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Evidence for regulation of growth, size and pattern in the developing chick limb bud

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SUMMARY

This paper examines the hypothesis that the developing chick limb bud has mechanisms for regulating the control of growth, size and pattern. The tests included: surgical removal of selected parts of the limb field, X-irradiation, temperature shock and the manipulation of known limb organizer regions (removal of the apical ectodermal ridge, or the addition of an extra zone of polarizing activity). The results strongly support the idea that there are regulatory mechanisms controlling both the pattern and the size of the limb and suggest that they involve regulation of the growth rate via control of cell division throughout the embryonic period. Possible mechanisms are discussed.

INTRODUCTION

In this communication I discuss the concepts of regulation and of regeneration, the differences between growth, size and pattern regulation, the evidence for each in the chick limb bud, and finally examine possible solutions to the problem of size regulation. The discussion is based on the concept of the limb bud as an embryonic field, explicitly as defined by Waddington (1956).

Regulative or mosaic?

Almost by convention the concept of regulation is now introduced by a reference to Driesch (1892, 1929). The very age of the reference confers a reassuring solidity. Yet the concept is not an easy one to define and it is arguable whether our understanding of the subject has advanced since the time of Driesch. He eventually succumbed to the difficulties of the problem and traced the downward path to philosophy via theoretical biology. He reasoned that the harmonious development achieved during regulation could not be explained by simple mechanistic rules and concluded that some higher spiritual component must guide the underlying life processes, this system he called *entelechy*, a return to primitive vitalism.

The debate on 'regulative or mosaic' was opened by Roux (1888) who used a hot needle to kill one of the first two blastomeres of the frog egg. The surviving

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cell produced only a half embryo during its early development. Thus it seemed that cells formed from different areas of the egg were non-uniform and therefore produced different parts of the embryo. This type of development Roux styled 'mosaic' or 'self-differentiation'.

Roux's results were soon challenged by other workers, the most compelling evidence coming from Driesch. He found that if one *separated* blastomeres of the sea urchin, each cell went on to complete embryonic development. This type of development he called regulative.

Despite Roux's experiment now being discounted, his concept has proved remarkably robust. Its meaning has widened beyond his original definition but it remains a sound empirical description of developmental phenomena. It may be simply defined as a developmental process in which part of a system can give rise only to those parts of the system that they would normally have produced had the system not been perturbed. It has subsequently been shown to be the dominant phenomenon in many systems and one could with some justice argue the generalization that all systems develop towards the mosaic state (Weiss, 1939). This generalization is seen at its most extreme in relation to cell lineage, where the division products of each cell may perform a rigidly determined programme of development (see Wilson, 1898); and the concept has been analysed recently in great depth for the nematode worm, *Caenorhabditis* (see for example Kimble, 1981).

Apart from any philosophic or mechanistic problems the concept of regulation, on the other hand, is operationally not straightforward. It is commonplace to find widely differing views on what it means. The careful author is therefore at pains to define his understanding of the concept before plunging into a discussion of ideas. A typical definition (paraphrased from Driesch, 1892) would be: a developmental process in which an abnormal complement of cells in an embryo (or part of an embryo) gives rise to a normal embryo (or part of an embryo).

The major problem with this is that while mosaic is still normally used in its absolute sense, regulative has come to describe all intermediate states between perfect mosaic and perfect regulator. The case of partial regulation may not agree with the original Driesch definition but one must suppose that they are likely to employ the same mechanisms.

There are two components that cooperate in regulative behaviour (clearly recognized by Driesch in his phrase 'harmonious equipotential system'). One concerns the intrinsic ability of the cell, the other concerns the ability of groups of cells to interact so as to modulate the intrinsic ability of the individual.

The intrinsic ability Driesch called the 'prospective potency'. In a regulative system the prospective potency of a cell is wide. Indeed, if the single cell is to produce a normal embryo, it must be all encompassing or totipotent. During development this intrinsic ability is progressively restricted so that the cell's possible mode of development is reduced. (The prospective potency is not to

be confused with the 'prospective significance', today normally called the presumptive fate.) In regulative systems fate is a trivial concept. It indicates only what a cell will do during normal development. It says nothing about the cell's state or about its intrinsic abilities. A cell does not know its fate (see Summerbell, 1976 for a discussion relevant to the development of the limb bud).

The second component, the ability of cells to interact, supposes that the field recognizes the perturbation and has mechanisms that modify the behaviour of the cells so as to compensate appropriately and harmoniously. It is an article of faith with most experimental biologists that the processes active in such compensatory cell behaviour are the same as those governing normal development.

Regeneration

Regulation and regeneration have much in common. Morgan (1901) saw no reasons to distinguish between them and no compelling counter argument has been made. He suggested that there are two general ways in which regulation/regeneration may take place. Morphallaxis involves transformation of a part of the field into a different part without proliferation at a particular surface. Epimorphosis involves the appearance of a growth zone which by division gives rise to new cells that develop into the replacement part. The subject has been extensively discussed and formalized in the French Flag Model (Wolpert, 1969, 1971).

With respect to growth control there is one immediate difference between the two modes. Epimorphosis *necessarily* involves cell division, morphallaxis need not do so. Again making extreme definitions one could insist that *perfect* regulation by *truly* epimorphic means would involve restitution of the correct pattern and of the correct cell number and size. Such a system would show perfect size control with growth intimately and necessarily linked to pattern. It has been suggested that many systems approximate to this extreme (French, Bryant & Bryant, 1976; Iten & Murphy, 1980; Javois & Iten, 1981) a positional discontinuity in the field stimulates cell division locally and the new cells adopt positional values so as to regenerate those that are missing (intercalation). However, whenever the hypothesis has been tested it seems that the change in positional value takes place either without cell division (Buliere, 1972); without a localized growth zone (Cooke & Summerbell, 1980; Honig, 1981; Summerbell, 1981*a*); or without the amount of cell division correlating with the size of the positional discrepancy (Maden, 1981, this volume).

Conversely, morphallaxis involves restitution of a normal pattern without cell division; the morphology is perfect but the overall size is wrong. Examples of this may be easier to find. There is the original Driesch (1892) experiment where separated 2-cell-stage blastomeres produce normal active blastulae 'of half size'. Similarly Cooke (1981) has demonstrated that experimentally produced small tadpoles show no extra cell division and regulate their proportions to maintain size-invariant pattern. Examples such as these *may* be representative

of extreme morphallaxis. However, other systems long quoted as examples of morphallaxis fail to demonstrate size invariance, e.g. *Hydra* (Bode & Bode, 1980; Wolpert, Hornbruch & Clark, 1974).

Only recently has interest been shown in intermediate cases (Maden, 1981, this volume; Summerbell, 1981 *a*) but it should be remembered that both Morgan and Wolpert were careful to state that they envisaged these two modes as extremes of a continuum.

Growth, size and pattern

When one examines a normal embryo it is clear that size is very accurately controlled. Perhaps the best example is the phenomenon of bilateral symmetry. When one compares the left and right wings of chick embryos at the end of the morphogenetic or embryonic period (day 10), then the lengths of left and right skeletal elements from the same embryo will equate very precisely (within $\pm 2\%$ in 67% of normal embryos, within $\pm 3\%$ in 95·7% of normal embryos, Summerbell & Wolpert, 1973).

This argues that during normal development either initial specification of the pattern is very accurate (Summerbell & Wolpert, 1973) and subsequent growth programmed and determinative (Lewis & Wolpert, 1976; Summerbell, 1976) or that mechanisms exist at later times to regulate or control the size of the field. The sea urchin must employ the former mechanism (at least up until blastula stage) since the cells continue to divide at the rate appropriate to the normal embryo even though it is only half size, i.e. there is regulation of pattern but not of growth or size.

I have already mentioned one extreme method of regulating size, the polar coordinate model (French *et al.* 1976), but it seems that it is not necessary to make the production of the pattern totally dependent on having a full complement of cells. It should be possible in principle to dissociate to some extent the regulation of growth, size and pattern. Such a development should not be surprising for there is ample evidence at fetal and adult stages for various mechanisms of growth control. Research in this problem has dealt almost exclusively with density-dependent control of cell division or with a search for various growth factors, humoral agents exerting either a positive or negative feedback on cell division (Summerbell & Wolpert, 1972; Stoker, 1978; Smith, 1981, this volume), while the phenomenon of catch-up growth is well documented at later stages (Williams, 1981, this volume).

However research on the embryonic period *in vivo* is scanty. Lawrence (1972) and Wolpert (1969) have both made suggestions for mechanisms controlling absolute size. More recently I have suggested a mechanism for growth control in chick limb development. Snow & Tam (1979) and Tam (1981, this volume) have direct evidence for compensatory growth control in mouse embryos.

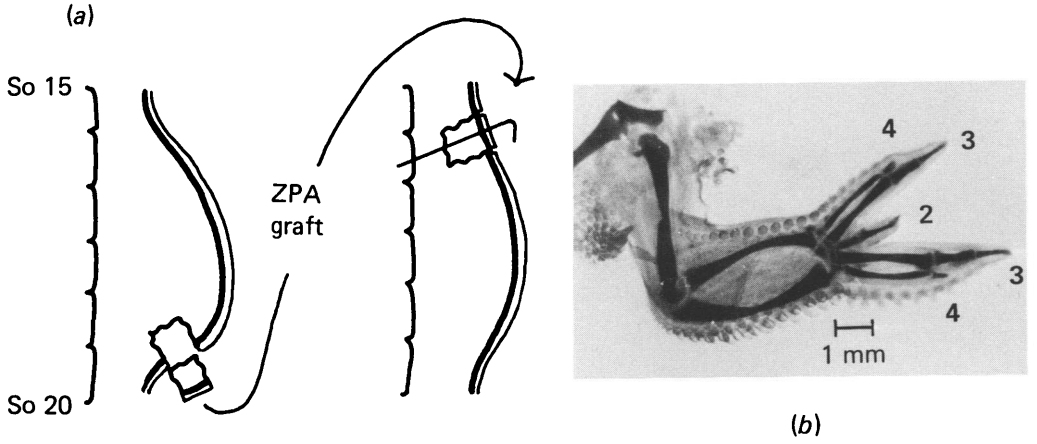

Fig. 1. The result of grafting the posterior limb organizer region (ZPA) to an anterior site on a host limb. (*a*) The operation. (*b*) Alcian-green-stained whole mount of resulting limb at day 10. The hand contains the digits **4 3 2 3 4**. In this example the 'normal' posterior digit is ~98 % the contralateral control length, and the anterior supernumerary digit is ~85 % the contralateral control length.

EVIDENCE FOR GROWTH CONTROL

This paper is based on data that has already been presented in a number of my publications, but the analysis shown in the figures is new. In some experiments I have added additional cases to those described in the original paper. Methods are not described in detail as they are already published. I include more detailed information where appropriate in the text and in the figure legends.

The zone of polarizing activity

The best direct evidence for growth control in the chick limb bud comes from experiments in which a polarizing region (ZPA) is grafted to the anterior margin of a host limb (Saunders & Gasseling, 1968). Such a graft alters the pattern so that it forms a mirror image reduplication of the host limb field causing the formation of a supernumerary limb of contralateral polarity (Fig. 1). The most immediate effect is an alteration in the growth rate, and this has now been documented at several levels.

The earliest indication (Cooke & Summerbell, 1980) is within 4–5 h after making the graft. Incubation of the embryo with [^{3}H]thymidine for 1 h shows a significant enhancement of the labelling index in the responding tissue adjacent to the graft. This is followed somewhere between 9–17 h by a significant increase in the mitotic index (number of nuclei in mitosis per 100 nuclei). The graft appears to have shortened the cell cycle time in responding tissue so that the tissue is growing very much faster.

I have examined growth of a defined limb field under the influence of the ZPA signal by grafting two polarizing regions to a host limb, one opposite somites 16/17 and the other at a measured distance away posteriorly (Summerbell, 1981*a*). By measuring the distance between the grafts at successive times

it is possible to estimate the rate of elongation of the anteroposterior axis of the developing mirror image supernumerary pair of limbs. The width starts to increase about 6 h after operating: the intrinsic rate of growth or widening (the rate of change of length per unit length per unit time) rapidly rises to a maximum about 12–16 h after operating and thereafter declines back to a level similar to the base rate in unoperated limbs. The data on growth fits well with data on cell cycle with respect to both growth rates and timing. Smith & Wolpert (1981) using a less sensitive measure of change of width confirm these results and also show that there is no particular sensitive stage. The limb responds similarly to the graft through stages 18–21 (about 24 h).

One might question the extent to which this is an organized and controlled response to a potential change in pattern rather than a non-specific enhancement of cell division by a mitogen-like substance coincidentally present in the graft. There are three separate lines of evidence that argue in favour of specific and controlled enhancement of growth.

(1) The number of digits formed between the two ZPA grafts can be predicted very accurately by the initial distance between the grafts. Using the sample in Summerbell (1981*a*), the prediction would be within ± 1 digit 95 % of the time, or $\pm \frac{1}{2}$ digit 67 % of the time. Much of this error probably lies in estimating the distances involved. The subsequent rate of change of length per unit length for the anteroposterior axis is the same for all initial distances between grafts (except for those $< 200\ \mu$m) so subsequent measurements of the distance between grafts gives an equally accurate estimate of the number of digits that will develop. Initial size, growth and final pattern are closely correlated.

(2) The length of the principal axis of the proximodistal dimension remains the same as on the contralateral control limb (data from Smith & Wolpert, 1981). The change of growth rate does not cause an indiscriminate increase in the length of this dimension.

(3) The proximodistal lengths of the extra skeletal elements produced approach, but never exceed the lengths of the equivalent elements on the contralateral control side (Summerbell, 1974*a*). The earlier the stage at which the ZPA graft is made then the more closely the lengths of the reduplicated elements approach the control side (Fig. 2). This again suggests that the enhanced cell division is not uncontrolled but tends to be at a rate that will produce skeletal elements of the correct length for the host cells from which they were derived. It also suggests that the mechanism is progressive requiring time. The gradual loss of accuracy at later stages is most compatible with systems that involve negative feedback between the controlling system and the size of the field.

The apical ectodermal ridge

There is a thickened ridge of ectoderm running along the distal rim of the developing limb bud called the apical ectodermal ridge (AER). The development of the correct pattern of tissue along the proximodistal axis seems to be

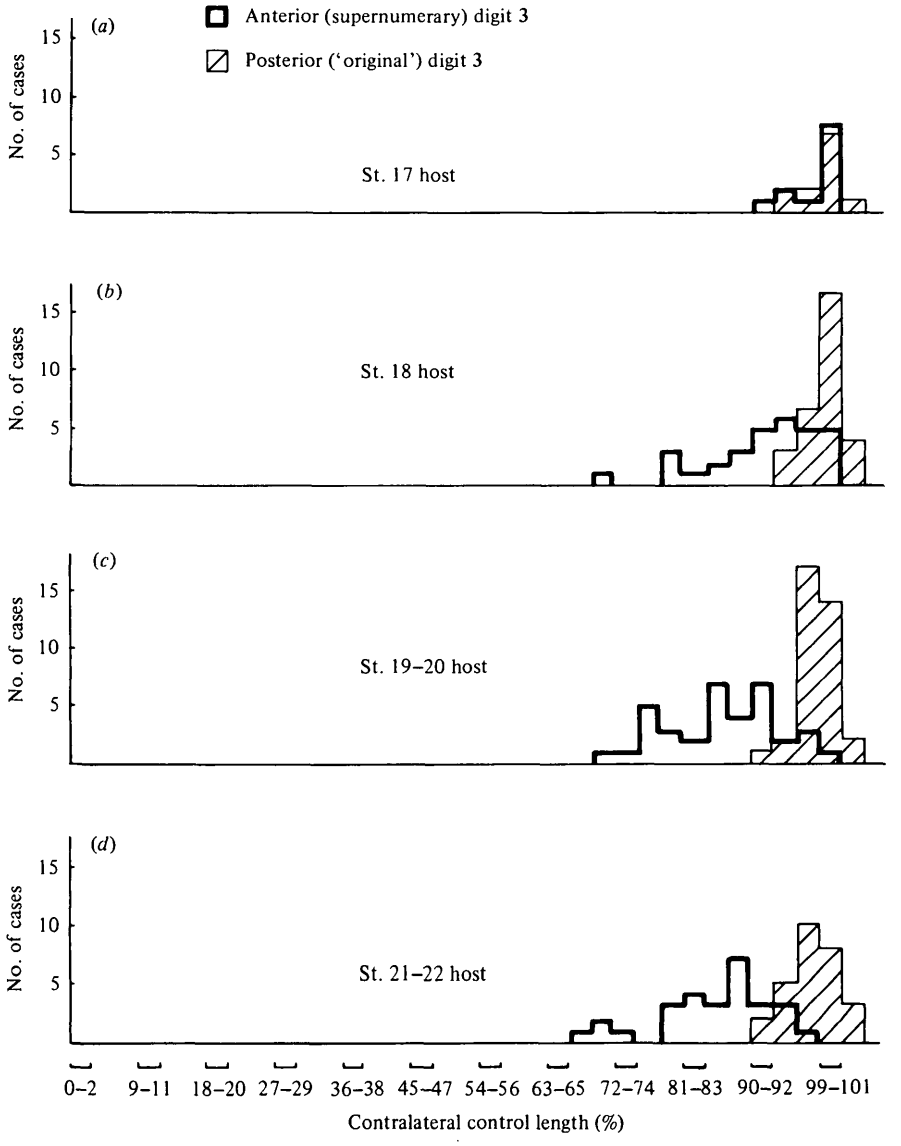

Fig. 2. Frequency distribution showing the number of cases that were measured as being the given percentage shorter than the contralateral control limb. The percentages have been grouped in blocks of 3 %. Each distribution shows both the anterior 'supernumerary' digit 3 and the posterior 'normal' digit 3, each compared with the same contralateral control. (*a*) St. 17 host: anterior digit 3 ($\bar{x}$ = 99, s = 2·4); posterior digit 3 ($\bar{x}$ = 99, s = 2·6). n = 11, or which 9 were published in Summerbell (1974*a*). (*b*) St. 18 host: anterior digit 3 ($\bar{x}$ = 91, s = 7·5); posterior digit 3 ($\bar{x}$ = 99, s = 2·5). n = 30, of which 19 were published in Summerbell (1974*a*). (*c*) St. 19–20 host: anterior digit 3 ($\bar{x}$ = 86, s = 7·5); posterior digit 3 ($\bar{x}$ = 98, s = 2·3. n = 36, of which 10 were published in Summerbell (1974*a*). (*d*) St. 21–22 host: anterior digit 3 ($\bar{x}$ = 84, s = 8·0); posterior digit 3 ($\bar{x}$ = 98, s = 3·3). n = 28, none of which were published in Summerbell (1974*a*).

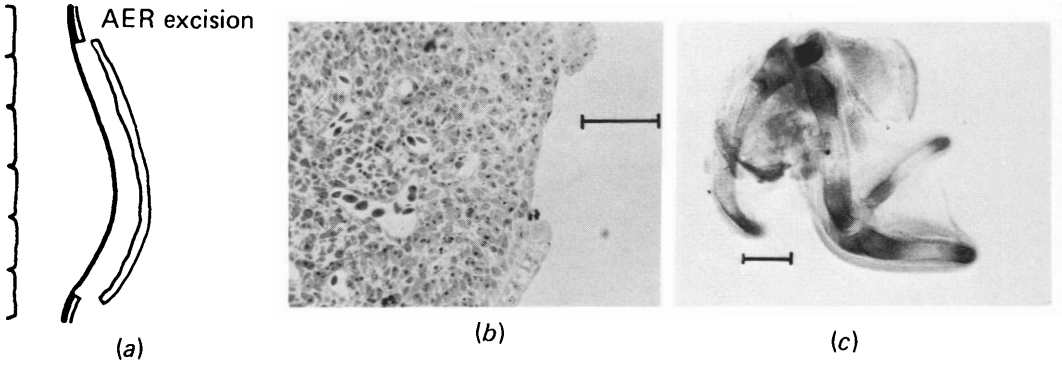

Fig. 3. Apical ectodermal ridge excision. (*a*) The operation, seen from dorsal surface. (*b*) Section of limb containing dorsoventral and anteriorposterior axes, showing missing section of ectoderm and apical ridge. This illustration is taken from Summerbell (1973). (*c*) Alcian-green-stained whole mount showing resulting limb at day 10. This illustration is taken from Summerbell (1973).

dependent on the continuing presence of the AER. If the AER is removed then the distal part of the limb will fail to develop (Saunders, 1948; Summerbell, 1974*b*; Fig. 3). The level of truncation is determined by the stage at which the operation is performed, older stages producing progressively smaller deficiencies. The cells of the AER itself do not form any durable structure and it has the general characteristics of an organizer region as defined by Spemann (1938). The only published rigorous explanation of its mode of action remains the 'progress zone model' (Summerbell, Lewis & Wolpert, 1973). Alternatively, Faber (1971) and Stocum (1975) have both suggested that it should be possible to consider the AER as the source of a morphogen, similar to that postulated for the ZPA and, by changing some of the parameters in the simulation of Summerbell (1979), it is possible to match most of the data, including the observation of truncation following AER removal. However, the loss of distal pattern does not account simply for the observed change in the rate of outgrowth of the bud. Without further assumptions neither model predicts any immediate effect on cell division or on the overall rate at which the limb grows. Furthermore, even if some adjustment is made so as to slow the overall rate of growth, both still suggest that the last programmed set of positional values will be specified over an abnormally large number of cells. Without the addition of some subsequent mechanism for growth control this would mean that the terminal element would be too big.

In practice removal of the AER causes a sharp increase in cell death and in cell cycle time (Janners & Searls, 1971; Summerbell, 1977*a*). Some cells are lost and the rate of proliferation is reduced. This appears to be a transitory effect of wounding and within 24 h the limb has resumed a programme of cell division close to normal levels, that, if extrapolated, would lead to a limb very much larger than would be appropriate for those parts of the pattern that will

be present. However over the next few days there is modulation of the rate of outgrowth and the reduction is such that the total length of the operated limb is exactly equivalent to the length of the same parts on the contralateral control side (Summerbell, 1977*a*). This gradual compensatory mechanism is very similar to the growth control observed in mouse embryos after treatment with mitomycin (Snow & Tam, 1979; Tam, 1981, this volume).

On examination, the terminal skeletal element may be found to be truncated at any point along its length. In those cases in which the element is just complete (it possesses an anatomically normal distal epiphysis but there is no indication of the next most distal element) then the length of the terminal element is within the normal limits of variation when compared to the contralateral control limb (Summerbell, 1974*b*).

This size equivalence, despite enormous perturbation and oscillations in the overall rates of growth and cell division in the operated limb can hardly be the result of pure chance. The data strongly argue the existence of mechanisms controlling at least the size and probably the growth rate.

Regulation of proximodistal deletions

The subject of regulation in the chick limb bud has been an area of high controversy for some time. Though the argument has normally been expressed in rigid rules (see for example, Wolpert, Lewis & Summerbell, 1975, with accompanying discussion by Sengel), the problems are more of detail. Embryology is not an exact science and survives on the generalization. A generalization, that I like, is: 'avian embryonic limb bud tissues are malleable up to a certain stage of development and can give rise to structures other than those they form in normal conditions. In other words, limb buds manifest regulative capacities' (Kieny, 1977). One can extend this generalization by saying that the younger the embryo, the more distal the position; and the smaller the amount of tissue removed, the better the regulation (Hornbruch, 1981; Summerbell, 1977*b*). When large amounts of tissue are removed from proximal levels at late stages the chick embryo limb bud behaves as a mosaic (Fig. 4*b*). There is a discrepancy in the pattern and its magnitude is linearly related to the proportion of the proximodistal axis that has been removed. Those parts of the pattern that are present are of normal size when compared to the contralateral control limb. The total length of the limb is determined by the proportion of the pattern that is present.

In contrast, when smaller amounts of tissue are removed from more distal levels or from younger embryos, then the anatomy of the skeleton at day 10 can often be normal. All of the skeletal elements are present (including the carpals) and characteristic knobs, bumps and joints are all present. There is regulation of the initial deficiency to produce a normal pattern (Fig. 4*a*). The mechanism organizing the regulation is not yet clear. The regenerated parts will include the progeny of both proximal (stump) cells and distal (tip) cells

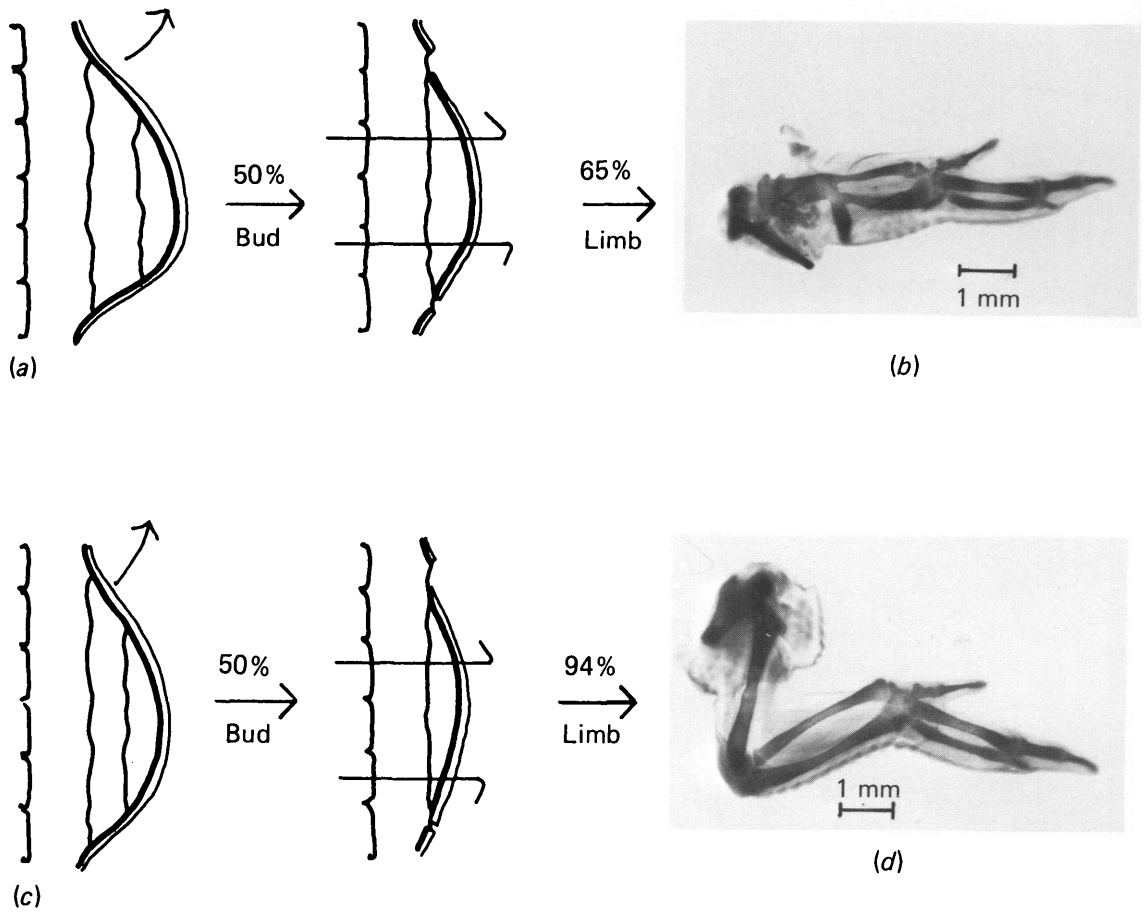

Fig. 4. Proximodistal deletion. (*a*) Removal of 50 % of the proximodistal axis of a stage-20 limb. (*b*) Alcian-green-stained whole mount of resulting limb at day 10. Result is typical of 'non-pattern regulating' cases with 65 % of the long axis of skeleton present. This limb was selected as an illustration from data first presented in Summerbell (1977*b*). (*c*) Removal of 50 % of the proximodistal axis of a stage-19 limb. (*d*) Alcian-green-stained whole mount of resulting limb at day 10. Result is typical of 'pattern regulating' cases at early stages with 94 % of the long axis of the skeleton present. This limb was selected as an illustration from data first presented in Summerbell (1977*b*).

(Kieny, 1977). Unlike the situation in amphibia or insects there is here no law of distality operating. It is possible that the regulation is purely epimorphic, but it cannot be purely morphallactic. Removal of a slice of tissue never gives a small limb with all the pattern proportionally reduced (so-called size independent). It seems most likely, extrapolating from the data on the zone of polarizing activity (ZPA, see above) that it involves something midway between the two. The regulation must involve at least some *replacement* of tissue and not only readjustment of proportions. As yet there is no direct data for changes

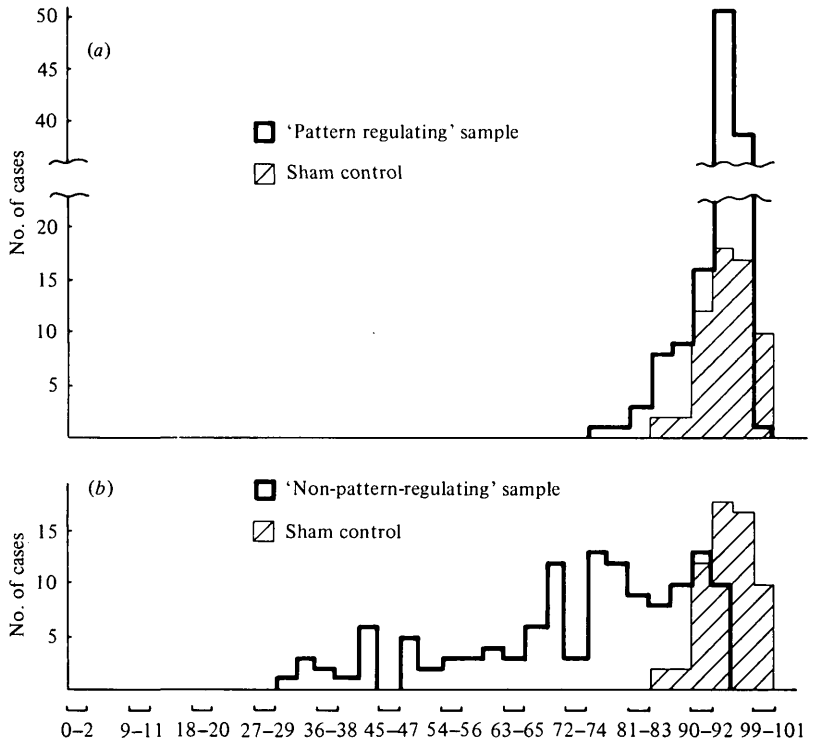

Fig. 5. Frequency distributions showing the number of cases that were measured as being given percentage shorter than the contralateral control limb. The length is the whole limb length. The percentages have been grouped in blocks of 3 %. Each distribution is compared with a set of sham control in which the tip was detached then put back without any deletion being made ($\bar{x}$ = 95, s = 3·7). (*a*) 'Pattern regulating' sample ($\bar{x}$ = 93, s = 4·3) n = 119, of which 52 were published in Summerbell (1977*b*). (*b*) 'Non-pattern' regulating sample. n = 129, of which 63 were published in Summerbell (1977*b*).

in the cell cycle time but it seems probable that the regulation will involve an increase in cell numbers. Again, despite the readjustment of positional values and changes in the intrinsic growth rate, no skeletal element significantly exceeded the length of the control side. Apart from restoration of the normal pattern it seems that the limb bud, despite the removal of a large proportion of the tissue present, was able to regulate the overall size of the limb fairly well. Figure 5 shows frequency distributions illustrating this regulation. The distribution shows the number of cases having a particular percentage difference from the contralateral control limb. This is compared with the results where the pattern was not regulated, and the results of a sham control.

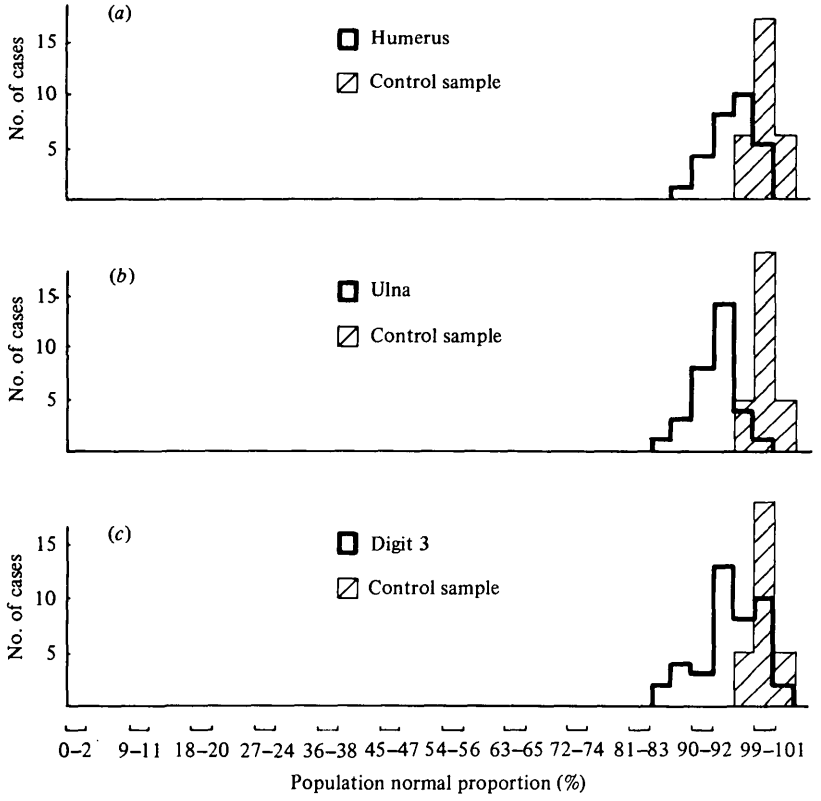

Fig. 6. Frequency distributions showing the number of cases that were measured as having a skeletal element, a given percentage shorter than the normal mean proportion of total limb length. The percentages have been grouped in blocks of 3 %. Each distribution is compared with the population distribution calculated from the mean and standard deviation of a large sample of normal embryos. The distributions contain all limbs in the teratogenic dose range that were 'pattern regulating', as defined in Summerbell (1981 *b*). (*a*) humerus ($\bar{x}$ = 96, s = 3·5) n = 31, all taken from Summerbell (1981 *b*). (*b*) ulna ($\bar{x}$ = 93, s = 3·1) n = 31, all taken from Summerbell (1981 *b*). (*c*) digit 3 ($\bar{x}$ = 95, s = 4·6). n = 42, all taken from Summerbell (1981 *b*).

X-irradiation

X-irradiation of the limb causes a complicated set of anomalies that have been explained in a number of different ways (Goff, 1962; Gumpel-Pinot, 1969; Summerbell, 1981 *b*; Wolff & Kieny, 1962; Wolpert, Tickle & Sampford, 1979). I will concentrate here on anomalies caused between stages 18–28 which in appearance are very similar to those caused by removing a slice of the proximodistal axis (see preceding section). Irradiation at a particular stage causes level-specific pattern defects of the skeleton. The tissue that seems to be most sensitive is the area just proximal to the distal tip, possibly the cells just emerging from the 'progress zone', Summerbell (1973, 1981 *b*). The problem with detailed analysis of the results of X-irradiation is that the experimental defects are

normally bilateral affecting each side more or less equally. There is no contralateral control. Wolpert *et al.* (1979) avoided this difficulty by comparing their experimental limb population with a non-irradiated but otherwise comparably treated control population. While the method was adequate to demonstrate that the magnitude of the defect varied directly with the dose, it was too inaccurate to demonstrate the kind of small discrepancies that we begin to realize are associated with pattern regulation and growth control. One needs a very sensitive assay to detect this phenomenon, such as is provided by the analysis of proportions method (Summerbell, 1978). I have already shown (see above) that there is very little variation in the lengths of skeletal elements when comparing left with right wings on the same embryo. Similarly, within a wing there is accurate control of the proportions of the skeleton. In a given strain each skeletal element has a length which is within $\pm 3\%$ of that for the mean population in 67% of embryos, and within $\pm 5\%$ in 95% of embryos (Summerbell, 1978). While this is less precise than the contralateral comparison it is much more accurate than using a control sample, and certainly sufficient to demonstrate that the effects of growth control are also detectable in this experiment. When there is a visible pattern error the proportions are also severely affected (Goff, 1962; Summerbell, 1981*a*, *b*; Wolpert *et al.* 1979), but when the gross pattern appears normal there are only small, though highly significant deficiencies in size. A summary of this latter data is shown in Fig. 6. The figure is derived from examining at day 10 embryos X-irradiated at various stages. It includes data from 104 limbs, irradiated with between 10–12 Gy (1000–1200 rads.), that appeared morphologically normal. Each distribution contains the limbs from stages where X-irradiation selectively affected the appropriate skeletal element. The results will be presented in detail elsewhere. Meantime they demonstrate the now familiar phenomenon that disturbed limbs with normal patterns approach normal size but do not quite make it exactly.

There is one added complication when considering growth and size, and this is that the effects of the X-irradiation include killing cells and temporarily halting cell division throughout the embryo. This results in a slowing of the overall increase in cell number and hence of the growth. This is in part offset by a retardation in the rate of development, but nevertheless the whole embryo is smaller than normal when it eventually reaches stage 35. This suggests that the putative mechanism for growth control is not necessarily tied to time. It does not act to produce a normal-sized embryo by a particular time (at least not before day 10); it acts to harmoniously maintain the proportions of the embryo within the normal range of variability.

Temperature shock

A short and rather unproductive experiment consisted of cooling eggs rapidly to ~ 4 °C for periods of a few hours to 36 h during the period between stages 18 and 28. Development was delayed appropriately and the embryos at ten days

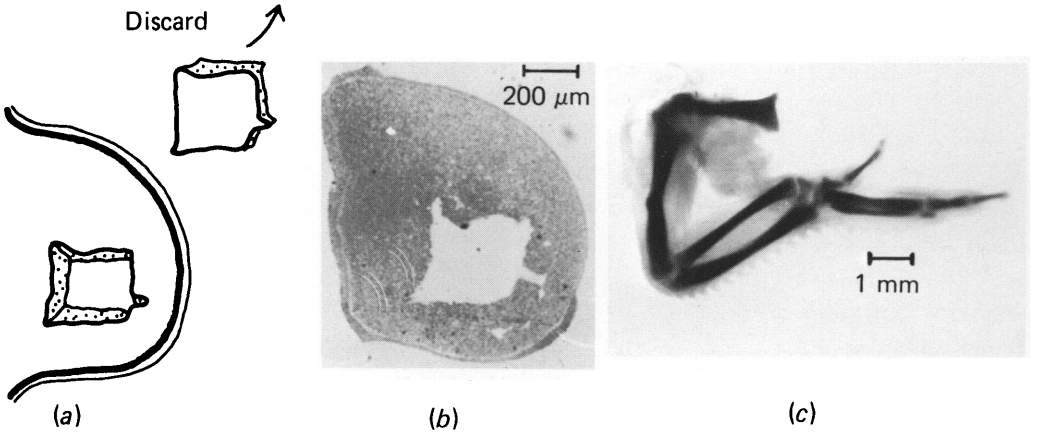

Fig. 7. Regulation of holes. A hole is cut through from dorsal to ventral surface and the tissue removed and discarded. (*a*) The operation on a stage-22 limb showing a typical position and size of hole. According to fate maps this would certainly remove the entire presumptive ulna region, and at least some of the radius, wrist and humerus. (*b*) A planar section showing an example of the profile of the hole, this illustration is taken from Summerbell (1973). (*c*) The result of a similar operation at stage 22 having normal pattern and size. This limb was selected as an illustration from data first presented in Summerbell (1973).

of incubation had limb skeletons shorter than the normal control population. However, the proportion of total wing length occupied by each skeletal element was not significantly different from the control population (*t* test on means, not significant 0·1 % level).

Regulation of holes through the dorsoventral axis

This final experiment was again designed to test the regulative abilities of the bud. In this case (Summerbell, 1973) a portion of mesenchyme was removed so as to produce a hole through the limb from dorsal to ventral surface, but without breaking the lateral or distal boundary (Fig. 7). The position of the hole was chosen so as to include predominantly areas fated to form skeleton (see fate maps of Stark & Searles, 1973; Summerbell, 1979). The size of the hole was variable, but never less than equivalent to the area of a single long bone or the whole of digit **3** as projected on to the dorsal surface as a fate map, and was often considerably larger. The total proportion of limb tissue removed averaged from approximately 30 % at stage 18 to about 10 % at stage 26. Similar experiments have been performed by Stark & Searles (1974) and by Barasa (1964) who frequently removed even larger proportions of the bud but who reported a high proportion of 'normal' wings resulting from the operation. The percentage of abnormal skeletons increased when the amount of tissue removed was increased (data from Stark & Searles), when the operations were performed on later stages (data from Stark & Searles, and Summerbell) or when it was at a more proximal level (data from Summerbell). Whenever there was a

pattern abnormality then the total length of the limb and of the affected skeletal elements was shorter than on the contralateral control side. The size deficiency in these abnormal limbs could be of any magnitude. The number of operations resulting in a limb with a pattern defect increased dramatically at stage 26 (25 %) and 27 (95 %). In this consideration of size regulation I have therefore included only those cases from stages 18 to 25 (Summerbell, 1973).

In a sample of 202 limbs I found that 10 % of the cases had a pattern defect affecting one or at most two proximodistally adjacent elements. A further 70 % had a normal pattern, but one or more skeletal elements were shorter than the contralateral control (> 2 S.D.). In most of these cases the anomaly was locally restricted (one, or at most two, proximodistally *adjacent* elements affected), but in 13 cases stylopod, zeugopod and autopod were all shorter than the contralateral control. Of this last group, 2 (both from stage 18) were candidates for size independence (morphallaxis); all skeletal elements were significantly shorter than on the contralateral control side (> 2 S.D.) but each was equally reduced so that the relative proportions occupied lay within the normal limit of variation (< 1 S.D.). The remaining 20 % had normal pattern and size (within 2 S.D. of contralateral control). Frequency distributions for a given percentage deficiency are shown in Fig. 8 for humerus, ulna and digit **3**. Each distribution contained only those cases in which the original operation (as judged by the fate maps) would have affected the particular skeletal element. Skeletal elements that should *not* have been affected have been excluded but where the hole overlapped two adjacent elements both are recorded independently.

DISCUSSION

This paper discusses a number of experiments that can be divided into three groups. The removal of some of the cells from an embryonic field (proximodistal deletions, X-irradiation, holes); the slowing of development (X-irradiation, temperature shock), and the addition or removal of a field organiser (ZPA grafts, AER excision). To compare these experiments I need make the assumption that the last group involves the modification of the field so that a different pattern will be expressed. The common feature then becomes that in each case the (modified) field has an abnormal complement of cells. The first question is whether there is *any* regulation.

An embryonic field develops so as to give a pattern and size that we recognize as lying within a certain 'normal' range. If development is perturbed, then there is either a detectable variation (size and/or pattern lies outside the normal range), or the field has regulated the defect. In assessing this regulation I have used four operational criteria: anatomical pattern, relative proportion, bilateral-size symmetry and age-related normal size and proportion. It is a fair generalization that these criteria nest (Fig. 9), each lying wholly within the boundary of the previous category.

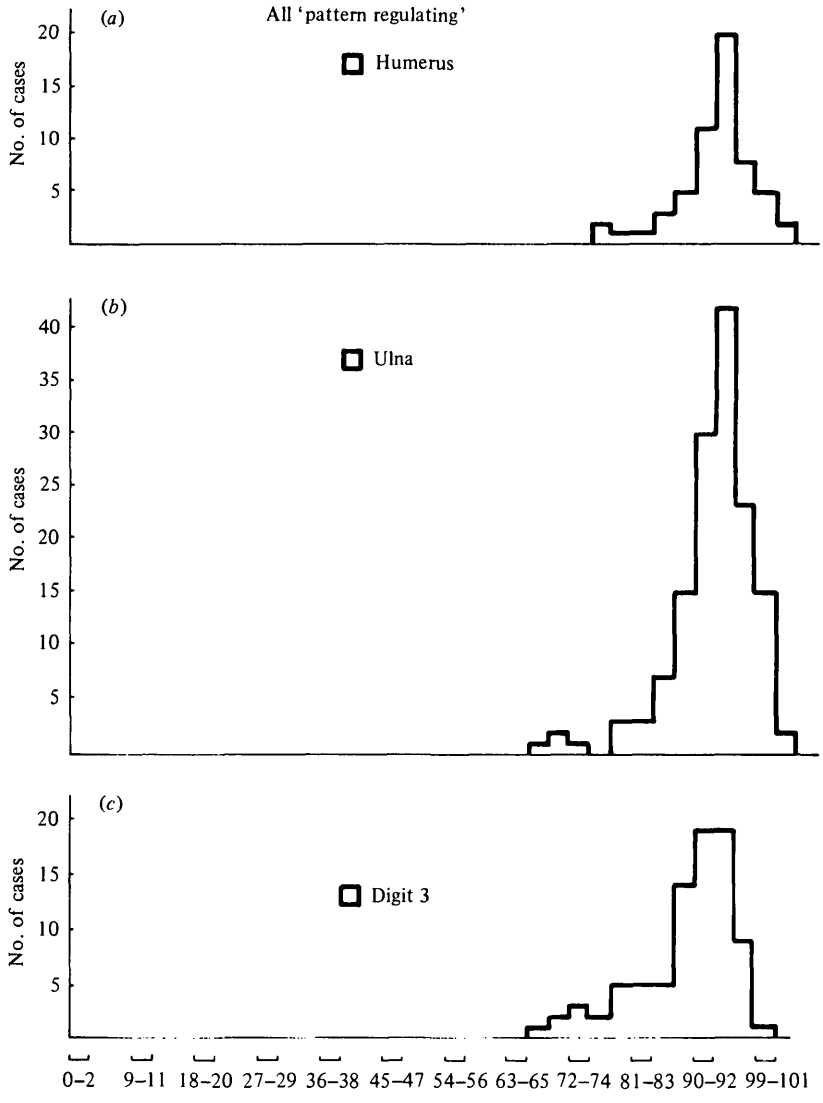

Fig. 8. Regulation of holes. Frequency distributions showing the number of cases that were measured as being given percentage shorter than the contralateral control limb. The percentages have been grouped in blocks of 3 %. Each distribution shows a particular skeletal element. The distributions contain all limbs that were pattern regulating. (*a*) humerus ($\bar{x}$ = 92·6, s = 5·7) n = 58, all taken from Summerbell (1973). (*b*) ulna ($\bar{x}$ = 91·8, s = 6·5) n = 149, all taken from Summerbell (1973). (*c*) digit 3 ($\bar{x}$ = 88·6, s = 7·2) n = 85, all taken from Summerbell (1973).

Anatomical pattern has provided the normal criterion for regulation. It is based on a more or less scrupulous examination of the structures produced from the field. It has most often considered only the skeleton though more recently other structures are beginning to be taken more into consideration (muscle, tendons; see Shellswell, 1977; Shellswell & Wolpert, 1977; feather germs; see

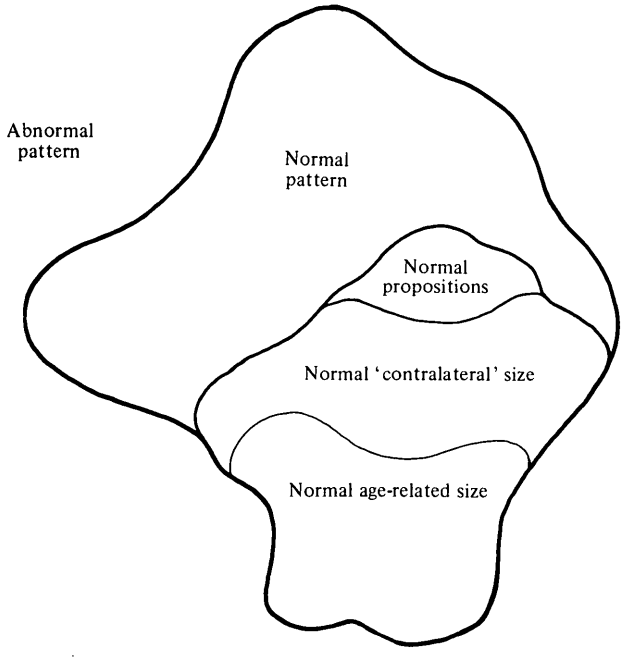

Fig. 9. Venn diagram illustrating the experiments described. The outer perimeter encloses the set of all limbs that were pattern regulating. Limbs with pattern defects lie outside this perimeter. The next lines enclose in sequence: all limbs of normal proportions, all limbs of normal bilateral size and all limbs of normal age related size. Note: (*a*) There are very few limbs with normal proportions that are not of normal bilateral size. (*b*) The sets nest, limbs of normal age related size lie inside the perimeter for normal bilateral size; limbs of normal bilateral size lie inside the perimeter for normal proportions; limbs of normal proportions lie inside the perimeter for normal pattern. Very few limbs broke this nesting rule.

McLachlan, 1980, 1981). The level of assessment is relatively arbitrary and subjective. However it seems that in the right circumstances the limb field can regulate major discrepancies, producing supernumerary limbs that involves reconstructing a large part of the skeletal pattern. This regulation is improved if occurring at early stages, or near the distal tip, or if the initial perturbation is relatively slight. If the anatomical pattern along a particular dimension is abnormal then the three size criteria will also be abnormal along the same dimension (see Fig. 9). *Pattern regulation is a prerequisite for size regulation.*

The criterion of normal relative proportions is of interest for two reasons. First, it is very useful as an adjunct to anatomical pattern markers when considering the effect of perturbations that are likely to be bilateral in their effect (X-irradiation, drugs). Second, it is the best estimate that we have for assessing the phenomenon of *size independence* (Wolpert, 1969). Size independence (the maintenance of normal pattern whatever the overall size of the field) is the main characteristic of morphallactic regeneration. If a system is regulating a

defect by truly morphallactic means then the relative proportions of the systems must be reconstituted whatever the size of the field. In the experiments in which it was possible to compare the lengths of stylopod (humerus), zeugopod (ulna) and autopod (digit 3), examples in which the proportions were regulated but in which the total length was wrong compared with the contralateral control side were extremely rare (< 1 %). The limb bud, at least along the proximodistal dimension *does not act as a morphallactic field*; the relative proportions of the pattern are not globally readjusted to compensate for a local deficiency.

The most accurate method of assaying deficiencies is to compare the perturbed limb with the unperturbed contralateral control. The rule of thumb (based on the standard deviations quote in the introduction) is that over 95 % of skeletal elements should lie with 3 % of the length of the contralateral control element (Summerbell & Wolpert, 1973). The initial length of a skeletal element at the time of differentiation is about 300 μm (Summerbell, 1976), equivalent to about 30 cell diameters. This means that whatever the details of the system, whether or not it involves size regulation, the effective length control mechanism in normal limbs is equivalent to $\pm$ 1 cell diameter ($\pm$ 3 %) at the time of differentiation. In the several experiments considered here, it is obvious that most perturbed limbs are unable to modify growth or size so as to achieve *perfect* regulation. However, in each experiment the majority of limbs, despite enormous initial deficits, regulates the abnormal complement of cells so that at the end of the period of morphogenesis the limbs are within 10 % of the normal length ($\simeq$ 3 cell diameter equivalents). Skeletal elements never exceeded the normal control length, even in the experiment (excision of AER) in which the field was manipulated to have an excess complement of cells for the pattern that actually appeared. *The case for some form of controlled regulation of size seems overwhelming.*

I have been unable to obtain evidence that time is an important factor in the control of growth or size. In those X-irradiated limbs that had normal proportions, the limbs were significantly smaller than the limbs of a control population that had been identically treated apart from the X-irradiation, but subjectively it seemed possible that the rate of development was retarded. This suggests that the *total size does not regulate to achieve a notional norm by a particular chronological time.* The cold shock experiment was fully compatible with this conclusion but one cannot exclude the possibility that an internal clock is stopped or slowed in step with the reduction in cell division and growth.

The essential argument of this paper is that cell division, growth and size are closely linked to pattern formation. It seems self-evident that the size of the limb and its component parts at the end of organogenesis will depend on the number of cells initially programmed to produce a specific structure and on the subsequent rate of growth. Direct studies on the cell cycle following AER excision (Summerbell, 1977*a*) and ZPA grafts (Cooke & Summerbell, 1980) have shown that interference with the principal organizing regions of limb field

both modify the specification of pattern and concurrently the rate of cell division. The change in the cell cycle is, to a first approximation, sufficient to explain changes in the rate of growth of the field immediately afterwards (Summerbell, 1977*a*, 1981*a*). Extrapolating from these two experiments I therefore reach the tentative conclusion that *changes in the rate of growth are driven by changes in the cell cycle.*

The obvious next question is whether the link between cell cycle and pattern is intimate. Is one observing a clear case of epimorphic regeneration in which a discontinuity in the pattern stimulates local cell division and in which the new cells adopt a new programme of development that will lead to replacement of the missing part? There is not yet direct data from any of the experiments involving the removal of cells (proximodistal deletions, holes, X-irradiation), but there is abundant evidence that in the case of ZPA grafts this cannot be the mechanism. The stimulation of cell division is not restricted to the area adjacent to the graft but is widespread throughout the limb (Cooke & Summerbell, 1980; 1981 (this volume)). Nor does the supernumerary limb develop as a blastema-like outgrowth at the discontinuity between graft and host, for it clearly involves specification of pattern across $\sim 300\ \mu m$ of the original field (Honig, 1981; Summerbell, 1981*a*; Summerbell & Honig, 1981). Thus it seems probable that *regulation in the chick wing is neither epimorphic nor morphallactic* (see also Summerbell, 1981*a*; Maden, 1981 this volume).

CONCLUSION

During normal development there are regulatory mechanisms that control both the pattern and size of the limb field. These do not involve an invariable and deterministic programme because experiments can still result in limbs of normal size and anatomical pattern. Nor is it an uncontrolled hypertrophy following intervention because the skeletal components of experimental animals are never too big. This harmonious interaction is not the result of morphallactic adjustment of the pattern so that it is harmoniously proportioned but smaller, nor is it an epimorphic intercalation of missing cells and pattern. It is possible that the mechanism involves an accurate programming of the correct number of cells for each part of the pattern prior to determination; but it seems more likely that there is compensatory regulation of the growth rate throughout the embryonic period.

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