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Reduction of the rate of outgrowth, cell density, and cell division following removal of the apical ectodermal ridge of the chick limb-bud

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SUMMARY

Removal of the apical ectodermal ridge causes a reduction in the rate of outgrowth of the wing-bud and the loss of distal parts. More specifically it causes a short-term increase in cell density and cell death and a decrease in the rate of cell proliferation. The evidence supports the hypothesis of density-dependent control of cell division and suggests that there may also be a mechanism regulating skeletal length at the time of differentiation. An informal model is presented to explain the observations.

INTRODUCTION

The early chick limb-bud comprises two main components, a central more or less homogeneous mesenchyme, and a peripheral regionally differentiated ectoderm. The main morphological feature of the latter is the apical ectodermal ridge (AER) which extends around the distal periphery of the limb-bud in the plane containing the antero-posterior and proximo-distal axes. The classical experiment of Saunders (1948) in which he removed the AER so as to produce limbs lacking distal elements remains one of the most important observations in the study of limb morphogenesis (see also Hampé, 1959; Barasa, 1960; Summerbell, 1974). This experiment together with its corollary, grafting a donor AER to a limb stump so as to produce a normal hand (to a host limb primordium) (Zwilling, 1956; Saunders & Gasseling, 1968; Hampé, 1959), amply demonstrates the crucial role played by the AER during the specification of positional value along the proximodistal axis. More recently, we (Summerbell, Lewis & Wolpert, 1973) have proposed a radically new model for limb development. We suggest that the function of the AER is to maintain the mesenchyme at the distal tip in a state of developmental lability, we call this region the 'progress zone'. Changes in positional value occur as an autonomous

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function of the cells in, and only in, the progress zone. The model explicitly accounts for the loss of distal form in the limb after the apical ridge has been removed (Summerbell, 1974), but it does not directly explain the reduction in the length of the limb. The latter phenomenon is dealt with in this paper.

The first observable change following excision of the AER is a reduction in the rate of outgrowth (Janners & Searles, 1971; Summerbell, 1974). Four factors might be involved in this phenomenon:

(1) There may be a change in the direction of outgrowth; from elongation of the proximo-distal axis to growth along the antero-posterior or dorso-ventral axis.

(2) Cells may be lost from the system by cells moving out of the limb (not a closed system), or by cell death.

(3) Cell density may change; packing the same number of cells into a smaller space.

(4) The rate of influx of cells into the system may be reduced: for example by lowering the rate of cell division.

In this study I examine each of these factors, and discuss their relative importance. The division cycle following apical ridge removal has been examined on two previous occasions but conflicting results were obtained. Janners & Searles (1971) were unable to detect any effect on the rate of cell division following extirpation of the AER, while Camosso, Jacobelli & Pappalettera (1960) claimed that cell division was somewhat reduced for a period of about 24 h. Both groups reported extensive cell death occurring a few hours after the operation.

Fortuitously the measurements of cell density and mitotic index for the above analysis may be used to test an earlier hypothesis of limb growth. We (Summerbell & Wolpert, 1972) suggested that the rate of cell division in the limb might be dependent on the cell density. We noted however that the correlation observed between these two variables was heavily weighted by the effect of cells pulling out of the mitotic cycle in lower density cartilage areas and this latter view has subsequently been supported by Ede, Flint & Teague (1975). The data in this paper are all collected before any precartilaginous or muscle areas appear and so provide a better test of the hypothesis.

The data presented in this paper are based on the use of 'thick' (1 μm) plastic sections as opposed to 5 μm wax sections in Hornbruch & Wolpert (1970) and Summerbell & Wolpert (1972). It is therefore not valid to attempt strict comparisons between the absolute values obtained in the two different experimental series.

METHODS

Fertilized White Leghorn eggs were incubated at 38 °C and windowed on the 4th day of development. Embryos were prepared at stage 19 (Hamburger & Hamilton, 1951). The apical ectodermal ridge was cut away from the

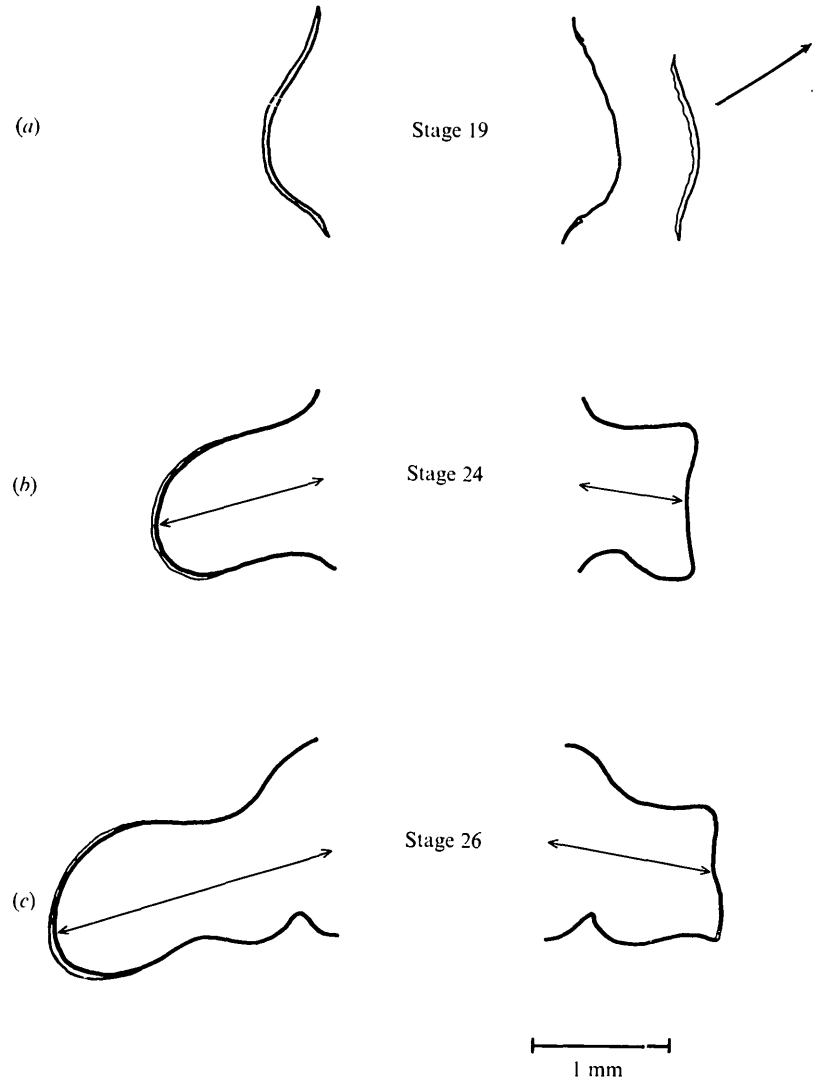

Fig. 1. The operation, its effect on the limb, and the measurement of limb length used for Fig. 3, for right (operated) and left (control) limbs. (*a*) The operation at stage 19. (*b*) The result after 24 h (stage 24 approximately). (*c*) The result after 36 h (stage 26 approximately).

mesenchyme along the entire length of the curved distal tip of the right limb-bud (see Fig. 1), using fine tungsten needles. The embryos were then returned to the incubator and were studied in one of three ways.

In one series, operated embryos were examined repeatedly during the first 48 h of development. The lengths of the operated and control wing-buds were measured from the base of the limb-bud to the distal tip (Fig. 1). By stage 26 this estimate of growth was discontinued as the developing elbow joint was

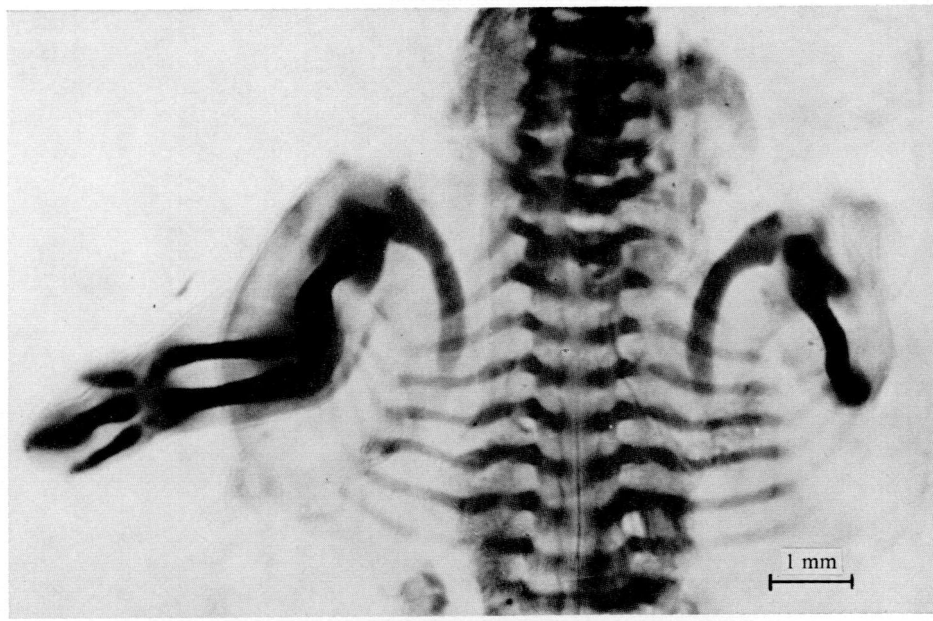

Fig. 2. The result after 96 h (stage 31 approximately). In this example only the humerus has been specified, the minimum obtained.

beginning to change the original simple outgrowth into a more complex shape with a bend.

In a second series, operated embryos were killed at approximately 24-h intervals after operating up until the 10th day of incubation. The embryos were killed and the wings from the operated (right) and control (left) sides fixed in 5 % trichloracetic acid, stained in 0·1 % Alcian green 2GX in 70 % alcohol with 1 % hydrochloric acid, dehydrated and cleared in methyl salicylate (see Summerbell & Wolpert, 1973). Operated and control limbs were examined and photographed using a Zeiss stereo IV dissection microscope (Fig. 2) and the lengths of the limb and its constituent cartilaginous skeletal elements measured (as described in Summerbell & Wolpert, 1973).

In a third series, operated embryos were sacrificed at 0, $3\frac{1}{2}$, 7, 11, 15, 18 and 24 h after operating, fixed in Karnovski's fixative, dehydrated and embedded in Araldite. Sections 1-μm thick were cut in a plane containing the proximo-distal and dorso-ventral axes of the limb, and alternate sections were stained in toluidine blue or by the Feulgen method. Whole Feulgen-stained sections from the mid-line of the antero-posterior axis of the limb and from 80 μm to each side were photographed using a Zeiss Photomicroscope I with a $\times 40$ planar lens. Cell nuclei were counted from photographs at a real magnification of $\times 800$. The positions of mitotic figures were noted on the photographs by identifying them in the original sections under oil immersion with a $\times 100$ planar lens. Cell density was counted as the number of cells in a square 50×50

μm and the figure converted to cells/1000 μm^2. The mitotic index was calculated as the mean number of mitotic figures per 100 nuclei. This method gave remarkably constant results when the cells and figures in the same sections were recounted after a time interval of several weeks and when they were counted by different individuals. The counts from all three sections of the limbs of five embryos at 0 and $3\frac{1}{2}$ h, three embryos at 7, 11 and 15 h, two embryos at 18 and 24 h were averaged to give the results shown in the figures.

RESULTS

Rate of outgrowth

Two estimates were made of the rate of outgrowth following apical ridge excision.

(1) In the first, left (control) and right (operated) limbs were measured during the first 48 h post-operatively. The length of the control limb was used to determine the 'age' of the embryo by reference to a normal growth curve (Summerbell, 1976). This curve measures the length of limb against time and makes it much easier to compare two limbs which are nominally the same age but in fact widely differ in size and stage. The length of the operated limb was then recorded (Fig. 3).

Following excision the rate of limb outgrowth is considerably reduced. The effect is temporary, however, and within about 12 h the limbs start to elongate rapidly having apparently recovered.

(2) The second estimate (Fig. 4) is made from fixed and stained material measured at more infrequent intervals but carried out during a longer period of development. This method was used because one can then measure the length of each skeletal element and use the total length of humerus, ulna and digit III (where present) so as to compensate for the bend at the elbow. The lengths as measured in this and in the preceding method were initially not comparable because of shrinkage caused by fixation. All results have therefore been converted into the equivalent *in vivo* length using the calibration curve developed by Summerbell (1976). Again the 'age' of the embryo is determined by the intersect of the control limb on the normal growth curve.

The lengths of the dorso-ventral and antero-posterior axes were also measured but after about 24 h they did not vary significantly from normal.

Cell density

The mean cell density at different levels along the proximo-distal axis is shown in Fig. 5. Operated and control side limbs are included in each graft for comparison. The control curves agree very well with those of Summerbell & Wolpert (1973). Operated and control limbs were very similar at $3\frac{1}{2}$ h and at 24 h after operating. Between these two times the density in the operated limb was higher than in the control limb. Proximally the difference was slight and by inspection of the marked standard deviations probably not significant. Distally

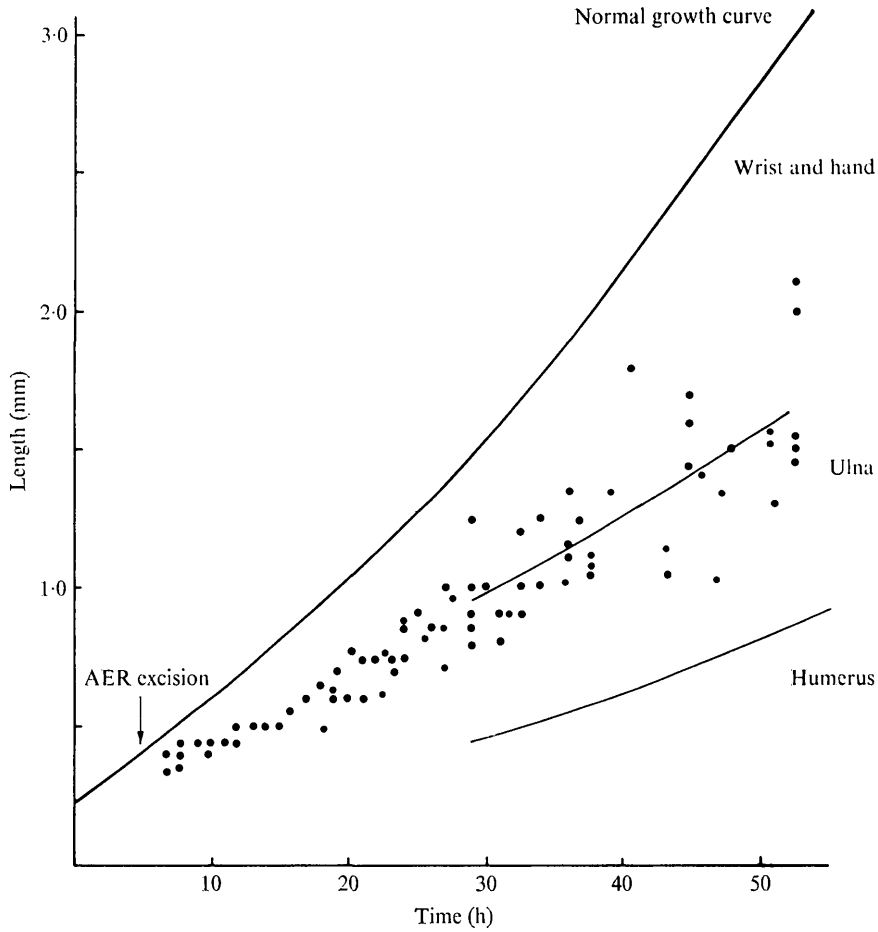

Fig. 3. The rate of outgrowth *in ovo*. The length of the control side, as measured in Fig. 1, is used to determine the 'age' of the embryo using the normal growth curve. The length of the operated limb is then plotted. Also shown are the proportions of the total wing length occupied by humerus and ulna/radius levels.

the difference was very marked. In the normal limb the density proximally rises steadily during this phase of development while at the tip it stays constant or even falls slightly. In the operated limb the cells at proximal levels behave normally, but cells at the tip seem to act as if they too were proximal cells and the density increases. Later the limb seems to recover and after 18 h the density at the tip returns to about the normal level (see Fig. 6).

Mitotic index

The mitotic index at different levels along the proximo-distal axis is shown in Fig. 5. There are much wider standard deviations for all individual points compared with the cell density figures and the curves drawn may therefore be considered less reliable. The control side curves agree very well with those of

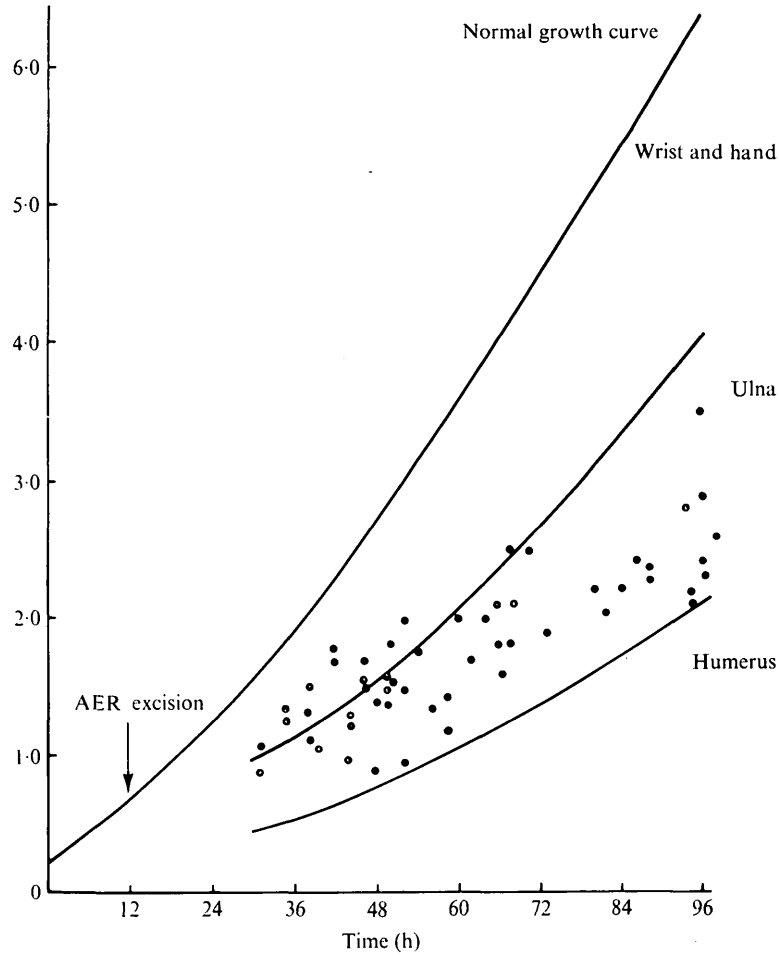

Fig. 4. The rate of outgrowth; summated skeletal lengths. As in Fig. 3 the length of the control limb is used to determine the 'age' from the normal growth curve. The length of the operated limb is then plotted. Again the distal boundaries of humerus and ulna/radius are shown.

Hornbruch & Wolpert (1970). Up until $3\frac{1}{2}$ h post-operatively the operated and control sides are not significantly different except at the most distal levels. During later stages the mitotic index as averaged over the entire operated limb is significantly lower than for the control limb, but in many cases because of the relatively small number of cells in mitosis at any one time, little confidence can be placed in the significance of differences between individual points. By 24 h the curve for the operated side approximates fairly well to that for the control side. Again the results have been summarized in Fig. 6.

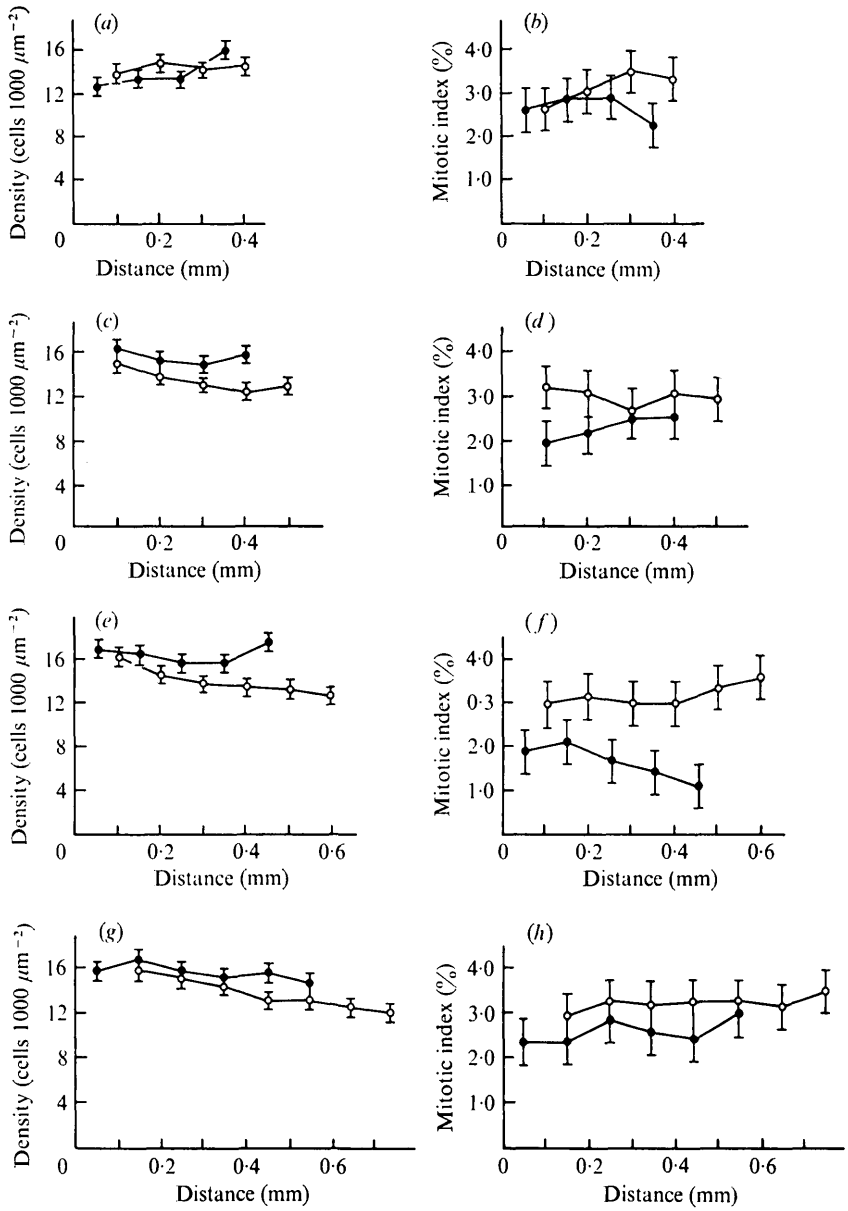

Fig. 5. Density and mitotic index. The cell density and the mitotic index are plotted for different levels along the proximo-distal axis. The error bars indicate one standard deviation. The distance is measured from the body wall as illustrated in Fig. 1. (*a*, *b*) 3½ h after operating (stage 20 approximately); (*c*, *d*) 7 h after operating (stage 21 approximately); (*e*, *f*) 11 h after operating (stage 22 approximately); (*i*, *j*) 18 h after operating (stage 23 approximately); (*k*, *l*) 24 h after operating (stage 24 approximately). —●—, Operated; —○—, control.

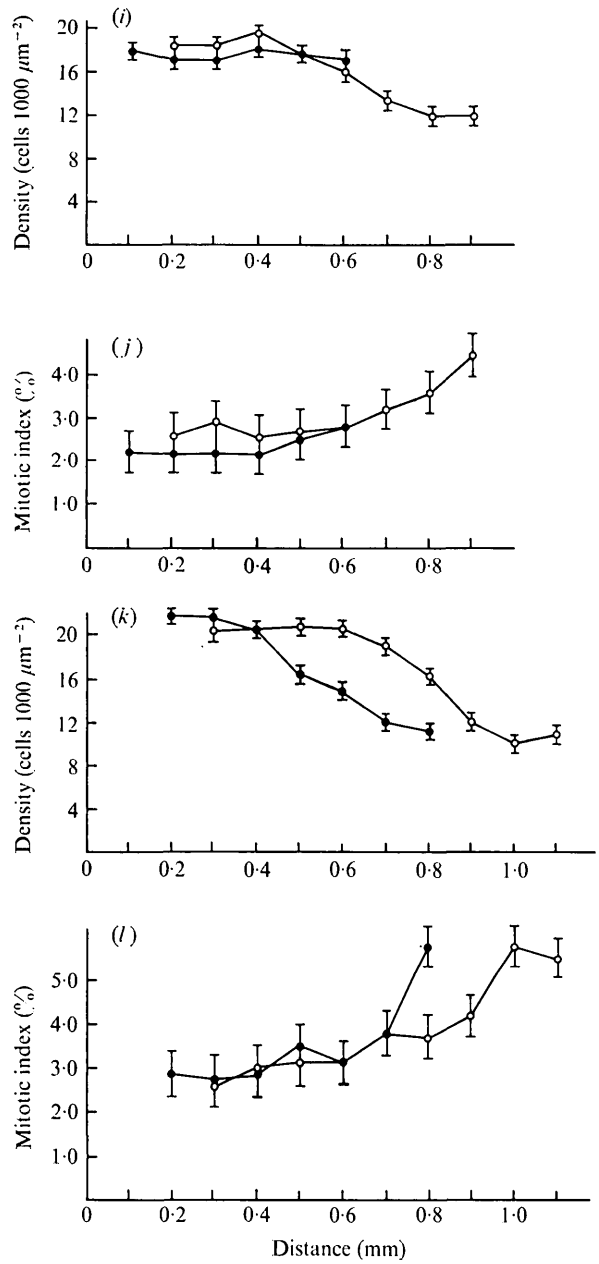

Fig. 5 (*i–l*)

Cell death

Cell death occurs only by the 10th hour after operation. There were dead cells throughout the limb but more particularly towards the distal ventral side of the mesenchyme. All evidence of cell death had disappeared by 24 h. In the sections showing cell death examined (11, 15, and 18 h) the mean incidence was about 50 cells per section or about 1·5 %. It is difficult to quantitate this

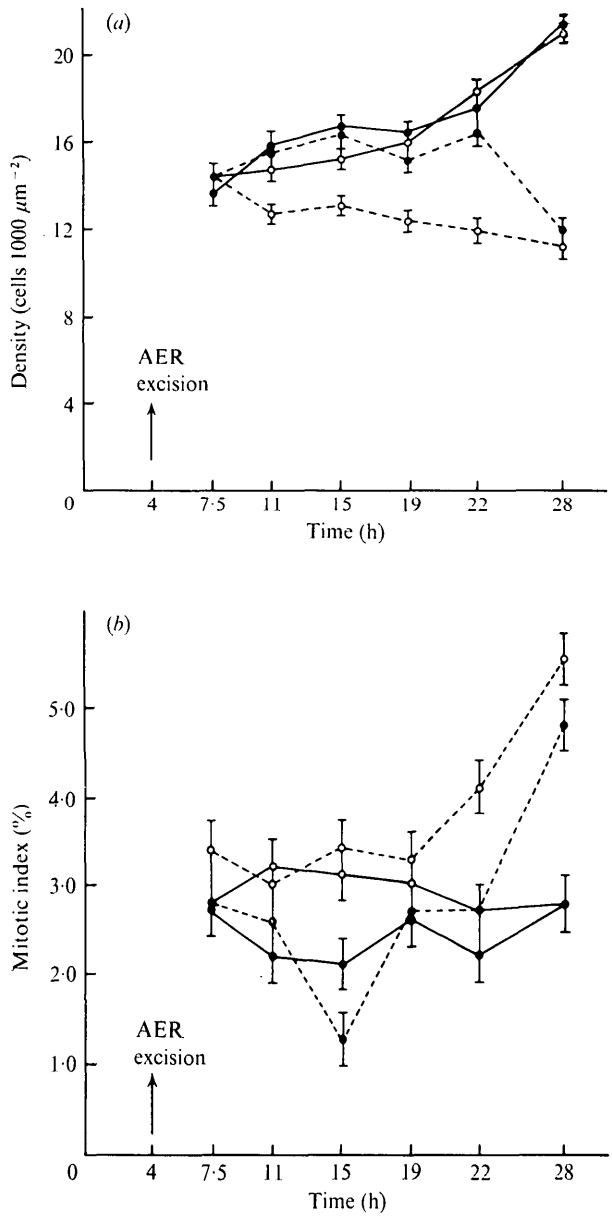

Fig. 6. Density and mitotic index at proximal and distal levels. The change of density and mitotic index with time for proximal and distal levels. The operation took place at 4 h on the horizontal axis (arrow). – – ○ – –, Distal control; —○—, proximal control; – – ● – –, distal operated; —●—, proximal operated.

parameter (because a single necrotic focus could represent one or more cells and because it is not known how long recognizable dead cells persist); thus the figure of 1·5 % may represent an incidence of 50 cells dying per section per hour or of only 50 cells dying per section for the entire period.

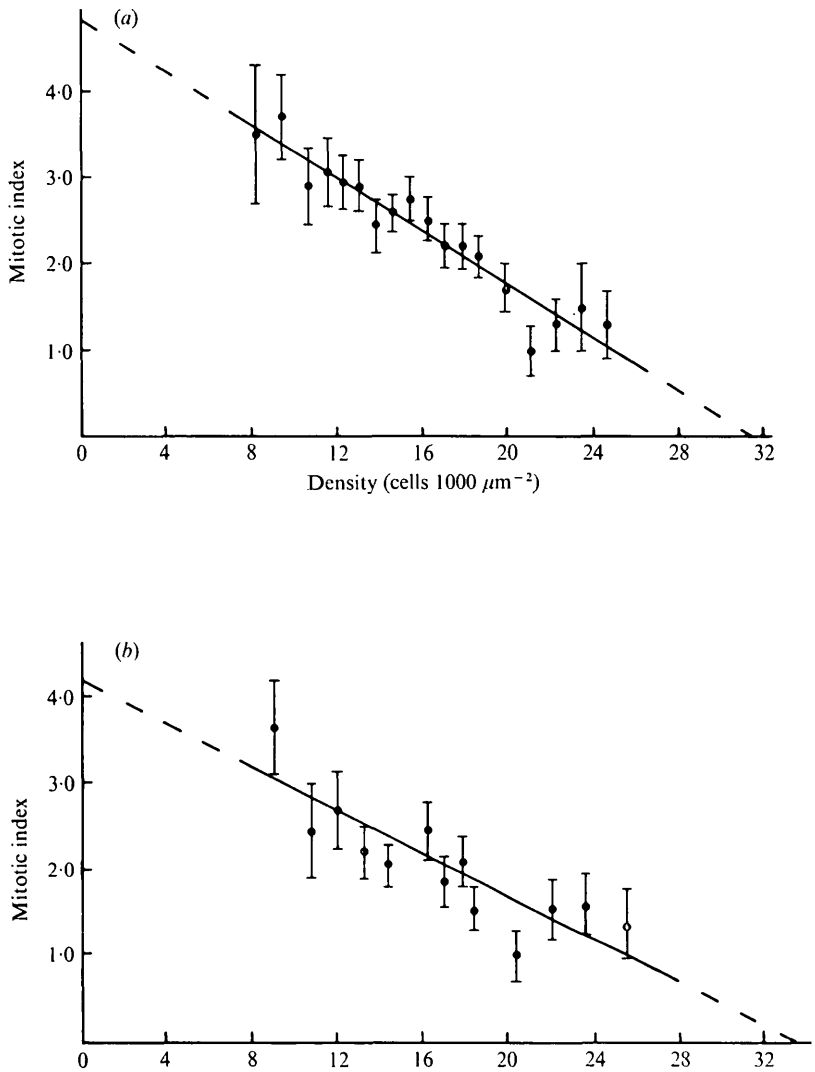

Fig. 7. The correlation between density and mitotic index. The sections were divided into square 50×50 μm. The density and mitotic index were counted for each square. The points represent the mean mitotic index for all squares of the same density and the error bars show one standard deviation. The regression lines (correlation coefficient) are calculated using every individual square and the observed mitotic index (i.e. not the mean mitotic index). (*a*) Control limb; (*b*) operated limb.

Correlation between mitotic index and cell density

There is a very close correlation between mitotic index and cell density. To calculate the regression line and correlation coefficient the 50×50 μm grid squares were used as the basic sampling unit of tissue 'area'. The number of cells and of mitotic figures for each square were counted. Correlation and

Table 1. *Regression analysis mitotic index against cell density*

	Control	Operated
Regression analysis	MI = 4·84 − 0·06x	MI = 4·2 − 0·05x
Standard error of b (slope)	0·01	0·01
Standard error of a (intercept)	0·61	0·49
Correlation coefficient (r)	0·87	0·76
Significance level of r	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Significance level between b_{operated} and b_{control}	Not significantly different	$P > 0{\cdot}2$
Significance level between a_{operated} and a_{control}	Not significantly different	$P > 0{\cdot}4$

regression coefficients were calculated from the raw data. Regression analysis assumed cell density as the independent variable. Because a number of values of MI were available for each cell density value, MI is given in the graphs as the mean and standard deviation (vertical bar) of these values (see Fig. 7). The results of the analysis are shown in Table 1.

DISCUSSION

The rate of outgrowth

When the apical ectodermal ridge is removed from the limb-bud there develops an adult limb in which distal parts are missing (Saunders, 1948). The parts which are present are of normal size and morphology, so that the limb as a whole is shorter along the proximo-distal axis (Summerbell, 1974). The excision not only prevents the specification of distal parts but it also, directly or indirectly, alters the pattern of outgrowth. My main concern is with the latter, i.e. to identify the factors causing the flattening of the growth rate curve shown in Fig. 3.

It was suggested earlier that four factors might be responsible for this early modification of outgrowth.

(1) No evidence was found for any long-term change in the gross proportions of the limb, it does not reduce its rate of outgrowth along the proximo-distal axis in favour of extension of the dorso-ventral or antero-posterior axes.

(2) There is an increase in the number of cells being lost to the system. Sections of limbs from 10 to 14 h after operation show a high incidence of necrotic cells throughout the limb (Fig. 2). It is difficult to quantitate the actual reduction in the cell population due to cell death as there is no means of ascertaining whether the average count of 1·5 % dead cells represents the total cell death occasioned by removal of the AER, or whether dead cells are rapidly broken down and removed from the system to replace other cells which also die. The figure of 1·5 % may represent a constant rate of cell loss, e.g. 1·5 % per hour for a period of 8–16 h. These two rather extreme estimates of the

effect of cell death have rather different consequences with respect to their importance in the observed reduction in the rate of outgrowth.

(3) There is an obvious difference in the way that cells are arranged in the operated as compared to the control limb. In the former, more cells are packed into the same space at the distal tip. Although this will have an effect on the rate of outgrowth in the short term, it cannot be of direct importance in the long term, as by 24 h the difference between cell densities in operated and control limbs is not significant.

(4) The mitotic index is on average lower in operated than in control limbs (Figs. 5 and 6) until the profiles were measured 24 h after operating. At this time the operated and control are sufficiently close for one to assume that the former has returned to the normal state. The mean depression of the mitotic index is $0{\cdot}75 \times$ the value of the control (standard error of mean $\pm 0{\cdot}03$) and this is effective over a fairly short time interval of about 18 h, starting shortly after operating. The reduction in the mitotic index is indicative of a change in the overall rate of proliferation, and this change is unlikely to be due to an increase in the length of mitosis itself (Mazia, 1961; Greulich, 1964; Cunningham *et al.* 1967; Grosset & Odartchenko, 1975). Therefore one would expect either that there is an increase in the length of the cell cycle or that some cells are pulling out of the division cycle.

Given the mitotic index (MI), and the mean cell cycle time (c), and if the time in mitosis (m) is short one can make the approximation:

$$\mathrm{MI} = 0{\cdot}69\, m/c \tag{1}$$

(Hoffman, 1949; Edwards *et al.* 1960), or if cells have only recently started to pull out of the mitotic cycle, equation (1) can be modified to:

$$\mathrm{MI} = 0{\cdot}69\, pm/c, \tag{2}$$

where p is the proliferative index and where m is again small.

One can assume that m is the same in operated and control limbs (see above). Therefore if the depression in mitotic index (MI) is caused wholly by an increase in the cell cycle time (p unchanged) then:

$$\frac{\mathrm{MI}_{\mathrm{operated}}}{\mathrm{MI}_{\mathrm{control}}} = \frac{c_{\mathrm{control}}}{c_{\mathrm{operated}}} = 0{\cdot}75 \tag{3}$$

or assuming that the change is caused wholly by cells pulling out of the division cycle and c is the same for both then:

$$\frac{\mathrm{MI}_{\mathrm{operated}}}{\mathrm{MI}_{\mathrm{control}}} = \frac{p_{\mathrm{operated}}}{p_{\mathrm{control}}} = 0{\cdot}75. \tag{4}$$

The number of cells in the limb Ns' at a stage S' depends on: the number of cells (Ns) at some earlier stage S, the proportion of cells that remain prolifera-

tive cycle (p), the time between s and s' (t) and the length of the division cycle (c) then:

$$\begin{aligned} NS' &= Ns(2p)^{t/c} + Ns(1-p)(2p)^0 + N(1p)(2p)^1 \ldots \quad + N(1-p)(2p)^{t/c} \\ &= Ns(2p)^{t/c} + Ns(1-p)[(2p)^0 + 1(2p)^1 + \ldots \quad + (2p)^{t/c}] \\ &= Ns\left[(2p)^{t/c} + (1-p)\frac{1-(2p)^{t/c}}{1-2p}\right]. \end{aligned} \tag{5}$$

We can solve this equation numerically and so find the number of cells in the limb (Ns') at s' in terms of the number of cells present (Ns) at time s. For the control limb I use: t = 18 h, c = 8 h, and p = 1 (Cairns, 1966; Summerbell & Lewis, 1975; Lewis, 1975). So:

$$Ns_{23\ \text{control}} = 4{\cdot}76 \times Ns_{19}.$$

Similarly we can calculate the change in cell numbers over the same stages for the operated limbs. There is a choice here. If the cell cycle time (c) remains constant and the change in mitotic index is due to a change in the proliferative index then from (4):

$$p_{\text{operated}} = 0{\cdot}75 \times p_{\text{control}},$$

substituting in equation (5) t = 18 h, c = 8 h, p = 0·75 gives:

$$Ns_{23\ \text{operated}} = 3{\cdot}24 \times Ns_{19}.$$

Alternatively if p remains unchanged and the cell cycle time becomes longer then from (3)

$$c_{\text{operated}} = 1{\cdot}33 \times c_{\text{control}},$$

substituting in equation (5) t = 18 h, c = 10·64, p = 1 gives:

$$Ns_{23\ \text{operated}} = 3{\cdot}23 Ns_{19}.$$

The length of the limb depends on the area of cross-section, the cell density, and the number of cells present (Lewis, 1975). At stage 23 the cross-sectional profiles of operated and control limbs are the same, within the limit of accuracy of the measurements. The mean cell density is also very similar (control = 16·8, operated = 16·7). We can therefore compare the operated (Ls'_{operated}) and control (Ls'_{control}) lengths:

$$\frac{Ls'_{\text{operated}}}{Ls'_{\text{control}}} = \frac{Ns'_{\text{operated}}}{Ns'_{\text{control}}} \tag{6}$$

and substituting our derived values for the number of cells in each limb gives:

$$\frac{Ls_{23\ \text{operated}}}{Ls_{23\ \text{control}}} = \frac{Ns_{23\ \text{operated}} \times Ns_{19}}{Ns_{23\ \text{control}} \times Ns_{19}}.$$

Using this ratio and Fig. 3 we can estimate the length of the operated limb from the length of the control limb as shown in Table 2, the difference between estimates assuming the change to be in the cell cycle time or the proliferative index is very small. Subsequently the operated and control limbs will maintain this ratio until there is a significant change in one of the parameters. The resulting predicted growth curve is shown in Fig. 8. The extrapolation back to

Table 2. *Estimates of growth curves*

	Length of operated limb, stage 23
Change due to increase in cell cycle time	$0{\cdot}68 \times L_{control}$
Change due to change in proliferative index	$0{\cdot}68 \times L_{control}$

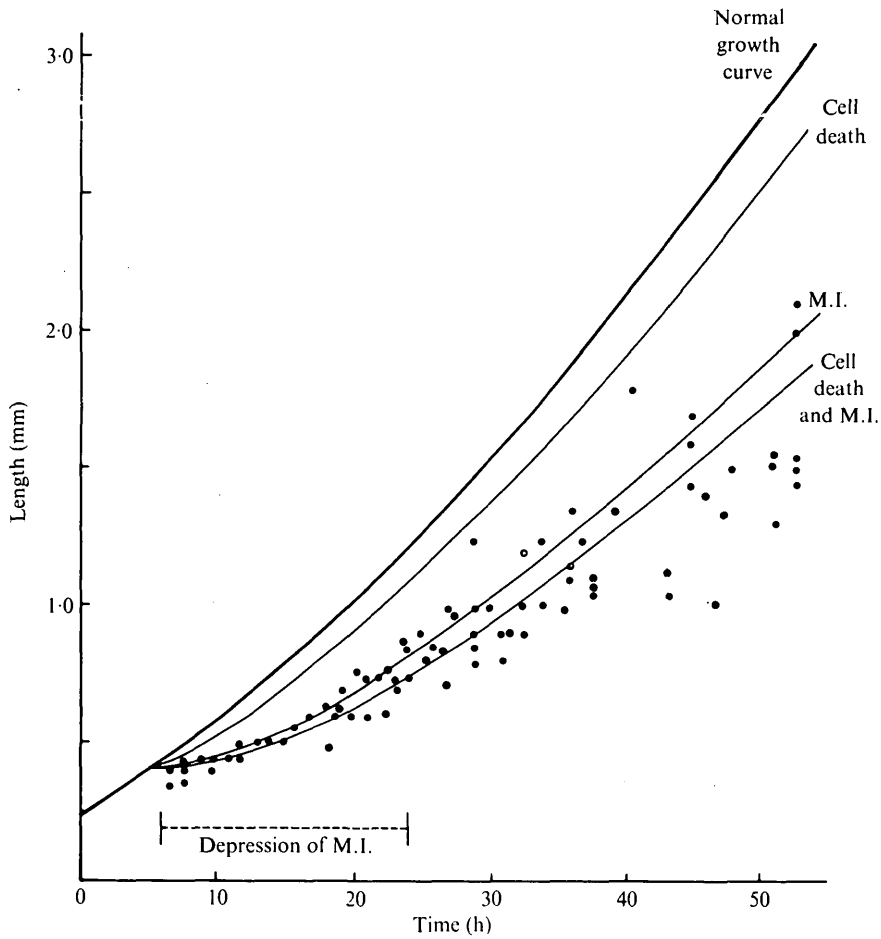

Fig. 8. The cause of early reduction in the rate of outgrowth. Estimated effects of the reduction in cell proliferation and of cell death are superimposed on Fig. 3 – the rate of outgrowth *in ovo*.

the time of operating is less certain because the length of the operated limb is very dependent on the mean cell density.

One can perform a similar calculation taking into account the effect of cell death. There is an extra problem here. It is not known whether the cell death counts represent $1\frac{1}{2}\%$ of the cells dying after the operation or perhaps continuing cell death of $1\frac{1}{2}\%$ per hour for about 8 h. Figure 8 includes estimates

using the latter parameter, exclusive of the effect of depression of the mitotic index, and as a combined curve. Even although the estimates must be considered as approximations, it is still pleasing that they are of the right order of magnitude to explain the observed results. It seems possible that the slowing in the rate of outgrowth of a limb from which the apical ridge has been removed may be explained as an effect of cell death and of a reduction in the rate of cell division.

Janners & Searles (1971) were unable to detect any change in the rate of cell division but did note considerable cell death. Despite this difference in the raw data it is interesting that if one examines their figure 4 and performs the same operation as illustrated in Fig. 8 then their experimental points also lie very neatly along the predicted rate of outgrowth.

Depression of mitotic index

The correlation coefficients obtained from both operated and control limbs were compatible with the hypothesis of density-dependent control of mitotic index (Summerbell & Wolpert, 1972). The methods used in this paper improved on the earlier paper in two ways:

(1) Ede *et al.* (1975) have pointed out that the correlation observed was heavily weighted by the effect of cells pulling out of the cell division cycle in high density precartilaginous condensations. In the present work the material was all taken from limbs between stages 19–22 so that the effect of this artifact is reduced.

(2) This study did not average large areas of varying cell density but used small areas of comparatively homogeneous cell distribution.

There are difficulties associated with this analysis, because the distribution of mitoses for a given density does not follow a strict gaussian curve. This problem can be overcome by the use of the regression technique which in this case also strongly supports the case for correlation between density and mitotic index. Perhaps the most significant feature of the regression analysis was the close agreement of the fitted curve for operated and control limbs.

Thus it seems that the depression in the mitotic index may be dependent on a temporary change in the density of packing of the cells in the distal tip of the limb. It is probable that the increase in density is caused by a simple wound healing response, cells in the vicinity of the wound adhere firmly together to form a dense aggregate (Summerbell, 1974).

The loss of distal parts

Although the excision of the AER immediately reduces the rate of outgrowth (Figs. 3, 8), the change is not sufficient to explain the results observed at day 10 (see Fig. 2). An extrapolation of any of the estimated curves which pass through the majority of points would clearly produce a limb which had either more distal segments present, or larger than normal proximal segments. The

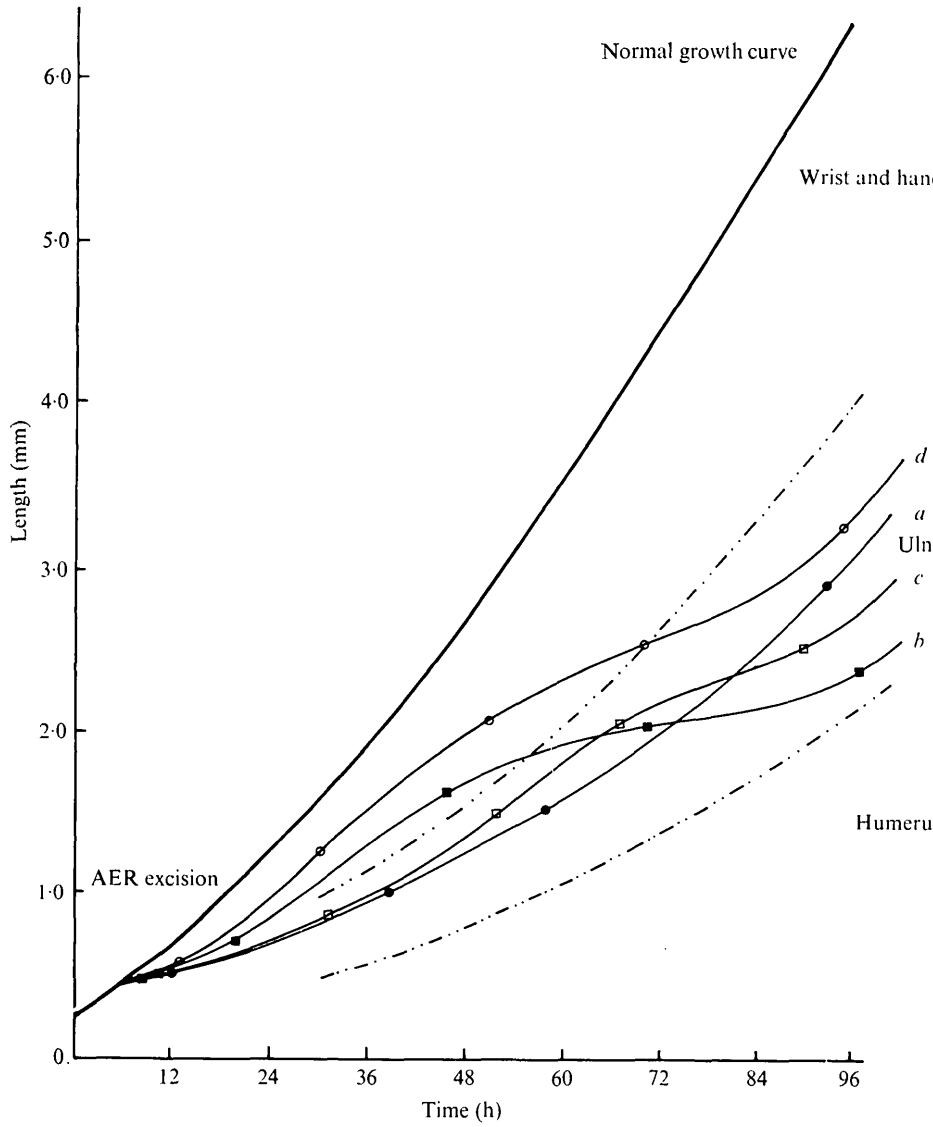

Fig. 9. Selected examples of the rate of outgrowth of individual operated limbs, compared with the control side. Each solid line shows the growth curve observed in a single embryo. Otherwise as in Fig. 4.

same caveat would apply to the analysis of Janners & Searles (1971). An answer to this problem was suggested by the results in Fig. 4.

It is possible to construct a map of normal development in which the contribution of each part of the skeleton to the total outgrowth is plotted (Summerbell, 1976). Such a map is shown in Figs. 3, 4 and 9. The summated length of the wing and the proportion contributed by stylopod (humerus), zeugopod (ulna/radius), and autopod (wrist and hand) are shown. It is quite clear from Figs. 3

and 8 and from the results of Janners & Searles (1971) that the reduction in the rate of outgrowth during the first 48 h produces a limb-bud long enough to contain at least half a zeugopod and at most half an autopod. It is equally clear that apical ridge excision at stage 19 normally produces at day 10 a limb with at most a whole zeugopod and at least most of the stylopod. The difference between the mean prediction and the mean result 48 h after operating is almost one-third the length of the limb (calculated using the fate maps of Lewis, 1975). Seventy-two hours after operating the results show (Fig. 4) that there has been a further reduction in the rate of outgrowth, and that the mean growth curve is on target for the mean observed result.

This suggests that the initial depression of the growth curve caused by cell death and the pause in cell division are not the only factors involved. Possibly at a time when cartilage cells have already started to differentiate and produce matrix, but have not yet formed discretely organized cartilage elements, there is a second event affecting the rate of outgrowth.

Fortuitously it was possible to examine this idea more closely with the existing data. Many of the limbs used in the first estimate of the rate of outgrowth (Fig. 3) were also carried over into the second set of measurements (Fig. 4) and fixed and stained at 3 or 4 days post-operation. It is therefore possible to examine the history of individual limbs throughout the crucial period. I have chosen a number of examples in Fig. 9 which represent the range of results obtained. In some cases (e.g. *a*) the first phase of reduction in the rate of outgrowth is sufficient to place the limb on target for the observed end result. At the other extreme the early change in the rate of outgrowth is very slight and there is subsequently a very marked second phase of change (e.g. *b*). The majority lay somewhere in between – usually closer to *b* than to *a* (e.g. *c* and *d*). This second event seems to be very real and important.

CONCLUSION

A general explanation

The following hypothesis is speculative and accounts for the observations. While it is not compelling it is adequate and perhaps attractive. It leans heavily on the only extant model explaining development of the proximo-distal axis (Summerbell *et al.* 1973), and owes a great deal to the ideas of Amprino (1965). It would also account for the observations of Janners & Searles (except for their estimate of the rate of cell division) and is compatible with their general conclusions. The observations on which the explanation is based are also supported by the work of Camosso *et al.* 1960 (depression of rate of cell division), Amprino, 1974 (cell density as a control of cell proliferation) and Cairns, 1975 (cell death following AER excision).

When the AER is removed from the limb, mesenchyme cells are exposed to the outside environment. The tissue responds by local contractions in the

vicinity of the wound. The cellular basis of the behaviour is the retraction of cell processes, the rounding up of cells, and the formation of long stretches of closely opposed membranes between cells (Summerbell, in preparation). The local contraction results in a local increase in cell density (the cells are packed into a smaller volume). This in turn affects the rate of cell division by a negative feedback mechanism (Summerbell & Wolpert, 1972; Amprino, 1974) so that the cells divide less frequently, and there is a reduction in the rate of elongation of the limb. This retardation is enhanced by widespread cell death (Janners & Searles, 1971; Cairns, 1975).

The ectoderm heals back over the wound within about 12 h. Once the limb envelope is renewed the cell density, and later the rate of cell division, returns to normal. During this pause the intrinsic rate of cell division within the limb falls (Janners & Searles, 1970; Summerbell & Wolpert, 1972; Summerbell, 1975). The subsequent growth is therefore slower and the limb is very much shorter than if it were just developing a few hours later. So far these phenomena can be explained very simply by the Amprino concept of the importance of the AER, removal of which causes reduction in outgrowth by simple physical effects (Amprino, 1965, 1974).

The explanation so far accounts for the early reduction in outgrowth but it does not account for all the loss, nor does it account for the change in morphology. 'Limbs that develop following ridge removal are not only short, but they are also lacking in distal limb elements', Janners & Searles (1971). What happens to the distal parts ? According to the progress zone model of Summerbell *et al.* (1973) the AER specifies a special mesenchymal region at the distal tip, the progress zone. Cells within the progress zone autonomously and gradually change their positional value towards a more distal level. When cells are pushed out of the progress zone due to proliferation their positional value becomes fixed at the level that they had reached at the time of exit. Thus cells at the proximal end of the limb left the progress zone very early and will differentiate to form proximal elements while cells which left later will form progressively more distal elements. Thus when the AER is cut off at stage 19/20 only the most proximal parts of the limb have been specified. The cells in the progress zone at that time abruptly find the AER influence removed and therefore also become specified at a proximal level, so that the limb will be truncated at a level near the elbow. Because of this sudden efflux of extra cells from the progress zone it seems likely that in fact too many cells will have been specified at the most distal level so far achieved and because this situation continues for about 48 h the stump actually becomes longer than it ought to be. To account for the subsequent normal length of the limb segment, I shall assume that, at about the time that the stump tissues begin to differentiate morphologically (as opposed to histochemically), a second event affects growth. The tissues regulate their growth rate so that all the parts which have been specified are at the correct length for that particular stage of development. Only those levels which have

been laid down are affected – for the limb does not regulate in a classical sense those parts which are missing. Truncated limbs which were too long for their morphology now gradually assume the correct length (Summerbell *et al.* 1973; Summerbell & Lewis, 1975).

This explanation successfully accounts for the effects of apical ridge excision. It uses both the ideas of Amprino (see review 1965 and 1974) and those of Saunders (see review Saunders & Gasseling, 1968; Saunders, 1972): the conflicting views that the AER is important both in growth control and in inductive activity may not be incompatible. Removal of the AER affects both the control of growth and the maintenance of progress zone cells as demonstrated in an immediate reduction in the rate of outgrowth and in the loss of distal parts.

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