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Blastemal kinetics and pattern formation during amphibian limb regeneration

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SUMMARY

To investigate whether the uniqueness of proximal and distal limb regenerates could be attributed simply to differing blastemal growth characteristics, their increase in volume, cell number and cell-cycle times were determined. With respect to these parameters proximal and distal blastemas were identical and, furthermore, no evidence could be found for the existence of separate growth zones such as an apical proliferation centre or a progress zone within the blastema. It was therefore concluded that level-specific properties of the blastemal cells play the major role in determining the structure of the regenerate, not their growth characteristics. The only discernible difference was in the cell number within the two types of blastema at the onset of cartilage redifferentiation – proximal regenerates had 60 % more cells. Thus it seems that the larger the pattern to be regenerated (the more proximal the amputation plane), the larger the primordium within which that pattern first appears. These two conclusions are discussed in relation to current theories of pattern formation during limb regeneration and development and a new way of envisaging the regeneration of pattern is described.

INTRODUCTION

Our knowledge of cell kinetics and cell interactions during amphibian limb regeneration has received little attention compared to progress in the understanding of similar phenomena during limb development. Perhaps partly responsible is the thought that the two are subject to different restraints and so each has little to offer the other. However, Faber (1971) and more recently Stocum (1975) have drawn parallels between limb development and regeneration, the latter author arguing for the conservation of developmental mechanisms through natural selection. Indeed, a contemporary theory of limb development, the progress zone theory (Summerbell, Lewis & Wolpert, 1973) also encompasses an explanation of the mechanics of regeneration.

If one accepts Stocum's argument, then the amphibian limb affords a unique opportunity to examine the controlling elements of level-specific differentiation during both development and regeneration through the simple expedient of amputating at different levels. In this manner one is provided with an

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unadulterated system, free from possible abnormalities induced by the manipulation of the tissues concerned and its attendant criticisms.

Most of the fundamental questions concerning the growth characteristics of upper and lower arm regenerates have yet to be answered satisfactorily. For instance, is there any difference in size between proximal and distal blastemas? Faber (1971) stated 'we know that blastemas growing on distal amputation levels are smaller than those growing on more proximal levels', whereas Iten & Bryant (1973) and Smith, Lewis, Crawley & Wolpert (1974) both considered blastemas of adult newts to be similar in size irrespective of the level of amputation. What are the growth rates, cell-cycle times and are all the cells dividing in proximal and distal blastemas? Spallanzani (1769) first observed that the same time was taken to regenerate either the toe or the whole leg of a salamander and since then, the 'law' – the greater the amount removed by amputation, the faster is the rate of regeneration – seems to have been upheld in several different species and appendages (see Tassava & Goss, 1966). Yet Iten & Bryant (1973) reported that blastemas from different levels of adult newt limbs grow at the same rate for the first 3 weeks and Smith *et al.* (1974) deduced that the intrinsic growth rate is the same for wrist, forearm and upper arm regenerates.

The main aim of this work is to describe the growth characteristics of upper and lower arm blastemas in greater detail than by simple length measurements, that is to determine the volume of the blastemas and the cell number and cycle time of their cells. If there are differences in the growth characteristics of proximal and distal blastemas which could be held solely responsible for their level-specific properties then we would be well on the way to an understanding of the concept of positional information in the limb. If their growth rates were the same however, we would then have to look elsewhere, and this study also attempts to answer these questions.

MATERIALS AND METHODS

The axolotl larvae, *Ambystoma mexicanum*, used in this study were kept throughout in individual bowls in an incubator at a water temperature of 20 °C. All operations were performed under anaesthetic using 0·1 % MS222 (Sandoz).

Histology. For this series, small larvae were chosen, around 30 mm long, to facilitate counting. The forelimbs of 14 larvae were amputated through the mid-stylopodium (giving 28 proximal regenerates) and 14 through the distal radius and ulna (giving 28 distal regenerates). At daily intervals from day 3 post-amputation three to four regenerates from each level were fixed in Bouin's. The samples were embedded in wax, sectioned at 12 μm and stained in haematoxylin and eosin. Whilst examining the sections the stages reached on any particular day were recorded and the total volume of each blastema

determined by counting the number of graticule squares covered by blastemal cells on every section and summing the values. The number of cells within each blastema was estimated by photographing a typical section of blastema, counting the number of cells within the photograph, calculating the density and relating this value to the previously determined volume. Axolotl blastemal cells have diameters of 10–15 μm, hence 12 μm was chosen for section thickness. In addition, 'mitotic maps' were constructed by recording the position of every blastemal mitosis in 10 consecutive sections on a graph paper drawing of the sections.

This entire experiment was repeated on 60–70 mm axolotls in order to provide a better comparison for the cell-cycle studies.

Label studies. For this experiment 60–70 mm axolotls were chosen so that the amount of available material was greater. The forelimbs of 17 larvae were amputated through the mid-stylopodium and 17 through the mid-zeugopodium. Six days later when small blastemas had appeared, the 34 proximal and 34 distal blastemas were used for the cell-cycle determinations in which each animal was injected intraperitoneally with 3 μCi [^{3}H]-6-thymidine (20·6 μCi/μg) in 10 μl saline. The labelling time was terminated 2 h later by the injection of a 2000 × chase (286 μg thymidine in 10 μl saline). Assuming equilibration in a 3 ml body volume the final concentration of thymidine was about 0·4 mM. To allow convenient sampling times over the next 72 h the injections were staggered at 10.00 and 22.00. At each sampling time one proximal and one distal blastema were removed and fixed in 3:1 ethanol:acetic acid. Subsequent treatment of the blastemas, adapted from the standard procedures for plant root-tips, have been described before (Wallace & Maden, 1976) and permit the easy scoring of mitotic figures. The slides were prepared for autoradiography with Ilford K2 emulsion and stored for 4–5 weeks. They were developed in Kodak D19 for 20 min, fixed, washed and mounted.

RESULTS

Dedifferentiation. After amputation through both levels muscle tissue retracted and caused the cartilage to protrude to a certain extent from the stump. The stumps were not trimmed partly so that more 'natural' conditions might prevail and also because Dearlove & Dresden (1976) have observed that trimming can cause the wound tissues to close in a purse-string fashion thus preventing epithelial/mesenchymal contact and inhibiting regeneration. The epidermis soon migrated over the exposed soft tissues, but was prevented from completely covering the cartilaginous projections for several days. It was most noticeable at proximal levels that the cartilage was attacked from the sides and the tip was most likely ejected; this seems to be the way in which cartilage normally regresses (Smith *et al.* 1974).

The onset of muscle dedifferentiation occurred at the same time from

Table 1. *The range of stages of regeneration from proximal and distal levels of 30 mm axolotl limbs seen on any particular day after amputation*

These were scored from histological sections and anticipated the same stage scored from external observation by several days. For example, 2–3 digits is equivalent to the notch stage externally.

Day	Distal	Proximal
3	Muscle dedifferentiation	Muscle dedifferentiation
4	Cartilage dedifferentiation	Muscle dedifferentiation
5	Early–medium bud	Early–medium bud Cartilage dedifferentiation
6	Medium–late bud	Medium–late bud
7	Radius and ulna redifferentiation	Late bud
8	Radius and ulna – 2 digits	Humerus redifferentiation
9	2–3 digits	Humerus – radius and ulna
10	4 digits	Radius and ulna – 2 digits
11	—	2–3 digits
12	—	4 digits

proximal and distal levels – on day 3 – and earlier than that of cartilage which seemed slightly delayed at proximal levels. This latter phenomenon would not be expected to have any significant effect on blastemal cell number (and did not in fact) since the contribution of cartilage to the blastema (at least in adult newts) is less than 10 % (Chalkley, 1954). The fact that the stumps were not trimmed is unlikely to be responsible for the proximal delay of cartilage dedifferentiation since both levels experienced the same treatment and muscle dedifferentiation began at the same time in both.

Blastemal growth stages. The first obvious accumulation of blastemal cells was present on day 4 at both amputation levels and by day 5 a recognizable small blastema had developed, approximating the early bud stage of Iten & Bryant (1973). On day 6 the blastemas were bigger (medium/late bud) and differences in shape between proximal and distal regenerates appeared. The former were more pointed and longer whilst the latter were wider and flatter – a result of the dissimilar stump shape. By the 7th day distal blastemas showed the first signs of cartilage redifferentiation – the radius and ulna, whereas the beginnings of humerus redifferentiation did not commence until day 8. Thereafter the rate of production of new elements was amazingly rapid – the start of digit formation on day 8, 2–3 digits on day 9 and 4 digits on day 10 from distal blastemas; radius and ulna on day 9, first appearance of digits on day 10, 2–3 digits on day 11 and 3–4 digits on day 12 from proximal blastemas. These events are summarized in Table 1. It is to be remembered that these stages are from sections, not from external observations. The notch stage was reached on day 9 in distal blastemas when internally 2–3 digits had developed.

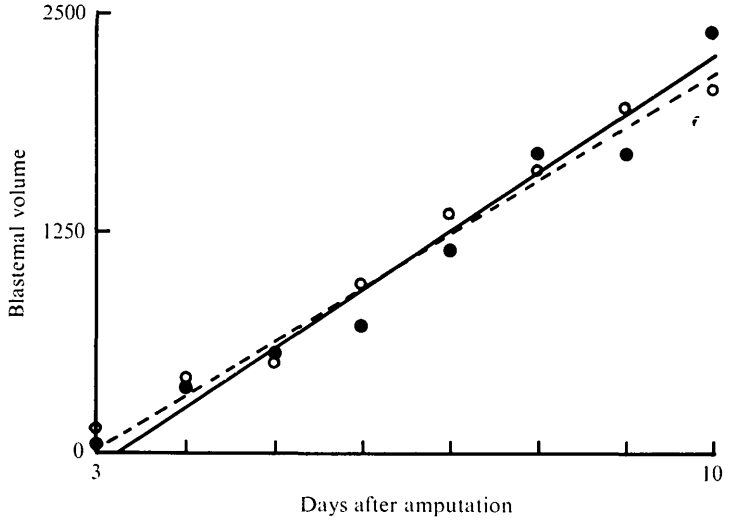

Fig. 1. The increase in volume (in arbitary units) of proximal (●) and distal (○) blastemas of 30 mm axolotls between days 3 and 10 post-amputation. The straight lines drawn here (solid line, proximal; broken line, distal) are the regression slopes calculated from the data points, each point being the average of 3–4 values. The coefficients of regression are not significantly different, thus blastemas from both limb levels expand in volume at the same rate.

It can be seen from Table 1 that after day 4 when blastemal cells could first be observed, a mere 8 days elapsed during which proximal blastemas replaced half the humerus, the radius and ulna, carpals, metacarpals and 4 primordial digits and 6 days were needed for distal blastemas to replace the radius and ulna ends, carpals, metacarpals and 4-digit primordia. Strictly speaking therefore, Spallanzani's observation (1769) that the time taken to regenerate a whole new leg or a toe is the same, cannot be taken literally as Smith *et al.* (1974) have pointed out. Whereas the comment of Goss (1969) that differentiation is slower in proximal regenerates has been confirmed. The conclusion of Iten & Bryant that the stage reached on any day after amputation at proximal and distal levels is the same does apply to the early blastema, but not to the stages of differentiation (Table 1). However these authors only scored external stages at 2-day intervals rather than the more precise method used here. The time lag between external stages and histological stages, as pointed out above, is considerable and might be the cause of this discrepancy; alternatively adult newts might behave differently, but Smith *et al.* (1974) who used these animals obtained the same results, in principle, as those reported here.

The above results were realized with 30 mm axolotls and as mentioned in the Methods this experiment has been repeated on 60–70 mm animals. Interestingly the relative stages for proximal and distal blastemas are exactly the same as above, but everything takes place one day later, for instance distal levels redifferentiate on day 8, proximal on day 9 etc.

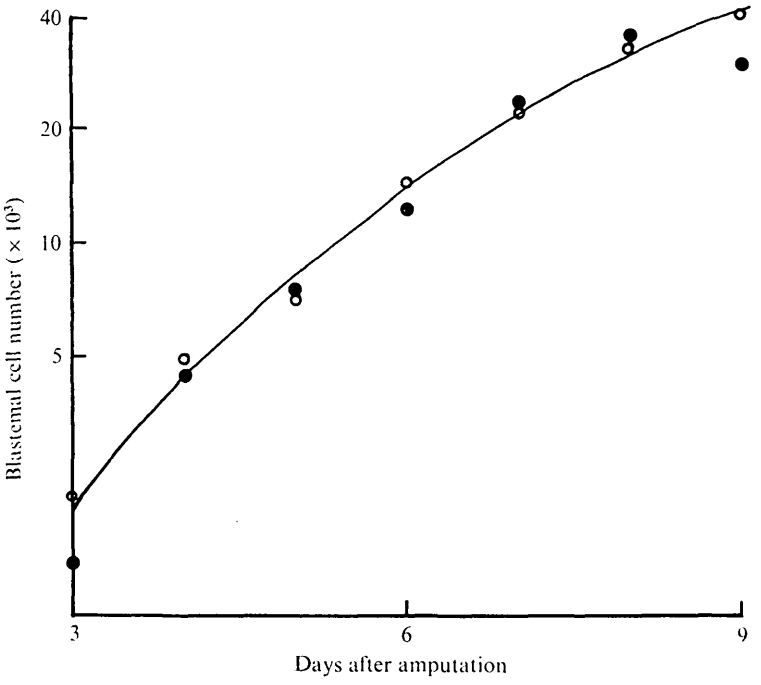

Fig. 2. Semi-log plot of the increase in cell number in proximal (●) and distal (○) blastemas of 30 mm axolotls between days 3 and 9 post-amputation. Each point represents the average of 3–4 values and since they are so close only one curve has been drawn. The population doubling time (Td) on any day is estimated from the tangent of the curve at that time (see text).

Blastemal growth rates. The increase in volume of proximal and distal blastemas is plotted in Fig. 1, both on the same axes for comparison. Each data point represents the average of three to four blastemas and it is quite clear that the regression lines through the points are almost identical. We can therefore conclude that on any day after amputation the volumes of proximal and distal blastemas are the same. It was mentioned above that distal blastemas were flatter and looked bigger than those on proximal levels; this was obviously an illusion.

Similarly Fig. 2 reveals that on any day after amputation proximal and distal blastemas have the same number of cells. Only one curve is drawn here as it could represent either set of points. Thus blastemas from different levels of the limb grow at the same rate. From the data in Fig. 2 we can determine the blastemal cell number when redifferentiation of cartilage elements first occurred. For distal blastemas the radius and ulna appeared on day 7 when the cell number was approximately 22000. When the humerus appeared one day later in proximal blastemas there were about 36000 cells; that is 60% more. From this comparison we may conclude that the greater the amount of

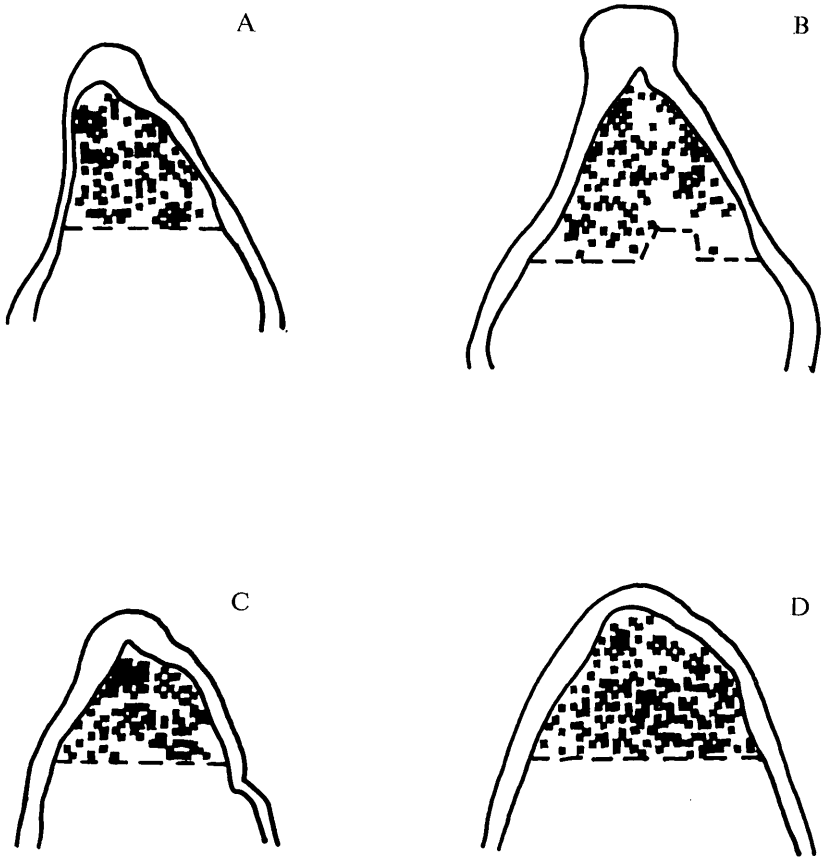

Fig. 3. Mitotic maps recording the position of every mitotic figure in 10 consecutive sections of: (A) proximal blastema day 7; (B) proximal blastema day 8; (C) distal blastema day 7; (D) distal blastema day 8; 60–70 mm axolotls.

tissue to be replaced (the more proximal the amputation plane) the larger the cell mass in which that pattern first redifferentiates.

From the slope of the curve in Fig. 2 on any one day, the following estimates of the population doubling time (Td) may be obtained. On day 4: Td = 1 day; d.5 = 1·25 d; d.6 = 1·4 d; d.7 = 1·5 d; d.8 = 2 d. The trend is clearly to a gradual increase in Td with increasing time (the curve flattens off), suggesting that more and more cells are being withdrawn from the cell cycle as redifferentiation progresses.

One other point is worth noting with regard to blastemal growth. Mitotic maps were prepared with a view to locating any area within the blastema which was associated with a higher rate of cell division. None was found, with the possible exception of the whole post-axial side which tended to have a greater cell density (cf. Singer, Ray & Peadon, 1964). Examples of these mitotic maps for both proximal and distal blastemas on two consecutive days are drawn in

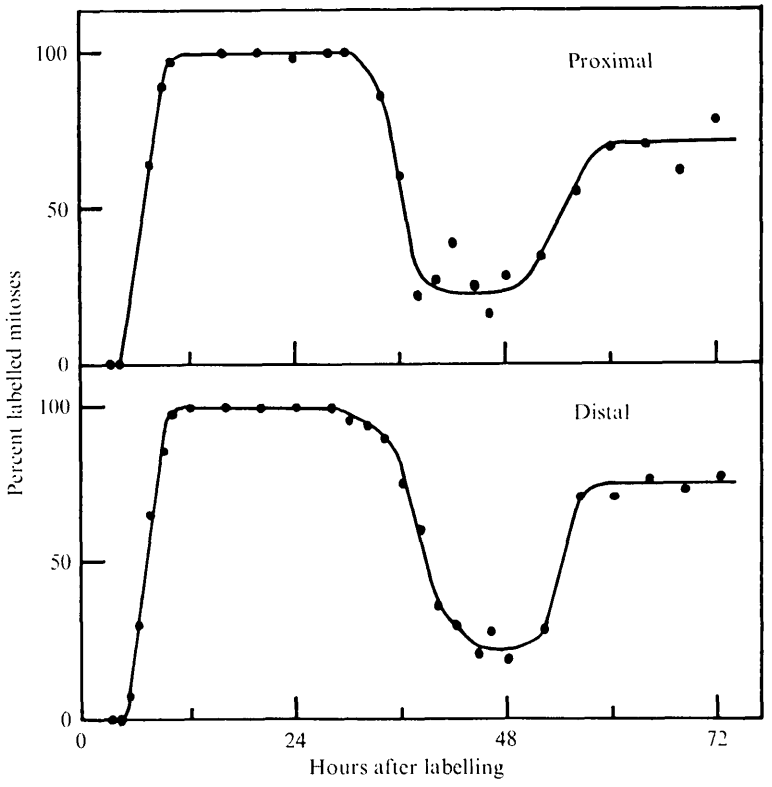

Fig. 4. Labelled mitoses curves of proximal and distal blastemas of 60–70 mm axolotls between days 6 and 9 post-amputation. These typical Quastler–Sherman plots give the same estimates of cell-cycle times – Table 2.

Fig. 3. Autoradiographic studies of DNA synthesis have likewise failed to support the idea of a distinct growth zone (Hay, 1966).

Cell-cycle times. These studies were performed on larger axolotls, 60–70 mm, so that more tissue would be available. A histological analysis was performed concurrently on this size of animal so that valid comparisons could be made. Some of these results have been referred to above. In these animals a small blastema was present by day 6 and redifferentiation began between days 8 and 9; consequently it was over this period that the cell-cycle determinations were made when all the cells would be expected to be dividing.

The percent labelled mitoses curves for both proximal and distal blastemal cells are typical Quastler–Sherman plots (Fig. 4) and from the 50 % intercepts, estimates of the average phase durations may be obtained (Table 2). It is immediately apparent that there is no difference in cell-cycle time (Tc) of proximal and distal blastemal cells, both being 48 h. Variations in the individual phases of the cell cycle, for instance the 2 h difference in S, are probably insignificant. The population doubling time over this period, obtained from the concurrent histological study by counting cell numbers as in Fig. 2, was

Table 2. *Duration of cell-cycle phases, in hours, of proximal and distal blastemas*

Blastema	G_1	S	G_2	M	Total cycle
Proximal	12·5	28	6·5	1	48
Distal	11	30	6	1	48

initially just over 1 day, then increased to 2 days between days 8 and 9. This implies that at first, cells were still entering the blastema from the continued dedifferentiation of stump tissues (Td < Tc), but by about day 8 this influx had ceased and the blastema then behaved as an isolated system in which all the cells were cycling (Td = Tc). This is an important confirmation of the earlier conclusion that the blastema, once established, is a homogeneous entity in which all the cells behave identically. The subsequent increase in Td implies that cells were being withdrawn from the cell cycle (Td > Tc).

An earlier estimate of the cell-cycle time of distal blastemas from slightly older axolotls resulted in values for G_1, S, G_2 and Tc of 9·5, 38, 4·5 and 53 h respectively (Wallace & Maden, 1976). The difference in Tc between that estimate and those in Table 2 is probably due to the fact that in the work reported here, the second peak of labelled mitoses did not reach 100 % as it did before. Perhaps lightly labelled cells scored during the first peak dropped below background value after one division or there was an influx of unlabelled, newly dedifferentiated cells during the sampling period (see above). The difference in S could be due to the greater chase concentration used here since the bigger the chase (up to the point where DNA synthesis is inhibited), the more precise is the Ts-Tl estimate. Indeed, the steepness of the drop in the first peak increases with increasing chase concentration (cf. Fig. 1 of Wallace & Maden with Fig. 4 here). It is therefore likely that the value for the length of S obtained here is more accurate.

DISCUSSION

Two important conclusions can be drawn from this study of the cellular kinetics of regeneration. Firstly, the growth characterics, that is volume, cell number and cell-cycle time of proximal and distal blastemas were identical. Therefore the explanation of the uniqueness of these regenerates lies not in these parameters. The information provided by the stump to the blastema in terms of growth rate is the same all along the limb.

Thus Faber was perhaps unjustified in stating that distal blastemas were smaller than those on more proximal levels unless he was comparing corresponding stages rather than corresponding times. For example at the 4-digit stage, proximal regenerates were twice the length of distal ones, but then

more cartilage elements had been replaced from proximal levels and they had taken 2 days longer. In both types of regenerate prior to the onset of redifferentiation, all the mesenchymal cells were in the cell cycle and the blastema may thus be considered a homogeneous entity. Mitotic maps, which recorded the positions of mitoses within the blastemas, confirmed this conclusion and denied the existence of a localized area of higher cell division rate such as an apical proliferation centre (Faber, 1965). Similarly, the concept of a progress zone of chick limb dimensions within the blastema (Smith *et al.* 1974; Wolpert, Lewis & Summerbell, 1975) is not supported by this demonstration of homogeneity; alternatively we might consider the whole blastema to be a progress zone.

The second conclusion that can be drawn from these results concerns the difference which was found between the two types of regenerate in the time of onset of, and hence cell number at the first redifferentiation of cartilage – proximal blastemas had a greater number of cells within when redifferentiation commenced. From this we may state that the larger the pattern that will eventually be manifest (the more proximal the amputation plane), the larger the primordium in which that pattern first appears.

Since this work concerns the kinetics of the development of the blastema it is important to consider whether contemporary theories of blastemal organization are compatible with these observations and conclusions. There seem to be four classes of theory, namely stump induction, proximal-distal, distal-proximal and non-sequential organization. Current opinion does not favour mechanisms based solely on stump inductions even though the phenomenon may occur both in cartilage (Goss, 1961) and muscle (Pietsch, 1962), because when grafted to non-limb sites undifferentiated blastemas can develop normally in the absence of the stump (Stocum, 1968). If this type of induction was responsible for blastemal organization it is difficult to see how the humerus could induce differentiation at a later stage than the radius and ulna (Table 1). Similarly, it is unlikely that the stump could dictate what structures were to be regenerated simply by controlling the size of the blastema (Faber, 1971) when proximal and distal blastemas are, at any one time, the same size.

A model for the development of the chick wing (Summerbell *et al.* 1973) has been used to explain the mechanics of regeneration (Smith *et al.* 1974) and is a proximal-distal type of theory. Briefly, the model assumes that the cells of the early blastema all have positional values corresponding to the level of amputation and as these proliferate further within a progress zone, their positional values become steadily more distal. It has already been stated that the existence of a separate zone which has 'a high proliferative rate' (Wolpert *et al.* 1975) has not been confirmed. However, it is predicted that the more proximal the positional value of the blastemal cells, the longer it will take them to attain their most distal value. The results described here clearly conformed to this in that any stage of digit formation in proximal regenerates

was consistently 2 days behind that of distal regenerates (cf. Spallanzani, 1769; Iten & Bryant, 1973). Furthermore, based only on measurements of regenerate length against time, Smith *et al.* (1974) reported that the 'intrinsic growth rate' of blastemas from the wrist, elbow and upper arm of adult newts was the same. If cell-cycle time can be considered as a measure of this parameter then their conclusion is confirmed as Tc was 48 h for both upper arm and lower arm regenerates.

Although the general features of this theory are compatible with the present observations, there are serious discrepancies in the detailed mechanisms. It has been suggested that positional values in the limb are assigned by a timing mechanism and that cell divisions are ticks of the clock, since it takes seven cell division cycles to lay down the seven segments of the chick wing. During regeneration from the upper arm it took four division cycles to produce 6 segments and from the lower arm it took three division cycles to produce 5 segments; the predicted cell-cycle time would be 30 h, the actual time 48 h. Furthermore, the rate of regeneration of young axolotls is twice as fast as that of older ones and it is unlikely that the cell-cycle time of older blastemal cells would be double that of younger ones. This sort of model, therefore, does not seem to be compatible with the present observations.

The third type of theory of blastemal organization comes from the demonstration by DeBoth (1970) of a direct relation between the amount of mesenchyme and the character of the pattern into which it redifferentiates. This observation was arrived at by grafting several blastemas together to a heterotopic site and he interpreted this to mean that the sequence of determination within the blastema occurs in a distal-proximal direction. These results have been the subject of some controversy in that they contravene the law of distal transformation (Stocum, 1975; Wolpert *et al.* 1975). Nevertheless a similar size effect was observed here – the more proximal the amputation plane the larger the blastema at the onset of redifferentiation. Faber (1976) has proposed a specific mechanism for this phenomenon based on the concept of a gradient of fixed slope. Thus the greater amount of tissue available the more of the gradient can be fitted in. Central to this proposal, however, is the assumption that the limb-bud and blastema are not size-independent. On-going work in my laboratory has shown that this is not the case, smaller and larger regenerates can be produced after suitable manipulations.

I wish to propose an alternative model for this size-effect of blastemas, although it must be admitted that DeBoth's results cannot be incorporated into this scheme. Stocum (1975) and Faber (1976) have presented what might be called non-sequential specification theories, the importance of which lies in the idea that the whole limb pattern is present at the earliest stages of blastema formation, albeit in latent form, with the proximal boundary value being represented by the amputation level (a level-specific property of the stump cells) and the apical epidermis re-establishing the distal boundary value. One

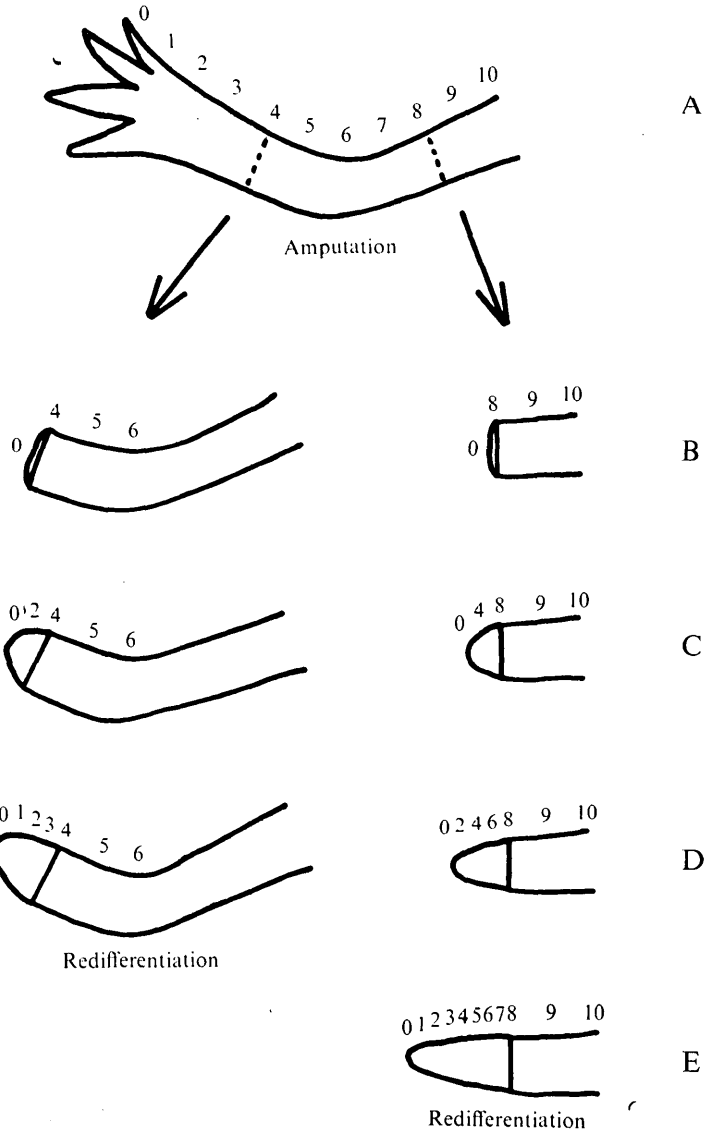

Fig. 5. (A) Hypothetical positional values of the normal limb. The distance between adjacent values has increased as the limb has grown from its embryonic size when the values were first established; no new numbers have been added. (B) Following amputation the epidermis migrates over the wound and re-establishes the distal boundary value 0. (C) The first cells to enter the blastema take up a positional value half-way between the two boundaries. (D) After a round of cell division from the two rows of cells in C, these new cells take up a positional value, once again, half-way between their proximal and distal neighbours. In this manner all the values have now been restored to the distal blastema which can therefore go ahead and redifferentiate. The proximal blastema has not yet regenerated all its values and must carry on growing. (E) After further growth, obeying the same rules, the positional values have now been restored to the proximal blastema and this can now redifferentiate. Since the rate of growth of the two types of blastema is the same one would expect the rate of replacement of positional values to be the same. Thus this model is consistent with all the observations reported here.

mechanism for intercalating the missing values between these two extremes, using a gradient of fixed slope, has been criticized above. Another is for the newly dedifferentiating blastemal cells to follow a simple averaging rule as follows. Let us label the limb from, say, 0–10 (Fig. 5A), where each number represents a positional value the width of which was fixed when the limb first developed (where width = distance between two adjacent values) and since then the values have simply grown apart rather than been added to. Upon amputation the epidermis re-establishes 0 and the proposal is that blastemal cells intercalate the missing values by taking up positional values half way between their proximal and distal neighbours (Fig. 5B–E). If the whole pattern has to be established before redifferentiation can commence then we can see how the size effect and time effect could come about since there are more values to be replaced from a proximal amputation plane than a distal one. In this scheme the organization of the blastema is strictly non-sequential and hence every intermediate growth stage has the potential to autonomously produce a complete regenerate. If the regenerated positional values are re-established in their embryonic configuration, that is with the minimum distance between two adjacent numbers, then the reason why the regenerate is so small at the earliest 4-digit stage is also apparent. Their gradual separation would constitute the phase 3 growth (of Iten & Bryant, 1973) during which the size of the regenerate (and the width of each positional value) gradually approaches that of the amputated portion, a process which can take anything up to a year in adult axolotls.

This scheme will be dealt with in greater detail in a subsequent publication; suffice it to say here that it is readily compatible with the observed behaviour of blastemas and results in a considerable simplification since additional postulates such as wound-hormones or the distalizing influence of the epidermis are not needed. The averaging law described above was not formulated with limb regeneration in mind, but has been borrowed from ideas currently being developed in this group to understand the general principles of pattern formation (Cummings, Goodwin & Prothero, in preparation). In this work such diverse phenomena as regeneration in hydroids, phyllotaxis in plants, feather patterns, somitogenesis and regeneration in insects are analysed in terms of a very elementary set of rules of which the averaging law is fundamental. Thus the further attractive feature of the scheme described above is that the mechanisms which might operate in amphibian limb regeneration can also be used to describe the development of pattern in a range of other systems.

I should like to express my sincere thanks to Dr J. Prothero, Dr B. Goodwin and Dr F. Cummings for many helpful discussions on the application of their ideas to amphibian limb regeneration. My thanks also to Dr H. Wallace for many gifts of axolotls and a critical reading of the manuscript, to Mrs Wendy Neilson for invaluable technical assistance and to the M.R.C. for financial support.

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