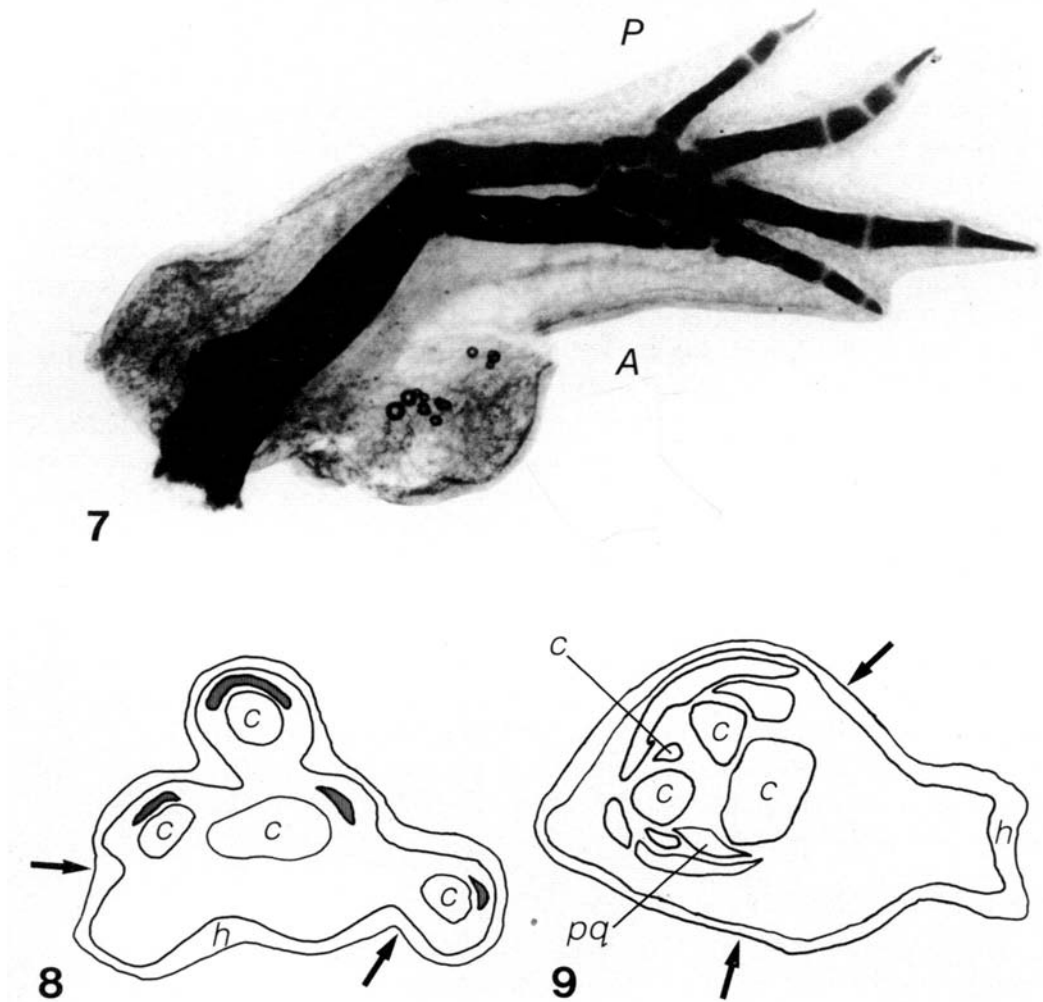


Fig. 7. Victoria-Blue-stained whole mount of a left arm showing the skeletal structures regenerating from a dorsal half stump. A, anterior side of limb. P, posterior side of limb. Magnification $\times 9$.

Fig. 8. Camera-lucida tracing of a transverse section at the distal metacarpal level of a 4-digit regenerate from a dorsal half stump. Only dorsal muscle was present at this level in this regenerate. Abbreviations: c, cartilage; Shaded areas, dorsal muscle. h marks the location of head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.

Fig. 9. Camera-lucida tracing of a transverse section at the distal forearm level of the same regenerate from a dorsal half stump as is shown in Fig. 8. Four forearm bones are present together with both dorsal and ventral muscle. Abbreviations: c, cartilage; pq, pronator quadratus muscle. h marks the location of head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.

cases muscle was confined to one side of the limb and in two cases there was an asymmetry in the distribution of Leydig cells within the epidermis.

Dorsal half limbs

Nine dorsal half limbs were made all of which produced a regenerate; seven of these had 4 digits and two had 3 digits (see Table 1). All the 4-digit limbs had a basically normal skeletal pattern (Fig. 7). Abnormalities were apparent in one 4-digit limb where one of the digits grew from the dorsal surface of the hand. In one other 4-digit limb two digits were fused in the phalangeal region and in another, two of the digits were terminally branched. In both the 3-digit limbs it appeared that the most anterior digit was missing.

Sections of these limbs also showed the characteristic fold of skin described for anterior and posterior half limbs but in dorsal half limbs, despite the head skin being sutured to the ventral surface of the upper arm, the head-skin-derived tissue was displaced to the anterior margin of the regenerate in more distal locations in all but one case. The exception was the above mentioned 4-digit limb which had a digit growing from the dorsal side of the hand. This limb, which was T shaped in section, had only dorsal muscle in the metacarpal region and head-skin-derived tissue occupied the ventral side of the limb and was not displaced anteriorly (Fig. 8). The distal forearm region of this limb contained four bones with an abnormal arrangement of muscles although a single *pq* muscle was identifiable (Fig. 9), but by the midforearm, a single radius and ulna with a normal muscle pattern was present. All other limbs had normal muscle patterns in the metacarpal region with a ventral vascular sinus and normal muscle patterns in the forearm region. The only abnormality appeared to be the relative absence of Leydig cells on either side of the limb at the metacarpal level.

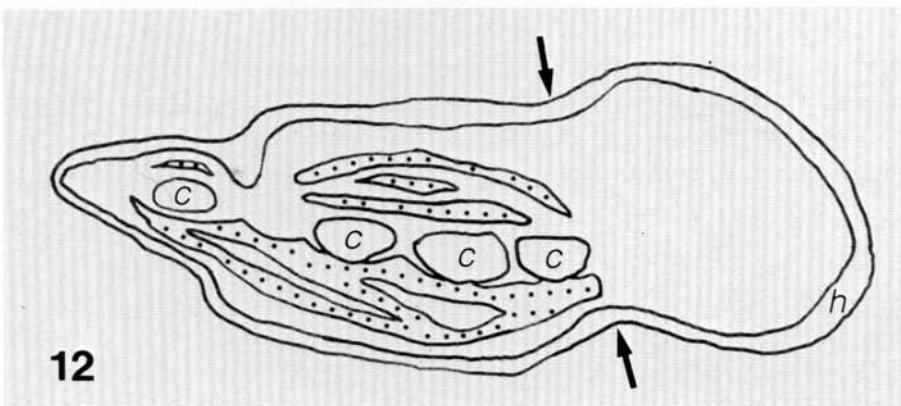
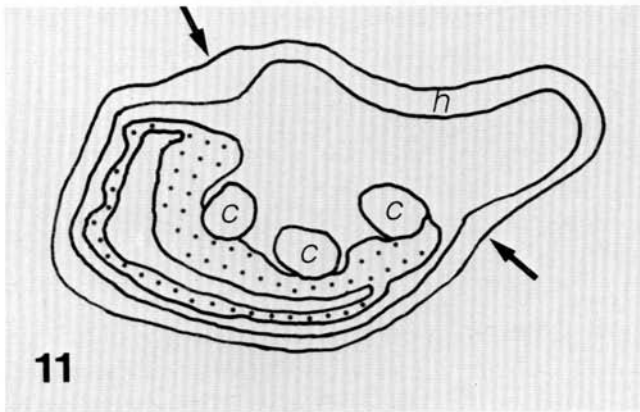
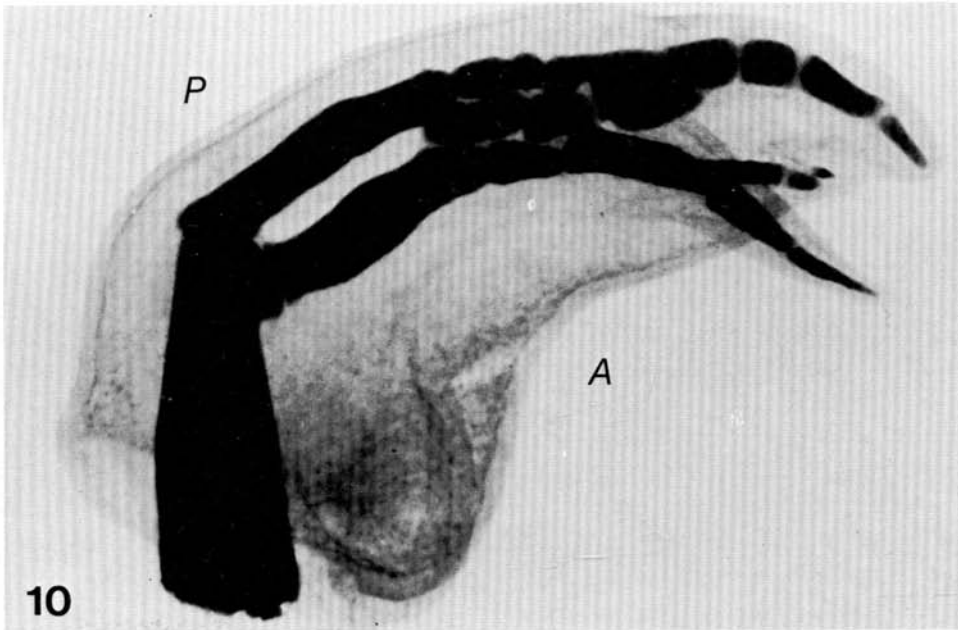
Ventral half limbs

Eleven ventral half limbs were made using the contralateral limbs of animals used to make dorsal half limbs. All ventral half limbs regenerated, (see Table 1), the most common regenerate (five limbs) contained 3 digits. There were also two

Fig. 10. Dorsal view of a Victoria-Blue-stained whole mount of a left limb showing the skeletal structures regenerating from a ventral half stump. Two forearm bones and three digits have regenerated but there is some disorganization of the normal pattern in the carpal and metacarpal regions. Abbreviations: A, anterior side of limb; P, posterior side of limb. Magnification $\times 9$.

Fig. 11. Camera-lucida tracing of a transverse section at the distal metacarpal level of a 3-digit regenerate from a ventral half stump. Ventral muscle occupies the ventral side of the limb while head-skin-derived tissue lies on the dorsal side. Abbreviations: c, cartilage; Starred areas, ventral muscle. *h* marks the location of head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.

Fig. 12. Camera-lucida tracing of a transverse section at the distal metacarpal level of a 4-digit regenerate from a ventral half stump. Ventral muscle was found on both dorsal and ventral sides of the hand and head-skin-derived tissue was displaced to the anterior side of the limb. Abbreviations: c, cartilage; Starred areas, ventral muscle. *h* marks the position of head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.



examples each with 4, 2 or 1 digits. Examination of the cartilage patterns showed that the two 4-digit limbs had a normal pattern. The digits of limbs containing 2 and 3 digits however were not arranged linearly in the anterior-posterior axis but were clustered around one another. The metacarpals often appeared to have abnormally large diameters and arose at slightly different proximodistal levels from those of adjacent digits (Fig. 10). All 2- and 3-digit limbs had both a radius and an ulna. The two single-digit limbs had a single forearm bone and appeared similar to those regenerating from anterior half limbs.

Sections of the limbs in this category showed that, in the forearm region, head-skin-derived tissue occupied an anterior position but, in the metacarpal region, graft-derived tissue was located dorsally when no muscle was present on the dorsal side of the hand (Fig. 11) and anteriorly when the regenerate contained muscle dorsal to the metacarpals (Fig. 12). Of the 4-digit limbs one had normal muscle patterns at both the metacarpal and forearm levels, the other 4-digit limb had ventral muscle and a vascular sinus on both dorsal and ventral sides of the metacarpals and few Leydig cells on either surface of the limb (Fig. 11). In the forearm region however the muscle pattern of this limb appeared normal with a single *pq* muscle on the ventral side of the limb. One 2-digit limb had double ventral muscle in the metacarpal region while the six remaining 2- and 3-digit limbs had ventral muscle on the ventral side of the limb only and no muscle on the dorsal side at this level (Fig. 12). These limbs had a ventral vascular sinus but few Leydig cells anywhere in the epidermis. At the forearm level four limbs (all with 3 digits) were double ventral having two *pq* muscles, one on either side of the radius and ulna, and in two cases a symmetrical ulna was present with the normally ventral spine on both sides of the bone (Fig. 13). The remaining three limbs which had 2 or 3 digits appeared to have normal muscle patterns in the forearm region. No muscle pattern could be deduced from the two limbs with single digits.

Controls

The controls consisted of ten posterior half limbs, ten anterior half limbs, five dorsal half limbs and five ventral half limbs. A few regenerating stumps appeared to have double blastemas early in development, this being particularly true of anterior half limbs. However all stumps regenerated basically normal 4-digit limbs with a radius and ulna. Minor abnormalities occurred in about one third of regenerates which showed fusion between two or more carpal bones and four regenerates (two posterior, and one dorsal and one ventral half) had distally branched phalanges. The muscle patterns of all control limbs were normal in both the metacarpal and forearm regions.

DISCUSSION

The present work was designed to examine differences in the regulative ability of different halves of the upper arm in both the anteroposterior and dorsoventral axes. All halves produced regenerates but there were marked differences in the

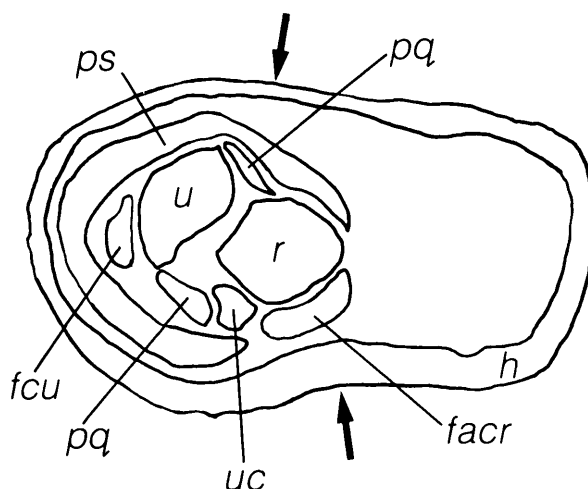


Fig. 13. Camera-lucida tracing of a transverse section at the midforearm level of a regenerate from a ventral half stump. Ventral muscle is present on both dorsal and ventral sides of the limb and the ulna is also symmetrically double ventral. Abbreviations: Bones: *r*, radius; *u*, ulna. Muscles: *pq*, pronator quadratus; *facr*, flexor antebrachii; *uc*, ulno carpalis; *fcu*, flexor carpi ulnaris; *ps*, palmaris superficialis. *h* marks the position of the head-skin-derived tissue and the arrows its limits. Magnification $\times 25$.

structure regenerating from different regions of the stump. The greatest difference in regeneration was between anterior and posterior half limbs where all posterior halves produced 3 or 4 digits while over 60 % of anterior half limbs produced one digit or less. These results are in line with those produced with symmetrical limbs (Holder *et al.* 1980) or by irradiation (Maden, 1979). In the former case double posterior stumps regenerated multiple digit limbs (often symmetrical in the anteroposterior axis) while double anterior stumps produced single digits, spikes or failed to regenerate. Irradiation of either the anterior or posterior half of the upper arm produced normal limbs from unirradiated posterior halves and severely hypomorphic limbs from unirradiated anterior halves. The agreement between all three different experimental protocols is strong evidence for a difference between anterior and posterior tissue in terms of their regenerative potential.

The anterior half limbs, despite having a markedly reduced pattern in the anteroposterior axis showed many features of a normal proximodistal organization even when only single digits regenerated. Such limbs had only a single forearm bone (in the case shown in Fig. 5 this bone is fused with the humerus) but had recognizable carpals, a single metacarpal and several phalanges.

Posterior and anterior half stumps contain both dorsal and ventral tissue and it was therefore not surprising to find a normal dorsoventral muscle pattern in posterior half limbs. The anterior half limbs also on occasions showed asymmetric distributions of muscle and Leydig cells. The origin of the 4- and 5- digit anterior

half regenerates is unknown but from their muscle patterns a possible explanation would be that the 5-digit regenerate came from a stump which was more dorsal than anterior while the 4-digit limb came from one that was more ventral.

Dorsal half stumps produced regenerates which were essentially similar to those from posterior halves, that is they were normal in both the anteroposterior and dorsoventral axes. In this case however the normal dorsoventral musculature of the regenerates was surprising given the exclusively dorsal origin of the stump tissues. Double dorsal symmetrical limbs have been surgically produced (Burton *et al.* 1985) 30 % of which regenerated 4-digit limbs and the majority of regenerates had double dorsal musculature. Irradiation of the ventral halves of upper hind limbs (Maden, 1979) produced a wide variety of regenerates which had a mean digit number of 4, but the muscle patterns were not analysed. It is possible that in the present study ventral muscle has regenerated from dorsal tissue but since this did not occur in double dorsal stumps (Burton *et al.* 1985) this appears unlikely. Two possible alternative explanations are likely. Firstly, that ventral tissue has somehow bypassed the head skin graft and enabled ventral muscle to be present in the regenerate, although it is unclear why this should have occurred only to dorsal half stumps and not to regenerates from ventral, posterior or anterior half stumps. The second alternative would be that the dorsoventral boundary lies dorsal to the humerus. If this were the case the present experimental protocol would have produced ventral half stumps with only ventral tissue but dorsal half stumps with both dorsal and ventral muscle present. This would lead to the regeneration of limbs with a normal dorsoventral axis from dorsal half stumps but limbs with only ventral tissue from ventral half stumps. In the light of this possibility it is interesting that one 4-digit limb, regenerating from a dorsal half stump, which had a digit growing from its dorsal surface lacked ventral muscle in the metacarpal region and had four bones in the forearm region. It is unclear whether this represents a distinct limb type, but it is possible that this limb is a truer indication of what dorsal tissue can produce than the normal limbs produced from most dorsal halves.

Apart from one 4-digit limb, which was normal, all of the regenerates from ventral half stumps, which produced more than one digit, either had no muscle at all on the dorsal side or had ventral muscle on both sides of the metacarpals. When no muscle was present on the dorsal side of the limb, head-skin-derived tissue occupied a dorsal location (Fig. 11). However when muscle was present on the dorsal side of the limb (*i.e.* when the regenerate was double ventral, Fig. 12), the graft-derived tissue was displaced to the anterior side of the limb. It is unclear whether this difference was due to an inhibition of muscle formation by the overlying head skin or whether it was the development of muscle which caused the graft to be displaced. Further work is underway to investigate this. Those limbs which lacked any muscle on the dorsal side of the metacarpals showed some disorganization in the anteroposterior axis with the hands tending to curl up to enclose the dorsal surface. It is likely that this disorganization of the hand was due to the absence of muscle on the dorsal side rather than to a failure in specifying the

anteroposterior axis. The forearm musculature was more difficult to analyse due to a reduction of muscle on the dorsal side but four limbs showed clear double ventral muscle at this level. Two of these also showed a double ventral ulna. The remaining limbs which produced more than a single digit had apparently normal forearm musculature. Double ventral limbs stumps (Burton *et al.* 1985) also produced double ventral limbs or limbs with only ventral muscle on one side of the metacarpals with abnormalities of the skeleton in the anterior-posterior axis. Irradiation of dorsal halves of hind limbs (Maden, 1979) also caused the regeneration of limbs with fewer digits than from irradiated ventral halves.

All control animals regenerated limbs normal in the dorsoventral and antero-posterior axes irrespective of which half of the limb had been removed. These operations are similar to the half limbs produced from the upper arms of newts (Bryant & Baca, 1978) which also regenerated normal limbs. These results show that without a mechanical barrier different parts of the limb can interact around the medial wound surface to form normal regenerates irrespective of the shape of the stump.

In the present study autografts of head skin readily healed into place on the limb with no incompatibility between head skin and limb tissues. These grafts were still visible due to their dark pigmentation as a bulge on the side of the limb 60 days later when the regenerates were amputated. Complete cuffs of head skin are known to inhibit regeneration (Jeffimoff, 1931 quoted in Polezajew & Faworina, 1935) but in the present work all four halves of the limb produced regenerates, many of them completely normal, indicating that 50 % of the limb is sufficient under these conditions for complete regeneration to occur. Tissues derived from the head skin graft grow distally with the regenerate and form a strip of tissue down one side of the limb. Head-skin-derived tissue is recognizable by the loose connective tissue beneath the skin and the presence of lateral line organs in the epidermis. The grafts showed no signs of being overgrown and the markedly different regenerates from different halves of the limb are strong evidence that the head skin effectively isolated half of the limb.

The results of the present study demonstrate a clear difference in regenerative potential across the anterior-posterior axis. Posterior half tissues appear to be essential for the formation of an anteroposterior axis containing more than a single digit. It is possible that in normal regeneration from whole limb stumps, the posterior half either contains all the positional information necessary for the regeneration of a complete limb or is the major source of cells to the blastema. A recent fate map for the anteroposterior axis (Holder & Burton, 1985) predicts that the anterior half of the upper arm should regenerate only the anterior edge of the radius and the anterior parts of the radiale and pre pollicis, the remaining limb pattern emanating from the posterior half of the upper arm. These predictions are largely fulfilled in the present study and the results suggest that regeneration from isolated posterior halves of the upper arm does not necessarily involve cellular interactions leading to the creation of new positional values, specifying the anteroposterior pattern that were not already present in the isolated stump region

at the time of amputation. Slight variations in the position of the medial cut made to isolate anterior and posterior half limbs can probably explain variations in the number of digits from anterior and posterior halves and account for the production of single digits from anterior half stumps. The fate map predicts an approximately equal distribution of regenerative potential in anterior and posterior half lower arms. In this case posterior halves should regenerate a restricted pattern and not a complete limb, and anterior halves should regenerate more of the anteroposterior pattern than an equivalent region in the upper arm. These experiments are being done at the present time. Unfortunately no fate map is yet available for the dorsal and ventral regions of upper or lower arm so we must reserve judgement about the potential of these regions and the likely position of a possible dorsal-ventral subfield boundary.

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