A quantitative study of blastemal growth and bone regression during limb regeneration in *Triturus cristatus*

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SUMMARY
Soft X-ray photographs show both the outlines of the soft parts of the regenerating limb and the bones. This enables one to make quantitative studies on bone regression and the growth of the regenerate, since good reference points are available. Bone regression of about 0.5 mm was observed to occur within about 20 days following amputation. Growth curves of regenerates cut at different levels confirm Spallanzani's old observation that the total time for regeneration is fairly constant for different levels of amputation—regenerates from proximal levels regenerate faster. However, the intrinsic growth rate curves are remarkably similar for different levels of amputation; the difference in final length arises because the short period of high intrinsic growth rate continues for slightly longer in regenerates from proximal levels.

In terms of the progress zone theory of limb development, three phases of regeneration are recognized: the formation of the blastema and progress zone; the laying down of the skeletal rudiments by the rapidly proliferating progress zone; and the subsequent slower growth of the rudiments. A quantitative model is put forward and it is shown that it can provide a good description of the observed growth curves. An important assumption is that the various skeletal rudiments are the same size when they leave the progress zone and the final differences in size reflect differences in the final growth phase. The progress zone model provides new quantitative insights into the relationship between the growth of the limb as a whole and the behaviour of individual cells.

INTRODUCTION
The initial purpose of this work was to make an X-ray study of bone regression in the amputated amphibian limb. However, our X-ray photographs showed not only the bones but also the outlines of the soft parts of the regenerating limb. Thus we could measure the rate of outgrowth during regeneration by a far more reliable technique than has previously been used. Such information is essential for a quantitative theory of limb regeneration, towards which there has been little progress since Spallanzani (1768) noted that the regeneration of the toe of a salamander took as long as a whole leg. In spite of the enormous literature on

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limb regeneration (see, for example, Schmidt, 1968) there seems to be a lack of the basic data required in order to begin to understand regeneration at the cellular level in terms of relatively crude parameters, such as cell division. It is our aim to account for limb regeneration in quantitative terms at the cellular level and in particular to explain the mechanism by which the spatial pattern of cellular differentiation is specified.

Pattern formation may be considered in terms of a mechanism involving the specification of positional information followed by appropriate differentiation (Wolpert, 1971). We have recently proposed a mechanism whereby positional information may be specified along the proximo-distal axis of the developing chick wing and have suggested that it may also be applicable to the regeneration of the amphibian limb (Summerbell, Lewis & Wolpert, 1973). For the chick limb we proposed that there is a region at the distal end, the progress zone, marked out by some influence from the apical ectodermal ridge. In the zone the positional value of the cells undergoes an autonomous change with time, possibly linked to cell division. After the cells leave the progress zone, which is a region of active growth, there is no further change in positional value. In terms of this mechanism, amphibian limb regeneration may be viewed as the setting up of a progress zone in the blastema. The positional value of the progress zone will, at the earliest stages, correspond to the positional value of the cells at the level of the cut. A formal model is presented in the following section. In this paper we will be primarily concerned with the gross growth characteristics of limbs cut at different levels, and with their interpretation in terms of the progress zone model. In a subsequent paper, basic histological data on cell division and the early pattern of differentiation will be provided.

Many previous studies on growth of the regenerating limb have concentrated on how it is affected by various parameters such as temperature (Schmidt, 1968), season of the year (Schauble, 1972), body size and age (Goodwin, 1946; Manner, Zapisek & Vallee, 1960; Pritchett & Dent, 1972), and starvation (Twitty & DeLanney, 1939). Much of this is reviewed in Schmidt (1968). An important new investigation is that of Iten & Bryant (1973) who have obtained results substantially similar to those to be reported here. Their work, like many other studies, employs a camera-lucida for taking measurements, but this has one major draw-back. It is very difficult to judge accurately the site of amputation, especially in the newt Triturus viridescens, where the pigmentation differences between the stump and blastema become very indistinct with growth. X-ray photographs have an obvious advantage here, in that measurements can be made from fixed reference points, such as bony elements located well into the stump tissues and easily visible. This, in effect, means that the proximal limit of the blastema is now accurately identifiable.

Bone regression has been observed histologically by several workers but again quantitative data are lacking (see Schmidt, 1968, for review). Various workers have suggested that the osteocytes and chondrocytes, released from their
Limb regeneration in Triturus

Fig. 1. The course of regeneration schematized according to the progress zone theory. The upper diagrams show the limb; the lower diagrams show the pattern of positional values $P$ (ordinate), along its length (abscissa). (A) The intact limb, before amputation. (B) The limb immediately after amputation at the level $l$ (marked by a dotted line). (C) The beginning of our ‘second phase’, corresponding to the small cone stage; the progress zone (shaded) has just been set up. (D) The middle of the second phase; the proximal rudiments of the regenerate have already been laid down, while the more distal rudiments have not yet emerged from the progress zone. (E) The end of the second phase; the progress zone has finished its work; all the rudiments are present, but have not yet grown to full size. (F) Regeneration complete: the rudiments have grown to full size, and the full normal pattern of positional values has been restored.

lacunae by matrix degradation, play an important role in contributing to the regeneration blastema, appearing to ‘dedifferentiate’ to their former fibroblastic state in the process (e.g. Goode, 1967).

A formal model

We wish to present here a formal and quantitative model of amphibian limb regeneration based on our progress zone model of the developing chick limb (Summerbell et al. 1973; Summerbell & Lewis, 1974). This leads to a very explicit formulation of the rules we would suggest for the control of regeneration, and will not only be used to interpret the data we will present in this paper but will, we hope, serve also as the theoretical basis for future papers.

We distinguish three phases in regeneration. First, a small blastema is formed by ‘dedifferentiation’ of stump tissues, so as to form the population of a progress zone beneath the ectodermal cap. Second, the cells in that progress zone proliferate rapidly so as to lay down the full set of skeletal rudiments. Third, those rudiments slowly grow to adult size. This growth may involve both cell division and matrix secretion by cartilage cells, pushing the cells further apart (Gould, Selwood, Day & Wolpert, 1974). The difference between the regenerates from different levels appears in the second phase: more rudiments have to be laid down in the regenerates from the more proximal levels. Further differences may arise in the third phase, if the rudiments of proximal and distal bones grow differently. These different phases are illustrated in Fig. 1.

We now wish to obtain an analytic expression for the length of the regenerate
at any time following amputation. We assume that mesodermal limb cells are equipped with positional values, i.e. internal records of their position in the limb (Fig. 1). At the end of the first phase of regeneration we have a small blastema of length \( w \), containing cells which all have the positional value corresponding to the level of amputation, \( l \). During the second phase, as these cells proliferate further, some of them change their positional values. The change is permitted only in a progress zone of length \( w \), extending inwards from the distal tip; in this zone the positional value becomes steadily more distal, as a result of some time-dependent process occurring autonomously in the cells there. The cells that overflow from the progress zone become fixed in positional value as they leave it. Thus the progress zone trails behind it a succession of cells with more and more distal positional values; these constitute the established skeletal rudiments. Eventually, after a time \( T \) from the beginning of the second phase, the cells in the progress zone achieve the most distal possible positional value, corresponding to the extreme tip of the developed limb. The changes of positional value come to a halt, the progress zone is extinguished, and the cells at the tip proceed to differentiate like those which have previously left the zone. The second phase of regeneration is complete.

The duration of this phase, \( T \), depends on the level of amputation, because it depends on the initial positional value of the cells which make up the blastema. The more proximal that value is, the longer those cells will take to reach the most distal possible positional value by proliferating in the progress zone. Conversely, we can specify the positional value \( P \) of any cell by giving the time \( T \) which must elapse after its emergence from the zone, before the final extinction of the zone. On this scale, the positional value at the level of amputation is \( P = T_h \) and at the tip of the developed limb, \( P = 0 \).

Now the programme of growth and differentiation after leaving the progress zone is a function of the positional value. A slice of tissue with positional value \( r \), whose length was \( dx \) at the time of leaving the zone, will have a length \( G(T, At) dx \), at a time \( At \) after that, where \( G(T, At) \) is a function which we shall call the local growth coefficient.

The length of the progress zone is \( w \); and let \( g \) be the intrinsic growth rate in it – that is, the rate of elongation per unit length. Then the length of the slice of tissue which leaves the zone in a time interval \( dt' \) will be

\[
dx = wg \, dt'.
\]

Suppose this slice of tissue leaves the progress zone at a time \( t' \) after the beginning of the second phase of regeneration. Then its positional value will be \( r_t - t' \). By the time \( t \), its length will have expanded to

\[
G(r_t - t', t - t')wg \, dt'.
\]

By summing the lengths of the expanded slices that have left the zone in successive time intervals, and adding the length of the zone itself, we get the total length of the regenerate. Thus if the limb was amputated at a level \( l \), the total
Limb regeneration in Triturus

length of the regenerate at any time $t$ during the second phase of regeneration will be

$$L_i(t) = w + \int_{0}^{t} G(\tau_i, t - t') \, w \, dt'$$  \hspace{1cm} (3)

if we take our time origin $t = 0$ to be the beginning of the second phase. During the third phase of regeneration, the progress zone has been extinguished, and no further positional values are laid down; but the tissue present at the end of the second phase continues to elongate according to its positional value. Thus for $t > \tau_i$ we have instead

$$L_i(t) = wG(0, t - \tau_i) + \int_{0}^{\tau_i} G(\tau_i - t', t - t') \, w \, dt'.$$  \hspace{1cm} (4)

Thus

$$L_i(t) = \begin{cases} 
  w + wG \int_{0}^{t} G(\tau_i - t', t - t') \, dt' & \text{for} \quad 0 < t < \tau_i, \\
  wG(0, t - \tau_i) + w \int_{0}^{\tau_i} G(\tau_i - t', t - t') \, dt' & \text{for} \quad t > \tau_i.
\end{cases}$$  \hspace{1cm} (5)

MATERIALS AND METHODS

The animals used in this experiment were adult *Triturus cristatus* (the Italian or Crested Newt). They were kept in tanks of tap water maintained at $25^\circ$C and fed weekly on minced heart. To minimize variation they were selected to be approximately the same size and all operations were carried out at the same time of year (late spring/early summer). They were all amputated through both forelimbs, at one of three levels: (1) humerus (midway between shoulder and elbow), (2) radius/ulna (midway between elbow and hand), or (3) carpals (wrist skinfolds region), under MS 222 anaesthesia. They were then allowed to regenerate normally; some, however, died and some regenerated abnormally. These were not used. At intervals during regeneration, the animals were X-rayed. They were anaesthetized and laid on the cassette containing the X-ray film. Care was taken to ensure that the limb was flat against the surface. A water-cooled Watson 50 kV laboratory X-ray unit was used (which has the special virtue of delivering a high intensity of soft X-rays). The X-ray source was a Machlett OEG 50 tube, with a thin beryllium window, which delivers 72 rads/minute at 20 mA and 25 kV. The newts were X-rayed for a 10 sec exposure, at 10 mA and 25 kV, giving a dose of 6 rads. (This is a low enough dose not to effect regeneration. It works out as an average of approximately 1 rad/day over the 100-day period of this study.) The X-ray film used was Industrex M, which was developed in Johnson's Contrast Developer for 4 min, and then fixed, washed and dried as usual.

Measurements were made of both bone length and blastema length, by
A. R. SMITH AND OTHERS

Fig. 2. (A) Animals amputated through the radius/ulna or wrist region were measured from the point where the proximal heads of the radius and ulna almost touch to the most distal margin of soft tissue. (B) Animals amputated through the humerus were measured from the articulation site of the proximal head of the humerus with the scapula to the most distal margin of soft tissue. The soft tissues are very well defined in our X-ray negatives, but it is difficult to show them in the photographs without over-exposing the bones of the arm. We show here three typical examples of over-exposed photographs showing the following stages: (C) Wound healing. (D) Small – large cone. (E) Four-digit.

projecting the X-ray photographs (used as negatives) on to a screen at a known magnification (×15). These measurements were made from a fixed reference point, chosen to be clearly visible in the X-ray photographs. Animals cut at the humerus level were measured from the proximal head of the humerus (at its articulation site with the scapula). Animals cut at the other two levels, however, were measured from the point where the proximal heads of the radius and ulna almost touch, as shown in Fig. 2. From these measurements, the change in the length of the growing blastema and the extent of bone regression were obtained over the 100-day period of this study.

RESULTS

Bone regression

The opacity of bones to X-rays is due to their mineral content. When the bone, or part of it, shows less opacity than normal, it can be deduced that the
Fig. 3. Bone regression in this animal, which has been amputated through the humerus, starts at day 8, and is well under way by day 14. The left-hand bone regressed (as observed by an X-ray transparent/translucent region appearing proximal to the distal cut surface), but the distal X-ray opaque piece of bone did not break away. The right-hand bone, however, does show the distal X-ray opaque piece of bone breaking away. It is presumably lost by day 27.
Limb regeneration in Triturus

Table 1. Quantitative data on bone regression

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Average initial length (mm)</th>
<th>Decrease in average length after regression (mm)</th>
<th>Cases showing regression</th>
<th>Mean % regression and S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humerus</td>
<td>13</td>
<td>4.0</td>
<td>0.5</td>
<td>11</td>
<td>12.5 (1.6)</td>
</tr>
<tr>
<td>Radius</td>
<td>11</td>
<td>2.4</td>
<td>0.3</td>
<td>8</td>
<td>12.7 (2.5)</td>
</tr>
<tr>
<td>Ulna</td>
<td>11</td>
<td>3.2</td>
<td>0.3</td>
<td>6</td>
<td>11.1 (1.6)</td>
</tr>
</tbody>
</table>

Bone contains less mineral than normal. Bone regression is observed in all animals as an X-ray transparent/translucent area along the normally X-ray opaque bone. In animals amputated at the humerus level or radius/ulna level the transparent/translucent area appears not at the distal cut surface itself, but just proximal to it. This region looks like a 'bridge' between two X-ray opaque areas, or as though the end of the bone is being 'pinched off'. Frequently, the more distal of these areas breaks off, and seems to migrate distally, but sometimes the 'bridge' may persist (see Fig. 3). When the cut is through the radius/ulna region these two bones do not always regress by the same amount. Bone regression is apparent by day 8, and is very advanced by day 14. Where a piece of bone breaks away, it does so by day 21, after which regression appears to cease for all levels of cut.

Wrist-level amputations pass through the carpals region (except in one case as in Fig. 4 where the cut was just proximal to the wrist elements yet distal to the radius and ulna). In these cases, regression occurs at the cut surface of the bone (not just proximal to it as in amputation of the long bones), often resulting in the complete disappearance of some of the carpal elements. However, in the one case mentioned above, where the amputation proved to be proximal to the carpal elements, regression was similar to that seen in the radius/ulna amputated limbs.

Quantitative data on regression are given in Table 1, where it can be seen that regression is about 10% of the initial length after amputation and is less than 0.5 mm.

**Figure 4**

Fig. 4. Bone regression in this animal, which was amputated through the wrist skinfolds region, occurs between days 8 and 14. The left arm has been cut through the carpals and the remaining carpal regresses to about half of its initial size. The right arm has been cut proximal to the carpals but distal to the radius and ulna. Here regression begins just proximal to the distal heads of the radius and ulna and the heads are then eaten away. This is very similar to an animal cut through the radius/ulna, in that only those bones regress. Regression of the radius and ulna was never seen in animals cut through the carpals.
Later, ossification of the regenerated skeletal elements of the replaced limb was also observed. The first ossification centres appear to be the long bones and digits, i.e. humerus, radius, ulna, metacarpals and phalanges, starting at about day 35. During the 100-day period studied, no ossification of the carpals was ever seen. The regenerated ossified long bones appeared to be a lot broader than the normal adult bones. This is similar to bone fracture repair, where the callus that forms around the fracture ossifies as an enlarged band around the bone, which does not get remodelled to the normal width until much later.

According to Ham (1969), bone resorption does not occur on surfaces that are covered, and hence protected, by osteogenic cells and/or osteoblasts, but only occurs on naked bony surfaces that are neither being protected nor built by the osteogenic cells or osteoblasts. Osteoclasts are usually present over the naked areas where bone is being resorbed. They are thought to be the specific agents of bone resorption (it is thought that they regard the naked bone surface as a foreign body and attack it with enzymes). This is puzzling because we see, in two of our levels of amputation, that regression takes place primarily not at the cut surface, but just proximal to this. At the carpals level, though, when the carpal elements have been transected, resorption does occur at the cut surface. The role of bone regression is not known but it may provide osteocytes for regeneration (Schmidt, 1968).

Growth of the regenerate

The average length of the regenerate, for each of the three levels of cut, is given in Fig. 5. The regenerating limb when cut through the humerus or radius and ulna passes through a sequence of morphologically recognizable stages. These have, in the literature, been given a variety of different names and no standard terminology exists (Schmidt, 1968; Iten & Bryant, 1974). The absence of such staging is very inconvenient and we have found it necessary to use yet another set of terms. These are based on external criteria only. They are given in Table 2 together with the approximate time they take to develop at 25 °C, measured in days from the time of amputation. The times between the later stages are less variable than the time between wound healing and small cone formation. The histology of the various stages together with the pattern of cell division will be reported in a subsequent paper.

The sequence of stages in the regeneration from the level of the carpals follows a slightly different pattern. In this case there is no real equivalent of small-cone, large-cone, flat-cone, and spatulate. Instead the digits arise directly as separate units from an oval blastema. With reference to our model, the first phase of regeneration is probably complete by the small-cone stage, and the second by the three-digit stage. The second and third phases overlap: the humerus rudiment, for example, may begin to differentiate and grow while more distal elements are still being laid down.

The growth curves have the typical sigmoid form (Fig. 5). Up to about day 20
Fig. 5. Growth curves of regenerating limbs cut at different levels. The theoretical curves (see equation (6)) are shown together with our experimental data – humerus (▲), radius/ulna (□), and carpals (●). The number of limbs used for each level was: humerus, 10; radius/ulna, 7; carpals, 5. S.E. at each time did not vary very much; typical S.E.s are 15% at day 14 and about 5% at day 70.

the lengths of regenerates from all three levels are about the same. After this time, which corresponds roughly to the spatulate stage, the curves diverge: the regenerate from the humerus level, for example, continuing to elongate at a faster rate (Fig. 5). It is difficult to speak about the end-point of regeneration in terms of growth, since all the regenerates show slow but continued growth even after 100 days. Using a coarse criterion based on the growth curves alone, one sees at a quick glance that these flatten off for all three levels of amputation round about day 30 or 40. In this sense, Spallanzani’s observations are confirmed. Choosing instead the four-digit stage as an end-point, we find that regenerates from the carpals level reach this on average in 21 days, whereas regenerates from the more proximal levels take longer – radius/ulna 27 days, and humerus 31 days. (These are only approximate as the relevant observations were only made at intervals of about a week.) There is thus a significant difference in the time required for regeneration from different levels. This is rather different from the conclusion of Iten & Bryant (1973), who found that regenerates from different levels reach the same stages at the same times. However, this disagreement relates to only a small fraction of the total time involved.

25-2
Table 2. Morphological stages in limb regeneration of Triturus

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Approximate time (days) from amputation (25° C)</th>
<th>Time since previous stage (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound healing</td>
<td>Ectoderm covers the exposed surface</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Small-cone</td>
<td>Small cone</td>
<td>10–14</td>
<td>Variable</td>
</tr>
<tr>
<td>Large-cone</td>
<td>Cone enlarged so its height is approximately equal to its base</td>
<td>12–16</td>
<td>2</td>
</tr>
<tr>
<td>Flat-cone</td>
<td>Cone flattened dorso-ventrally</td>
<td>14–18</td>
<td>2</td>
</tr>
<tr>
<td>Spatulate</td>
<td>Flattened cone elongates</td>
<td>15–19</td>
<td>1</td>
</tr>
<tr>
<td>Two-digits</td>
<td>An indentation appears along distal margin</td>
<td>18–22</td>
<td>3</td>
</tr>
<tr>
<td>Three-digits</td>
<td>A second indentation appears along the distal margin</td>
<td>20–26</td>
<td>3</td>
</tr>
<tr>
<td>Four-digits</td>
<td>A third indentation appears along the distal margin lateral to the first two</td>
<td>21–31</td>
<td>3</td>
</tr>
</tbody>
</table>

One feature of the growth curves is the more rapid elongation of regenerates from proximal levels during the later phases of outgrowth (Fig. 6). We wish now to try to understand this at the cellular level. Does the faster rate of elongation of the humerus-level regenerate necessarily reflect an intrinsic difference in the behaviour of its cell, or is it for most of the time just a geometrical consequence of its greater size? And in the latter case, how and when does this proximal regenerate first come to be bigger than the distal regenerates? To answer, we must look not at the gross rate of elongation but at the ‘intrinsic growth rate’ – that is, the rate of elongation divided by the length (Fig. 7). The intrinsic growth rate is effectively the rate of elongation of the ‘unit cell’. Our graphs show that the intrinsic growth rate is high at first, and decreases later. The striking feature is that the intrinsic growth rate for all three levels follows a very similar pattern; the heights of the maxima are virtually the same, but the period of high intrinsic growth rate lasts a few days longer in the regenerates from the more proximal levels. This gives a bigger primordium, and so can largely account for both the greater eventual rate of elongation and the greater final length of regenerates from a more proximal level. The few extra days at a high intrinsic growth rate are, however, only a small fraction of the total time of regeneration, which thus seems to be nearly the same whatever the level of amputation, as Spallanzani (1768) remarked.

We can now consider these results in terms of our model. This involves substituting a formula for $G(t, \tau)$ in equation (5) and so calculating the length $L$ of the regenerate as a function of time, for different levels of amputation. Thus
we get curves to compare these with the data in Fig. 5. Our formula for \( G(\tau, t) \) will be a guess. We aim only to show that the data can be accounted for very plausibly in terms of our progress zone theory. We do not claim that our formula for \( G(\tau, t) \) is accurate, or, for example, that \( w \) and \( g \) are exactly constant, as we have implicitly assumed above.

The expression that we choose is

\[
G(\tau, t) = \exp g \frac{(\alpha + \beta \tau)t}{\alpha + \beta \tau + t}
\]

where intrinsic growth rate, \( g = 0.16 \) days\(^{-1} \), \( \alpha = 6.8 \) days, \( \beta = 0.2 \). The constant \( \alpha \) determines the total amount of growth (including both cell division and matrix secretion) undergone by the most distal tissue, with positional value \( \tau = 0 \), after it leaves the progress zone. In this somewhat arbitrary model, we suppose that more proximal rudiments undergo more growth after leaving the zone: we take this to be the chief reason why the humerus ends up about six times as long as the third phalanx. The constant \( \beta \) specifies the steepness of this variation of growth with positional value (see Fig. 8). It is important to recognize
Fig. 7. The intrinsic growth rate of the regeneration blastema after amputation through the humerus (▽), the forearm (■), and the wrist (○). We use the formula
\[
\frac{1}{L} \frac{dL}{dt} = \frac{d \ln L}{dt}
\]
and plot the points \( \left( \frac{t_{n-1} + t_{n+1}}{2}, \frac{\ln L_{n+1} - \ln L_{n-1}}{t_{n+1} - t_{n-1}} \right) \),
where \((t_n, L_n)\) are the points of the original growth curve.

the meaning of the assumption made here; this is that the size of the rudiments of the main elements of the limb – humerus, radius and ulna, carpals, and phalanges – is the same for each one as it leaves the progress zone, and that the final differences in size reflect differences in growth in phase 3.

To fit our curves to the data, we suppose that the second phase of regeneration begins 12 days after amputation, and that the progress zone is maintained thereafter for 20.8, 10.8 and 8.6 days respectively, in the regenerates from cuts through the humerus, the forearm and the wrist. The theoretical curves are shown together with the experimental points in Fig. 5. The fit is good, and though the number of disposable parameters is large, they are all assigned values compatible with rough estimates from other sources. Some of these estimates will be considered in a subsequent paper, though they may not be precise enough to give a much more stringent test of the theory. For example, the intrinsic growth rate, \(g\), in the progress zone, and the constants \(\alpha\) and \(\beta\) describing the amount of growth of the various rudiments after they leave the progress zone, can be found from measurement of cell division rates and matrix secretion during regeneration. It is more difficult to determine the start and duration \(\tau_t\) of the second phase and the length of progress zone \(w\). However, we are attempting to
do this using techniques similar to those used for the developing chick limb (Summerbell et al. 1973).

It is clear that the progress zone theory, and the quantitative analysis it enables us to make, can give new insight into the process of regeneration and suggest what cellular parameters require investigation. The applicability of the theory to the newt is far from being established, though we are currently trying to gather the necessary evidence. The theory does, for example, provide an immediate explanation of the law of distal transformation (Summerbell et al. 1973): by spending time in a progress zone, cells can acquire a more distal, but never a more proximal, character. At the very least the quantitative approach we have adopted here for following limb regeneration will provide a crucial baseline for further studies. It is perhaps one of the few attempts that have been made to account for the overall growth curves in terms of cellular activities.

We would like to thank Dr G. Farrer-Brown for drawing our attention to the virtues of soft X-rays and for the use of his X-ray machine, and Mr M. Tarbit for technical assistance. This work was supported by the Medical Research Council and the Science Research Council supports one of us, J.L.
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