The control of growth and the development of pattern across the anteroposterior axis of the chick limb bud

By DENNIS SUMMERBELL

From The National Institute for Medical Research, London

SUMMARY

Two grafts of zone of polarizing activity (ZPA) were made to host limb buds. The grafts define the width of the shared responding field lying between them. They cause a change in the growth pattern of the bud so that there is an increase in the width of the tissue between the grafts. Concurrently they redefine (respecify) cell states in the responding tissue so as to cause formation of a mirror-image reduplicate hand between them. The number and type of digits formed depends on the initial distance between the grafts.

The results suggest that the initial presumptive hand field is very small (~ 300 μm), that it is not a classical morphallactic system, and that it is able to regulate its growth pattern. A point-source diffusion model is presented.

INTRODUCTION

During the last decade, the dominant conceptual framework of pattern formation in development, has been the theory of positional information (Wolpert, 1969, 1971). Principally this theory aided the resurgence of interest (Lawrence, 1966; Locke, 1967; Webster, 1966a, b; Stumpf, 1966, 1968) in classical field gradients (extensively reviewed by Child, 1941; Huxley & de Beer, 1934). Among systems that seem particularly amenable to this type of explanation is the developing chick limb bud, and there now exists good evidence for a gradient across the anteroposterior (craniocaudal) axis (Wolpert, Lewis & Summerbell, 1975; Tickle, Summerbell & Wolpert, 1975; MacCabe & Parker, 1976; Summerbell & Tickle, 1977; J. A. MacCabe, Lyle & Lence, 1979; Summerbell, 1979).

A favourite set of mechanisms for producing a gradient can be described by the generic term of source-sink diffusion models (e.g. Crick, 1970; Lawrence, Crick & Munro, 1972; Wolpert, Hornbruch & Clarke, 1974). Apart from the wide application in the literature the general idea has strong virtues: it has a clear-cut and plausible mechanism which is readily understood, it would work, and, it is easy to simulate quantitatively; most important, it constitutes an

1 Author's address: The National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.
excellent paradigm for many other averaging models that set up positional information. This paper was stimulated by problems experienced in adapting the source-sink diffusion paradigm to the limb-bud system. While a model based on these principles satisfies the general requirements of the experimental data (Wolpert et al. 1975) there are serious difficulties in demonstrating that it works quantitatively. Specifically, it is necessary to propose that the field organizer must control growth as well as pattern (Tickle et al. 1975; Summerbell & Tickle, 1977).

When tissue from the caudal lateral edge of the developing chick limb bud is grafted to the anterior margin it causes the formation of supernumerary limb parts with double posterior mirror-image symmetry (Saunders & Gasseling, 1968; A. B. MacCabe, Gasseling & Saunders, 1973; MacCabe & Abbott, 1974; Summerbell, 1974a, b; Fallon & Crosby, 1975a, b; Crosby & Fallon, 1975; Tickle et al. 1975; Fallon & Crosby, 1977; Summerbell & Tickle, 1977; Smith, Tickle & Wolpert 1978; Smith, 1979, 1980).

While it is clear that the grafted tissue, the so-called zone of polarizing activity (ZPA) is provoking the reduplication, its role in normal development is uncertain and recently discussion has been enlivened by Saunders (1977), Fallon & Crosby (1977), J. A. MacCabe, Calandra & Parker (1977), Smith (1979, 1980), Summerbell (1979), Iten & Murphy (1980), Javois & Iten (1980). Prior to this it was generally assumed that both host and graft ZPA were active and that the limb bud between them constituted a single field with two symmetrical signalling regions (Wolpert, 1969; Saunders, 1972; Wolpert et al. 1975).

The problem arises when trying to link this latter interpretation with a source-sink diffusion model. When the two concentration profiles generated by the organizing regions intersect, the resulting summed concentration in the centre is too high to be consistent with the digits obtained between the organizers. The obvious solution is to propose that the graft, simultaneous with changing the pattern, changes the growth rate so as to increase the distance between the organizers. The experiments in this paper were designed to test this prediction, and to provide parameters for growth allowing one to finally construct a quantitative simulation. The results show that there is indeed a strong connection between pattern specification and growth. Coincidentally they exclude the simpler forms of intercalation's models and more surprisingly they suggest that the initial anteroposterior hand field may be much narrower than has generally been assumed.

METHODS

Fertilised embryos from a local flock (Needle Farm) were incubated at 38 °C and windowed on the third day of development. The windows were sealed with Sellotape and returned to the incubator. Embryos at stages 18–20 (Hamburger & Hamilton, 1951) were selected as hosts. A piece of tissue including the ectoderm, mesoderm and AER was removed from the right wing bud opposite somite 16 or
Fig. 1 Camera-lucida drawings of embryo /640: 0 h, appearance of limb bud immediately after grafting, \( w_0 \) measured between cut surfaces; 14 h, the positions of the grafts (G) are marked by indentations, the pins, and by the appearance of the tissue (opaque white, indicating extensive cell death); 24 h, the major outgrowth between the grafts has widened considerably \( W_t/W_0 = 1.33 \) (lower than average); 40 h, major outgrowth continues to widen \( W_t/W_0 = 2.4 \) (about average), minor outgrowth (between host and graft ZPA) is regressing. Note the very marked apical ectodermal ridge with very sharp boundaries near the graft, this appearance was typical and greatly facilitated measuring the width.

somite 17 as shown in Fig. 1. The excised fragment measured about 200 \( \mu m \) squared on the dorsal surface, and as far as I could judge kept the same profile through to the ventral side. A second similar fragment was then removed from a more caudal position. The distance between the two graft sites was then measured using an eyepiece graticule calibrated in units of 25 \( \mu m \). A stage-21 embryo was used as the donor and two equivalent pieces of tissue (200 \( \times \) 200 \( \mu m \times \) depth of D–V axis) were removed from the posterior lateral edge of left and right wing buds. The two pieces of graft tissue were transferred to the host egg and fastened into the prepared graft sites with platinum pins (25 \( \mu m \) diameter). The three bare mesenchymal surfaces of the graft contacted the three bare mesenchymal surfaces of the host but no attempt was made to otherwise preserve particular
orientation of grafts or order of grafting. Sham controls were performed in the same way as the main series except that equivalent anterior limb margin was used in place of ZPA tissue.

Camera-lucida drawings were made of the completed operation, and at varying intervals for two days. Not all experimental animals were treated in this way because the handling of the embryos necessary to obtain good camera-lucida drawings markedly lowers the survival rate. Measurement of subsequent changes in the width of the limb bud between the grafts were all taken from camera-lucida drawings. The majority of the animals for which no camera-lucida drawings were made was still examined either one or two days after operating. This was to determine the general shape of the limb bud (see Results). All surviving embryos were fixed in 5% aqueous trichloracetic acid on day 9 or 10 for 3 h. The staining schedule was as follows: (1) rinse distilled water; (2) wash 70% alcohol, 10 min; (3) wash 70% acid alcohol (ethyl alcohol 70 ml, distilled water 30 ml, hydrochloric acid 1 ml), 10 min; (4) stain in 0.1% Alcian green 2Gx (Gurr) in acid alcohol, 3 h; (5) differentiate 70% acid alcohol, overnight; (6) wash 70% alcohol, 10 min; (7) dehydrate in two changes absolute alcohol, 30 min each; (8) mount between glass coverslips in methacrylate (Phillpotts, Data Sheet 11, Taab Laboratories, 52 Kidmore End Rd, Reading, U.K.).

In a few cases quail embryos of stage 21 by chick criteria were used as donors. The hosts were fixed in half-strength Karnovsky’s fixative after 24 h and embedded in methyl methacrylate. Samples of 2 μm transverse sections (containing dorsoventral and anteroposterior axes of limb) were taken at intervals of 50 μm, stained by the Feulgen technique and mounted. Three good sections from each level were photographed using a Zeiss photomicroscope with a ×10 objective and at a final print magnification of ×50. The sections were then re-examined under the microscope using a ×40 planachromat objective and the position of quail cells marked on the appropriate photographs. A computer generated three-dimensional reconstruction of the limb bud with the distribution of quail and chick cells was made using the techniques developed by Perkins, Barrett, Green & Reynolds (1979).

RESULTS

A normal wing bud has three digits in the sequence 234(Z) (anterior to posterior). In principle, given complete interaction, one might expect a single graft of caudal tissue to interact with the host to produce a maximum digit sequence 234G43234(Z) and a double graft to produce 234G432234G432-234(Z) (cranial to caudal) where G represents graft and (Z) the putative host ZPA.

I originally performed double ZPA grafts on 125 host embryos of which 99 (80%) survived to day 10. Two of these survivors had reduced hands and I was
Table 1. **Digits formed between the two grafts. Somite number indicates the position of the anterior graft**

<table>
<thead>
<tr>
<th>Somite</th>
<th>Total</th>
<th>G234G</th>
<th>G2234G</th>
<th>G3(223)4G</th>
<th>G(3223)4G</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>70</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>55</td>
</tr>
<tr>
<td>17</td>
<td>27</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>97</strong></td>
<td><strong>4</strong></td>
<td><strong>4</strong></td>
<td><strong>14</strong></td>
<td><strong>75</strong></td>
</tr>
</tbody>
</table>

(Digits enclosed by brackets were not always present.)

unable to identify which digits were present; the results are therefore based on the remaining sample of 97 embryos. The limbs never possessed the maximum possible complement of digits, the most that I obtained was seven (see Table 1), the same number as that reported in Tickle et al. (1973). I obtained a digit anterior to a somite-16 graft only once (4G4323G(Z)) but somite-17 grafts normally had anterior digits; 4G..., 18 cases; 23G..., 3 cases; G..., 6 cases. These anterior digits were always of the same handedness as the host, as they did not lie between two graft regions I have not considered them further.

The digits between the grafts were usually arranged in mirror image symmetry about the mid-line, with a digit 4 adjacent to each graft, though in 22 cases out of 97 the symmetry was incomplete, most frequently because one of the digits 4 was missing (see Table 1). There was no obvious systematic reason for these ‘incomplete’ reduplications and they occur at about the same frequency in other experiments involving ZPA grafts. Summerbell & Tickle (1977) suggest that it is indicative of the effective ‘strength of activity’ of the ZPA graft and more recently Honig, Smith, Hornbruch & Wolpert (1980) have proposed a more discriminating method of assessing this effective strength. The digit nearest the graft is scored on an integer scale from 3 to 0 with ‘digit 4’ high (= 3) and ‘no digits’ low (= 0). The ‘strength’ is calculated as the percentage of the possible maximum score for the sample. Using this method the average score was 94% with no significant variation for anterior or posterior graft or for the position of the anterior graft (somite 16 or 17).

**Graft cells do not participate in outgrowth**

Serial reconstructions of double quail ZPA grafts to chick hosts were used to determine the contributions of quail cells to the reduplicated bud between the grafts. Though I cannot exclude the presence of occasional isolated quail cells, it appeared that essentially no graft cells were incorporated into the distal part of the outgrowth and that there was only a very small contribution to the proximal part. I estimate the largest quail component in any of the eight cases reconstructed as less than 10% of the total volume of the outgrowth. A typical case is shown in Fig. 2.
Fig. 2. Three-dimensional reconstruction of serial sections of limb bud 24 h after grafting. The stippling indicates the maximum extent of quail cells (summed after each section had been superimposed). Neither graft extends into the tip of the outgrowth. Volumes were estimated using a base line drawn arbitrarily to maximise the proportions of the grafts: anterior outgrowth (between grafts) 58 units, posterior outgrowth 54 units, anterior graft 7 units, posterior graft 6.5 units (50 units ~ 0.14 mm³).

Because of this observation I have not included any allowance for graft tissue in my estimate of the initial width of the field across which the two grafts act. This parameter was measured as the width of host tissue, as described in the methods.

Which digit belongs to which field?

During the second day after operating it is possible to determine by observation how the bud has responded to the three organizers (two grafts and one host). The new bud fields are distinctive outgrowths, separated from each other by indentations seen over the ZPA tissue (see Figs 1 and 2). The graft itself is whiter and more opaque than the host tissue, and usually the platinum pin is still visible. This, aided by the morphology of the hand at day 10, enables one to assign a position to each graft relative to the digits present. In many cases this preliminary observation was crucial in determining which digits were present between the grafts. I have where necessary used the notations 2, 3, 4 and G (for graft) to identify the graft sites amongst digits.
Fig. 3. Relationship between the initial width of the shared field and the digits obtained between the grafts. 3$^3$3 indicates digits 3 fused together across the midline, 3 indicates a reduced digit 3 on the midline. Circles indicate anterior graft opposite somite 16, squares indicate anterior graft opposite somite 17, half filled indicates cases with at least one reduced digit 4.

Wide fields make many digits

Figure 3 shows the relationship between the initial distance between the grafted ZPA's (± 25 μm) and the digits obtained between the grafts. The digit score was based on the number and type of digits present, a pair of digits fused across the mid-line (e.g. 4 3$^3$3 4) or an unpaired reduced digit on the mid-line (e.g. 4 3 2 3 4) were each scored as a half a digit as shown in the figure. Apart from this digits 4 were frequently reduced, often lacking the phalange. Nevertheless if the metacarpal was recognizable as belonging to digit 4 then it was scored as a full digit, unless it fell within the fusion across mid-line scoring convention. Figure 4 shows cleared and stained 10-day limbs. The purpose of this figure is to indicate how particular limbs were scored (see legend) and the examples chosen concentrate on the type of marginal decision described above.

Only limbs from the last column of the table (digit 4 adjacent to both grafts) have been included in Figs 3 and 5–7. Results from anterior graft opposite somites 16 and 17 are included in Fig. 3, the two groups seem to behave very
Fig. 5. Relationship between the initial width of the shared field between the grafts and the digits obtained between the posterior graft and the host ZPA. Conventions as Fig. 3.

similarly and I could detect no difference in these results other than those caused by the restrictions imposed on the possible initial field widths. Circles indicate somite 16 results, squares indicate somite 17 results, half solid characters indicate cases in which digit 4 was reduced.

There appears to be a linear relationship between the distance between grafts and the digits obtained save perhaps for distances below about 200 $\mu$m when the slope perhaps increases.

For completeness the digits obtained between the posterior graft and host ZPA are shown in Fig. 5. The distance is again the graft–graft distance and not an estimate of the posterior graft–host ZPA distance. The graph therefore

---

Fig. 4. Stained and cleared 10-day limbs, score represents digits between grafts in Fig. 3, bar ~ 1 mm. (a) $W_0 = 450 \mu$m, good mirror image $G\, 4\, 3\, 3\, 4\, G$, scores 4. (b) $W_0 = 400 \mu$m, digits fused across midline, $G\, 4\, 3\, 3\, 4\, G$, scores 3½. (c) $W_0 = 550 \mu$m, unfused reduced digit in midline, $G\, 4\, 3\, 2\, 3\, 4\, G\, 4$, scores 4½. (d) $W_0 = 550 \mu$m, digits 4 reduced, $4\, 3\, 2\, 3\, 4\, G$, scores 5. (e) $W_0 = 575 \mu$m, missing digit 4, $G\, 4\, 3\, 2\, 3\, G$, not counted in Fig. 3. (f) $W_0 = 625 \mu$m, missing digits 3 and $4\, G\, 2\, 2\, 3\, 4\, G$, not counted in Fig. 3. (g) $W_0 = 150 \mu$m, ant = s16, an unusual result, $4\, G\, 4\, G\, 4$ (post 4 may be 4*4), scores 1. (h) normal control limb.
suggests the reverse slope. Results from anterior grafts opposite somite 17 are not included in this figure as the majority did not develop digits between posterior graft and host ZPA. In four cases where the distance between the grafts was 150 μm or less this responding field produced a digit 4 (e.g. Fig. 4g).

**ZPA grafts increase the growth rate**

Figure 6 shows the change in the distance between the two grafts (width of field) with time (Δw/t). Because of variation in the original width I have shown this change as the ratio of the width at time t to the original width. Initially the host tissue contracted slightly, presumably a response to operative trauma (Summerbell, 1977). Within 7–10 h the buds had recovered sufficiently for the ratio to have returned to the starting value. The region then developed into a
distinct outgrowth, leaving the two graft areas proximally (see Figs 1 and 2). The outgrowth normally had more or less parallel sides and a distinct distal edge with a well developed apical ridge. The measurements of width were taken from camera-lucida drawings. It is difficult to be consistent in making this estimate but repeat measurements indicate that it is probably reproducible to ±50 μm so that my base unit of 25 μm gives a false impression of the accuracy. Measurements were made up to 48 h, after which changes in the outline of the limb made it difficult to know where to measure the width.

The open circles indicate results from fields with an initial width of 200 μm or more. Within this group I was unable to detect any relationship between the initial width and the ratio at later times, but all limbs showed a rapid increase in the width between the grafts.

The closed circles represent results from fields with an initial width of less than 200 μm. Though this width too increased it did so markedly less than in the preceding group.

The crosses indicate results of the sham controls with anterior margin grafts instead of ZPA (the accuracy of these measurements at 48 h is rather low). These showed very small increases in the width. Both grafts were left proximally on a single normal developing outgrowth.
Figure 7 shows the intrinsic growth rate for the anteroposterior axis, that is the rate of change of width per unit width per time \((\Delta W/W.\Delta t)\) against time \((t)\). The figure includes only those results with a starting width of 200 \(\mu m\) or more. The initial contraction, the rapid increase in intrinsic growth rate followed by a slow fall towards zero are all obvious. The dotted line shows the intrinsic growth rate for the proximodistal axis during the same period.

**DISCUSSION**

Changes in the width are caused by changes in the cell-cycle time

During early normal development the bud elongates proximodistally but maintains roughly constant dimensions across its other two axis. All of the cells at the tip are apparently in the division cycle (Summerbell & Lewis, 1975) and the cell cycle time has been estimated at between 6 h (Cairns, 1977) to 13 h (Janners & Searles, 1970). Following the juxtaposition of anterior mesenchyme with mesenchyme from the posterior lateral edge, Camosso & Roncali (1968) found that limbs examined after 13 h already showed an enhanced mitotic index. More specifically (Cooke & Summerbell, 1980), we have found that a ZPA graft to anterior tissue increases the labelling index in the host following incubation in \[^{3}H\]thymidine for 1 h. There was a significant enhancement at 5, 9 and 17 h after grafting. The mitotic index was marginally higher at 9 h after operating and almost doubled after 17 h. (The relatively low number of mitotic figures in the limb means that there have to be large relative changes before differences become significant.) As all cells are dividing it seems therefore that the presence of the graft shortens the mean cell cycle time of cells in the adjacent mesenchyme by raising the rate of entry into ‘S’ phase within a few hours of the operation. The full effect, in terms of cell division itself is not apparent at 9 h but is great at 17.

This correlates well with the results presented in this paper. One cannot compare the growth rate directly with the cell cycle time since the latter is equivalent to the intrinsic rate of growth (or widening: \(\Delta W/W.\Delta t\)), as shown in Fig. 7. Following the double ZPA grafts at 0 h there is an initial contraction of host tissue between the grafts. This is probably the result of trauma: a similar reaction occurs following the removal of the apical ectodermal ridge (Summerbell, 1977). Within 4–6 h the intrinsic growth rate measured across the anterioposterior axis begins to increase (passes 0). It reaches a maximum at 12–16 h. This fits well with the cell cycle data where the change in the cell cycle time is small at 9 h and great at 17 h. It may appear from this that the change in intrinsic growth rate is rather low compared with the change in cell cycle time, but one must remember there is also expansion of the proximodistal axis, and changes in cell-packing density. In fact one can make a rough estimate of the change in cell cycle time necessary to produce the observed growth rate taking into account these parameters. I have used values for the mitotic index taken
from Hornbruch & Wolpert (1970), the cell packing density taken from Summerbell & Wolpert (1972), the growth rate for the proximodistal axis taken from Summerbell (1976); and the calculation was performed as described in Summerbell (1977). The mean cell cycle time required for the period between 12 and 26h post grafting would be 13·3 h for the control limb and 7·7 h for the operated limb giving an enhancement of the mitotic index of 1.7. Because the parameters come from various sources and the limb is heterogeneous one should treat these estimates with caution. Nevertheless it is useful to know that they compare well with the enhancement of mitotic index reported in Cooke & Summerbell (1980) (1·6–2·2 depending on position) and fall within the range of cell cycle times suggested by Cairns (1977) and Janner & Searles (1980). The spatial correlation is not so good, but this will be discussed elsewhere.

How to control the rate of growth

In previous papers (Tickle et al. 1975; Summerbell & Tickle, 1977), we presented a model in which the position of digits across the anteroposterior axis was specified with respect to the zone of polarizing activity. The problem with the model was that it did not fit the data without a major ad hoc assumption: that the grafted region modified the growth of the host bud so as to increase the width of the anteroposterior axis. The results in this paper provide decisive justification of this assumption.

It raises in turn the problem of how to control this change in size. The mechanism that I will now discuss is based on an idea for size control suggested by Lawrence (1972). He suggested that the length of the field could be controlled by allowing cell division to continue until the gradient of positional value across the field reached the correct slope. The field then having reached the correct length, cell division would stop. My modification again measures the slope but uses it to control the growth rate rather than the absolute size.

I start with the source-sink diffusion paradigm for interactive models described in the appendix to Summerbell (1979), a model satisfying the requirements outlined in Lewis, Slack & Wolpert (1977). The ZPA acts as the source of a morphogen, the local concentration of which is held relatively constant. The morphogen is free to diffuse into adjacent mesenchyme cells where it is broken down. This gives a concentration profile with an exponential form with the high point at the posterior edge. The position of a cell relative to the ZPA is specified by the local concentration of the morphogen, and the cell uses this information to program its differentiation. There are two additional assumptions:

(a) cells are able to measure the slope \( m \) of the concentration profile, either by monitoring the flux or by comparing the concentration \( c \) in nearest neighbours:

\[
m_i = \frac{c_{i+1} + c_{i-1}}{2x},
\]

where \( x \) is the distance between cells;
Fig. 8. (a) The equilibrium concentration profile and its slope for the parameters used in the simulation for a normal ZPA. The simulation was performed as described in Summerbell (1979). I previously omitted to specify the degradation term, the simulation is relatively insensitive to variation in this parameter and a value of $c' = 0.96c$ (where $c$ is the concentration) gives a fair fit to the data, the fit is slightly improved by using any function giving a relatively higher rate of degradation at higher concentrations. (b) The concentration profiles between two ZPA grafts at successive times. The anterior graft is opposite somite 16, and the initial width of the shared field is 500μm. The grafts were made at stage 18/19 and the 48 h curve would be equivalent to about stage 27/28. The predictions made by the curve for points on Figs 6 and 7 are within the limit of variation of the observed results.
(b) at equilibrium the slope at any point will be a function of the concentration, and all cells have programmed within them the equilibrium slope for all positions along the concentration profile. During development cells compare their observed slope with the expected equilibrium slope. Discrepancies cause a modification of the cell cycle time (\( T_c \)):

\[ T_c \propto (m - m_{eq}). \]

If the slope is too steep, the field is not long enough, so the cells divide faster. If the slope is too shallow the field is too long, so the cells stop dividing. A simulation of the model for double ZPA grafts with anterior graft opposite somite 16 is shown in Fig. 8.

**Epillaxis or morphamorphosis**

From the outset the theory of positional information has been afflicted by the awkward dichotomy of epimorphosis (the regulation of pattern by the sequential addition of ‘new’ cells with progressively different positional values at a boundary), and morphallaxis (the regulation of pattern by the reassignment of positional values to existing cells between two boundaries). This was unintentional as Wolpert (1971) initially pointed out that in many cases pattern formation would involve both. Nevertheless it has led to the situation (Wolpert et al. 1975) in which one could refer to the proximodistal axis of the limbs as being of the epimorphic type while the anteroposterior axis was morphallactic. The results reported here demonstrate that at least in the latter case the mechanism is not quite so straightforward. The process of specifying new positional values in the reduplicated hands cannot proceed to completion without cell division, but simultaneously there are changes in positional value taking place across a significant width of tissue indicating long-range signalling. The system shows characteristics of both types of pattern regulation and it seems that in this case at least these twin concepts are misleading and unhelpful.

**Other models also have their problems**

The idea of using twin ZPA grafts to define a shared responding field was suggested to me by Lewis Wolpert and Amata Hornbruch. They were using the technique to compare the predictions of the source-sink diffusion and an intercalation model based on the type of cell interaction used in the polar coordinate model of French, Bryant & Bryant (1976) as applied to the chick wing bud. Iten has recently argued in favour of the latter interpretation (Iten & Murphy, 1980a, b; Javois & Iten, 1980) and the experiment was designed as a test of her working hypothesis.

The principle is that the tissues across the limb carry positional values in a graded series. When tissues with disparate values are placed next to each other, cell division is stimulated at the junction and ‘new’ cells adopt values intermediate to nearest neighbours (i.e. intercalate) until the difference between
neighbours falls to a minimum step size equivalent to the equilibrium or normal state. The posterior margin is therefore not an area of particular significance, but is only dissimilar to tissue at the anterior margin. The intercalation hypothesis predicts that following double grafts of posterior margin to the anterior margin, the digits formed by the responding tissue should be a function of the position of the responding tissue along the anteroposterior axis, and not of the width of the responding tissue between the grafts.

A comparison of the results when the anterior graft was opposite somite 16 or somite 17 (Fig. 3) shows that the digits formed between the grafts depended on the distance between the grafts and not the position of the anterior graft. Wolpert & Hornbruch (1980) have performed a much more thorough study of this aspect with a wider range of anterior graft positions and I confirm their conclusions. They thoroughly analyse and discuss their data and it seems unnecessary for me to do likewise.

Iten & Murphy (1980) and Javois & Iten (1980) have also performed similar experiments using two ZPA grafts. However, the position of their anterior graft was always kept constant so that they effectively varied only the distance between grafts. They obtained results compatible with Wolpert & Hornbruch (1981) and with mine.

There is an unfortunate mystique concerning the reaction-diffusion model (Gierer & Meinhardt, 1972) perhaps encouraged by the formal method of presentation. Basically the model creates a concentration profile across a field. It is no better and no worse at this than, for example, the source-sink diffusion model. Some idiosyncrasy of the model may on occasion provide a particularly apt explanation for a particular observation, but then some other observation will require a particularly clumsy extension to the model.

The major strength of the model, compared with any averaging model (e.g. source-sink diffusion, Wolpert et al. 1974; intercalation, French et al. 1976; or the particularly simple and elegant averaging model of Maden, 1977) is the way in which the reaction-diffusion mechanism can be used to determine which genes are turned on for a given position along the gradient. A similar mechanism can also be used to control switching in the genome. This feature is quite independent of the specification of positional information, and interpretation by reaction-diffusion sits as well on the shoulders of source-sink diffusion as it does on its near relative.

To return to the chick limb bud, Meinhardt (1977, 1978a, b) has suggested that the reaction diffusion model provides a good natural explanation of the observations reported in Tickle et al. (1975), particularly the phenomenon of ‘distal deepening’. The explanation depends on a peculiarity of the model: that two sources in close proximity cause mutual inhibition and a consequent overall reduction in the concentration profile. This allows central deepening of the concentration profile without a change in width and hence a more normal complement of digits. It is not intuitively obvious how the reaction-diffusion
model will adapt to the changing data and further simulation will be necessary. The main difficulty will probably not be the lateral inhibition, but rather the cause and effect of growth. The model is particularly sensitive when applied to growing systems, requiring continual adjustment to the source strength of the organiser.

The limb field is very small

The most surprising outcome of this project concerns the initial size of the limb field.

First it was unexpected to discover from the quail grafts illustrated in Fig. 2 that the graft does not contribute appreciably to the limb. This observation lends limited support to the argument of Smith (1979) that continued activity of the ZPA is not necessary to produce a hand.

Second it was unexpected that so little distance is required to specify a whole hand. Working from Fig. 3, the width of tissue needed to make two full hands can be estimated as 575–675 μm. This suggests that the initial width of the field necessary to produce a half reduplication must be no more than about 300 μm. This tissue then expands under the influence of the ZPA so that it will measure (from Fig. 6) 500 μm at stage 24 and 650 μm at stage 25/26 – the time at which the hand plate starts to differentiate. This compares well with estimates for normal development of 550 μm at stage 24 taken from Figs 6 and 7 in Stark & Searles (1973) and 700 μm at stage 26 taken from Fig. 7c in Summerbell (1976). It suggests a slight inaccuracy in my simulation (Summerbell, 1979) when the width of the presumptive hand plate was taken as 550 μm at stage 25/26. It also makes a rather profound difference to some of the other parameters in this simulation. One of the problems was reconciling the very fast propagation of the signal necessary for it to spread across the whole limb bud during initial development, with the rather slow changes that follow perturbation of the field later in development. This incompatibility was solved by adopting a very long time for the initial concentration profile to develop. I allowed the ZPA to function as early as stage 10/11, the time at which the craniocaudal axis of the limb becomes autonomous (Chaube, 1959). However, the earliest stage at which there is any direct evidence of ZPA activity is stage 15 (A. B. MacCabe et al. 1973), which is some 16 h later. By extending the growth regulation model used in this paper to the normal case, it is possible to establish a concentration profile similar to that found in the original simulation at stage 20, assuming ZPA activity to commence at stage 15.

I suggest therefore that one can think of the normal limb as having a presumptive anteroposterior presumptive digit field of about 300 μm at stage 15. The field gradually expands during development until it reaches something approaching equilibrium, with a width of about 650 μm, at stage 25/26 just prior to differentiation.
My thanks for advice and help go to John Asante, Jonathan Cooke, Mike Gaze, Larry Honig, Amata Hornbruch, Lauri Iten, Malcolm Maden, Jonathan Slack, Victoria Stirling, Cheryl Tickle, David Willshaw and Lewis Wolpert.

REFERENCES


Growth and pattern in chick limb bud


(Received 28 July 1980, revised 22 September 1980)