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The zone of polarizing activity: evidence for a role in normal chick limb morphogenesis

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SUMMARY

When an impermeable barrier is placed so as to divide the early chick limb-bud into anterior and posterior parts then development continues only on one side of the barrier. The detailed results are inconsistent with mosaic development. They can readily be explained by supposing that pattern is specified by the concentration of a diffusible morphogen controlled by the zone of polarizing activity. A simulation of appropriate concentration profiles is presented and its relevance to similar experiments published elsewhere is discussed. It seems probable that the zone of polarizing activity is active during normal development.

INTRODUCTION

When tissue from the posterior lateral edge of the developing chick limb-bud is placed at the anterior lateral edge, it causes mirror-image reduplication of the anterior-posterior axis (Saunders & Gasseling, 1968; MacCabe, Gasseling & Saunders, 1973; MacCabe & Abbott, 1974; Summerbell, 1974*a*; Fallon & Crosby, 1975*a*, *b*; Crosby & Fallon, 1975; Tickle, Summerbell & Wolpert, 1975; Fallon & Crosby, 1977; Summerbell & Tickle, 1977; Smith, Tickle & Wolpert, 1978). Despite this impressive property (one of the most striking in developmental biology) many of these authors question the role of the ZPA in normal development. In this paper I present evidence that a primary effect of the ZPA can be detected, and that its action is compatible with a source-sink diffusion model.

The two opposing view points are perhaps best represented by Wolpert (1969, 1971) who considers that the ZPA controls specification of the posterior axis by positional information, and Saunders (1977) who now considers that development can proceed normally without it (but see Saunders, 1972). The key issue is the behaviour of the limb-bud following removal of the ZPA. It is commonly stated that removal of the region of ZPA activity in the early limb-bud can be followed by development of a normal limb (MacCabe *et al.*, 1973; Fallon & Crosby, 1975*a*). Yet the evidence is far from certain. MacCabe *et al.* (1973) removed all of the ZPA at stages 17–18 and 19–24. In the first experiment all

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15 of the limbs showed posterior defects but in the second, 7 out of 15 were essentially normal. Similarly Fallon & Crosby (1975*a*) removed the high point of ZPA activity from 47 wings at stage 20/21. Thirty % of the results were normal but the remainder had posterior defects. It is worth noting that the figure illustrating the normal group has a slightly reduced digit IV.

The question of whether or not a ZPA is necessary for normal development therefore still seems very open.

If one takes fate maps of the skeleton into consideration the situation becomes even more confusing and a simple interpretation of this problem becomes impossible. Clearly the most important presumptive fate maps for the anterior-posterior axis are those of Stark & Searls (1973), who in an elegant study provided data of unprecedented accuracy. A comparison of their maps with those of MacCabe *et al.* (1973) shows that the ZPA apparently overlaps the posterior edge of the presumptive skeletal area. One will always therefore face the problem that opponents of a functional ZPA will claim that deficient limbs are a result of intruding onto the skeletal fate maps whilst removing ZPA. Protagonists can always claim that normal limbs result from failure to totally remove ZPA, 'induction' of ZPA activity in more distal cells, or, that specification of cell-state by the ZPA involves a complex homeostatic mechanism whereby cells can remember their positional value along the posterior-anterior axis and can only change their positional value towards a level nearer the ZPA (Summerbell & Tickle, 1977).

Because of these problems Saunders (1977) has warned against attempts to construct models of limb morphogenesis involving the ZPA as a source of morphogenetic substances. Yet the behaviour of such models is often subtle and difficult to anticipate. In this paper I will show that it is possible to detect the influence of the ZPA during normal development by inserting barriers impermeable to communication along the anterior-posterior axis. A reduced effect is produced when a permeable (Millipore filter) barrier is used. The experiments will incidentally show how one can reconcile the important experiments of Amprino (1976, 1977) who obtained skeletal elements from anterior tissue, with the fate maps of Stark & Searles (1973) who showed that normally nothing should develop there.

METHODS

Fertilized White Leghorn embryos 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 16–19 and 21–22 (Hamburger & Hamilton, 1951) were selected for operating (Fig. 1). A slit was cut from the lateral edge of the somites to the distal tip through the entire dorsal-ventral thickness of the bud and parallel to the proximal-distal axis. A small sheet of 8 μm thick tantalum foil or of 0·8 μm pore size Millipore filter was inserted into the slit so that it projected dorsally, ventrally and distally. The barrier was

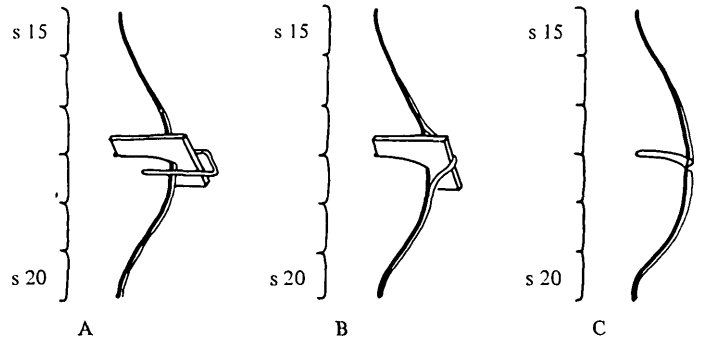

Fig. 1. Diagrams of the three types of operation. (A) Normal operation. (B) Barrier inserted under apical ectodermal ridge. (C) No barrier inserted. B and C gave mainly normal limbs.

held in place by means of a U-shaped platinum pin. In some control cases no barrier was inserted. In other control cases the slit was not continued through the apical ectodermal ridge (AER) so that the barrier projected dorsally and ventrally but not distally. The embryos were normally examined after 24 h, and in some cases 48 h. The comparatively few embryos in which the barrier had been lost or displaced so as not to divide the limb completely were discarded. Barriers correctly positioned at 24 h were invariably still in place at 48 h. In some cases the limb could not be examined at 24 h so a few embryos may have been retained which had lost their barriers. In many cases camera lucida drawings were made during these examinations.

All surviving embryos were fixed, stained, then cleared on day 10 of development using the method of Summerbell & Wolpert (1973).

RESULTS

In the 30 cases in which no barrier had been inserted the limb-buds looked normal after 24 h and 48 h and produced apparently normal limbs in 27 (90 %) of the embryos. The remaining three (10 %) had minor defects of the digits. Fifteen embryos had the barrier inserted without breaking the AER. In all cases the barrier was left at a proximal level as the limb-bud grew out. Seven embryos (47 %) developed normally with the barrier finishing in soft tissues. In eight cases (53 %) the barrier finished in close juxtaposition to the humerus. In some of these cases the humerus was doubled, in others partially doubled, and in the remainder had minor abnormalities. These two sets of results will not be discussed further.

The results for the main experiments are presented in the following manner. The position of the barrier along the antero-posterior axis is recorded by reference to the somite against which its proximal edge rested. Thus the barrier can occupy one of seven positions: S 16/17, S 17, S 17/18, S 18, S 18/19, S 19,

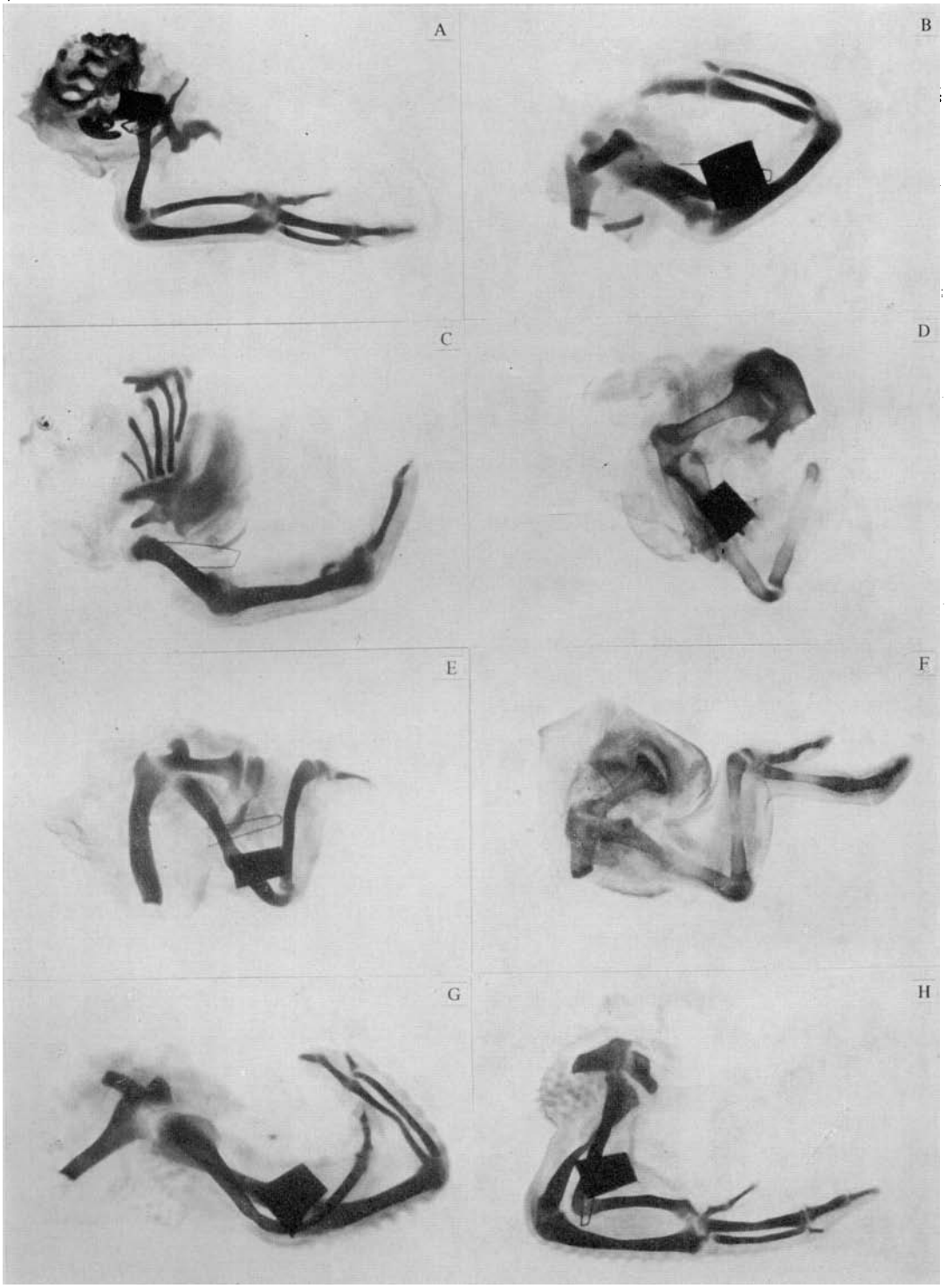

Fig. 2. Photographs of the main types of result obtained. (A) Somite 17: barrier – normal. (B) Somite 17/18: barrier – ulna, III and IV. (C) Somite 18: barrier – ulna and IV. (D) Somite 18/19: radius – barrier. (E) Somite 19: radius and II – barrier. (F) Somite 19/20: radius, II and III – barrier. (G) A variant with radius and II – barrier – ulna, III and IV. (H) Control: barrier under AER – normal.

Table 1. *Tantalum foil barriers inserted at stages 16–18*

		Position of barrier relative to somites						
	Result	16/17	17	17/18	18	18/19	19	19/20
				(*a*) Effect on the zeugopod				
Basic range	/ R U	10	2					
	/ U	3	2	17	4	2		
	R /					8	4	1
	R U /							6
Variants	R/U			1	2			
				(*b*) Effect on the autopod				
Basic range	/ Normal	12	4	7	1			
	/ III IV	1		11	3	2		
	/ IV				2			
	/					6	1	
	II /					2	3	1
	Normal /							6
Variants	II/III IV							
	II / IV							
	II III /							

Table 2. *Tantalum foil barriers inserted at stages 20–22*

		Position of barrier relative to somites						
	Result	16/17	17	17/18	18	18/19	19	19/20
				(*a*) Effect on the zeugopod				
Basic range	/ R U	4	3					
	/ U		2	2				
	R /							
	R U /					1	3	5
Variants	R/U			3	7	5		
				(*b*) Effect on the autopod				
Basic range	/ Normal	4	3	1				
	/ III IV		2	3	3			
	/ IV				1	1		
	II /				1	2		
	II III /					2	2	
	Normal /						1	5
Variants	II/III IV			1	2			
	II / IV					1		

Table 3. *0·8 μm pore size millipore filter inserted at stages 16–18*

		Position of barrier relative to somites						
	Result	16/17	17	17/18	18	18/19	19	19/20
				(*a*) Effect on the zeugopod				
Basic range	/ R U	9						
	/ U	3		11				
	R /					7		
	R U /							6
Variants	R/U			9		7		
				(*b*) Effect on the autopod				
Basic range	/ Normal	12		4				
	/ III IV			14		5		
	/ IV					1		
	/					4		
	II /					2		
	Normal /							6
Variants	II/III IV			1		1		
	II / IV			1				
	II III /					1		

S 19/20. Each position represents a change of about 150 μm from the preceding position. The operation produced a restricted number of typical results. The range of results is illustrated in Fig. 2. The type of result correlated well with the position of the barrier. In analysing the results the zeugopod (ulna/radius) and the autopod (hand) are considered separately; the stylopod (humerus) and wrist are not analysed in detail.

Table 1 shows the effect of inserting tantalum foil barriers at stages 16–18. This causes a well-defined sequence of results at both zeugopod and autopod levels which correlate with the original position of the barrier. Almost all of the results fell into 'basic' categories with development on only one side of the barrier. In three cases out of 62 there were 'variant' results (see Table).

Table 2 shows the effect of inserting tantalum foil barriers at stages 20–22. The results at the level of the autopod are very similar to those in Table 1 (except that two of the basic results were slightly different, see Table), showing the same well-defined sequence. At zeugopod level many results were 'variants' so that 31 cases out of 34 gave both ulna and radius (91 %).

Table 3 shows the effect of inserting 0·8 μm Millipore filters as barriers at stages 16–18. The results are very different from those for tantalum foil with a high proportion of 'variant' results. This is most evident at the zeugopod level where both radius and ulna are frequently present. The differences are less spectacular in the autopod but still show greater variability than the corresponding experiment at earlier stages.

DISCUSSION

Regulation

Warren (1934) has described a very similar operation in which he divided the limb-bud into roughly equal anterior and posterior halves. He concluded: that normally one third of the limb skeleton develops from the anterior half and two thirds from the posterior half; that the sum of the development of the two halves never exceeds that of a normal limb; and that often fewer parts are formed, particularly anterior to the barrier. This indicated 'no subsequent regenerative or regulative capacity'. The results reported here fully support his observations and conclusions.

Mosaic development

A simple interpretation of these experiments would be to assume mosaic development coupled with a rule that normal growth can take place only on one side of the barrier. (A possible mechanism for the rule could be the position in which the axial (subclavian) artery develops. If the barrier is placed anterior to the blood vessel then only the posterior half thrives; if the barrier is placed posterior to the vessel only the anterior half.)

Close examination shows that this simple model is not adequate to explain even the simplest series of results, the tantalum foil barriers at early stages.

I have illustrated in Fig. 3 a simple hypothetical fate map which gives the best fit to the results observed from the tantalum foil experiments. The model is clearly unable to explain the observed results without additional assumptions. It predicts well Fig. 3A, B, C and F but is unable to give the results found for Fig. 3D and E.

Changing the presumptive fate map to a more posterior position would give a closer fit to the fate map of Stark & Searls (1973) but gives a much poorer fit to the observations presented in this paper. One could make the additional assumption that insertion of the barrier causes trauma so that effectively one element is lost to each side of the barrier. This gives a fair fit to the data but the assumption is not supported by the control experiments. Cutting a slit without barrier insertion, and barrier insertion without cutting the apical ridge both give normal limbs.

Interactive development

One is naturally drawn towards an interactive mechanism for normal development because of the clear evidence for interaction between a grafted ZPA and the host (Saunders & Gasseling, 1968; Wolpert, 1969, 1971; Saunders, 1972; Wolpert, 1978). A possible mechanism is that the ZPA acts as the source of a morphogen which is held locally at a fixed concentration. 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

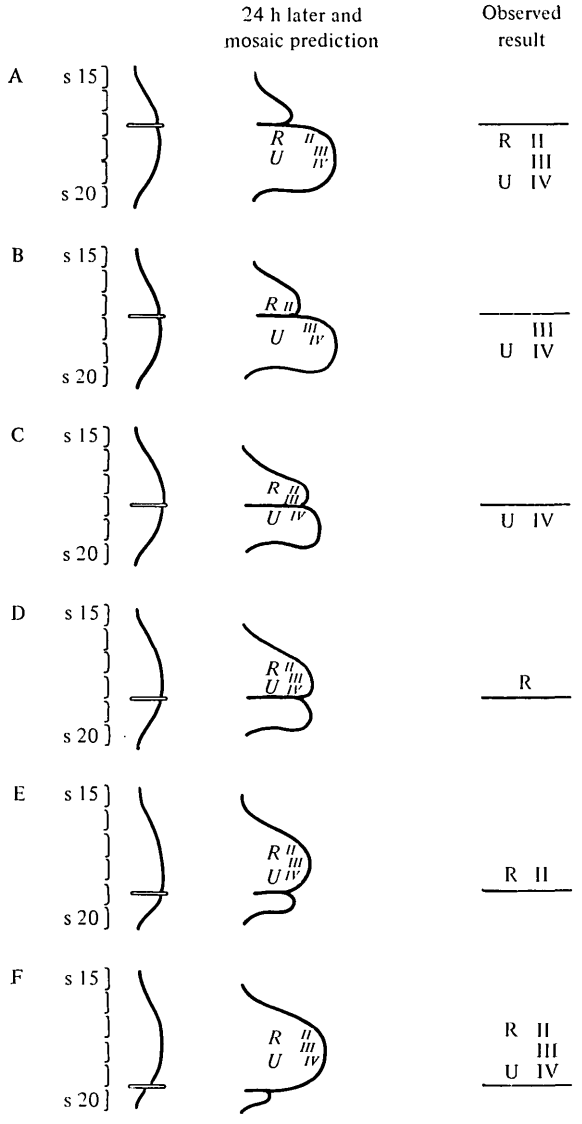

Fig. 3. The effect of inserting a tantalum foil barrier at stages 16–18. A presumptive fate map designed to give the best fit to the observed data is superimposed on the limb-bud taken from camera lucida drawings 24 h after operating. Normal observed results are shown in the adjacent column.

the ZPA (Tickle *et al.*, 1975; Summerbell & Tickle, 1977). The position of a cell relative to the ZPA is specified by the local concentration of the morphogen and the cells use this positional information to determine how they differentiate (Wolpert, 1969, 1971).

The parameters chosen for the stimulation of diffusion are as follows. The effective diffusion constant is $2{\cdot}7 \times 10^{-9}$ cm^{2}sec^{-1}, a value compatible with

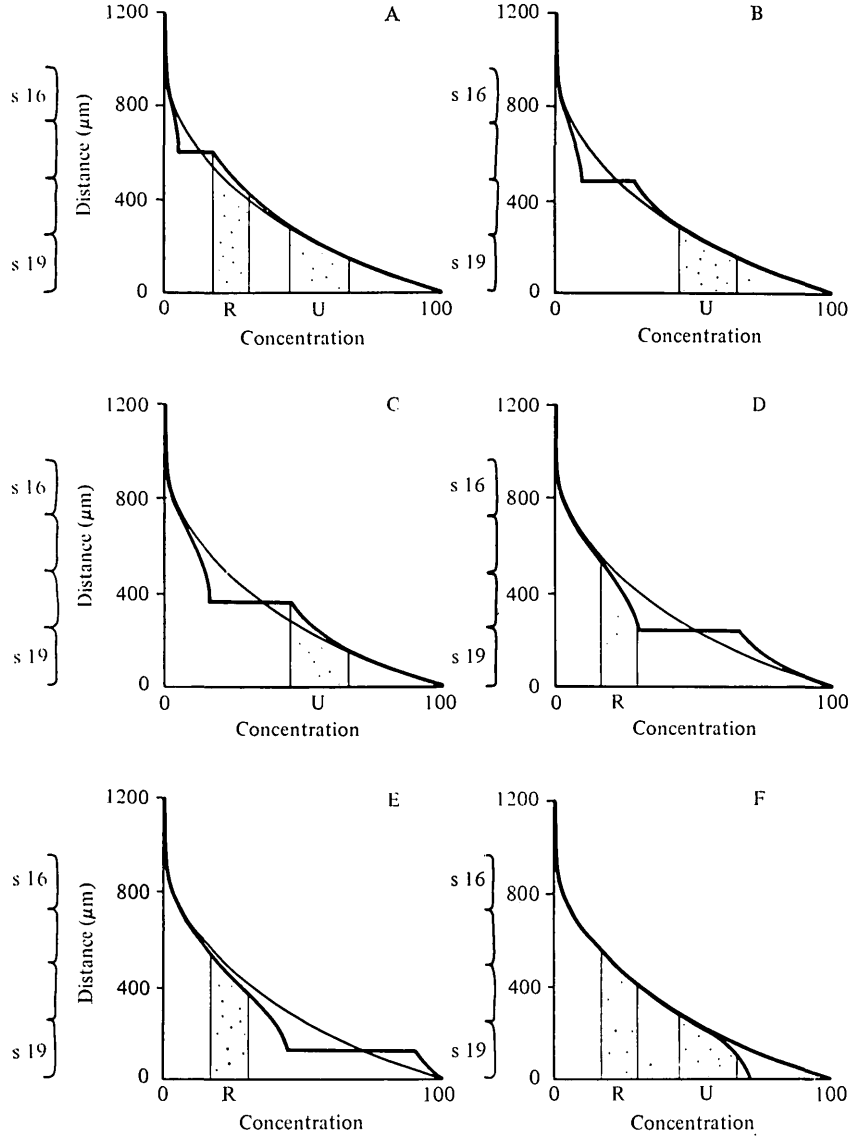

Fig. 4. Concentration profiles at stage 20 following barrier insertion at stage 17. The discontinuity in the heavy line shows the position of the barrier. The light line shows the profile for a normal limb without barrier at the same stage. Stipple shows the concentration range specifying a skeletal element. Figure 4F has two heavy lines. The upper is the profile when some ZPA lies anterior to the barrier. The lower shows the profile when the barrier just misses the ZPA.

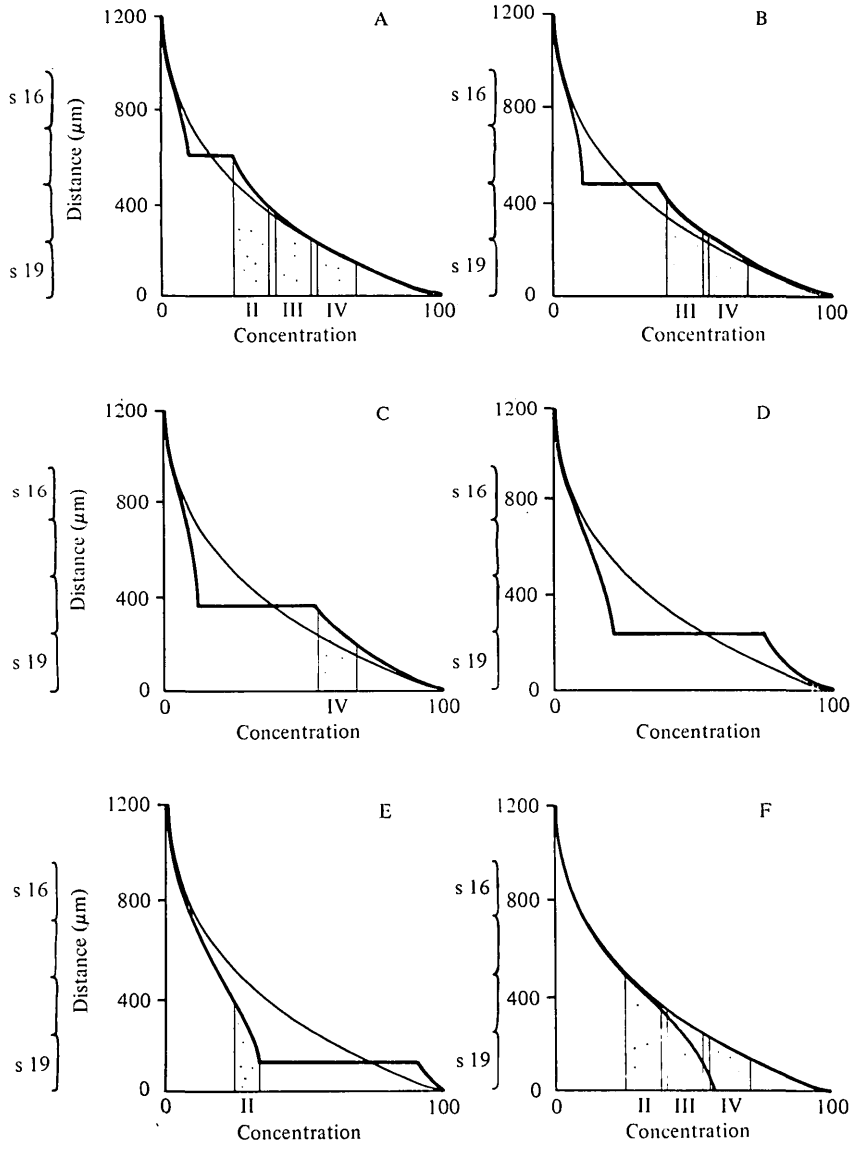

Fig. 5. Concentration profiles at stage 25–26 following barrier insertion at stage 17. The discontinuity in the heavy line shows the position of the barrier. The light line shows the profile for a normal limb without barrier at the same stage. Stipple shows the concentration range specifying a skeletal element. Figure 5F has two heavy lines. The upper is the profile when some ZPA lies anterior to the barrier. The lower shows the profile when the barrier just misses the ZPA.

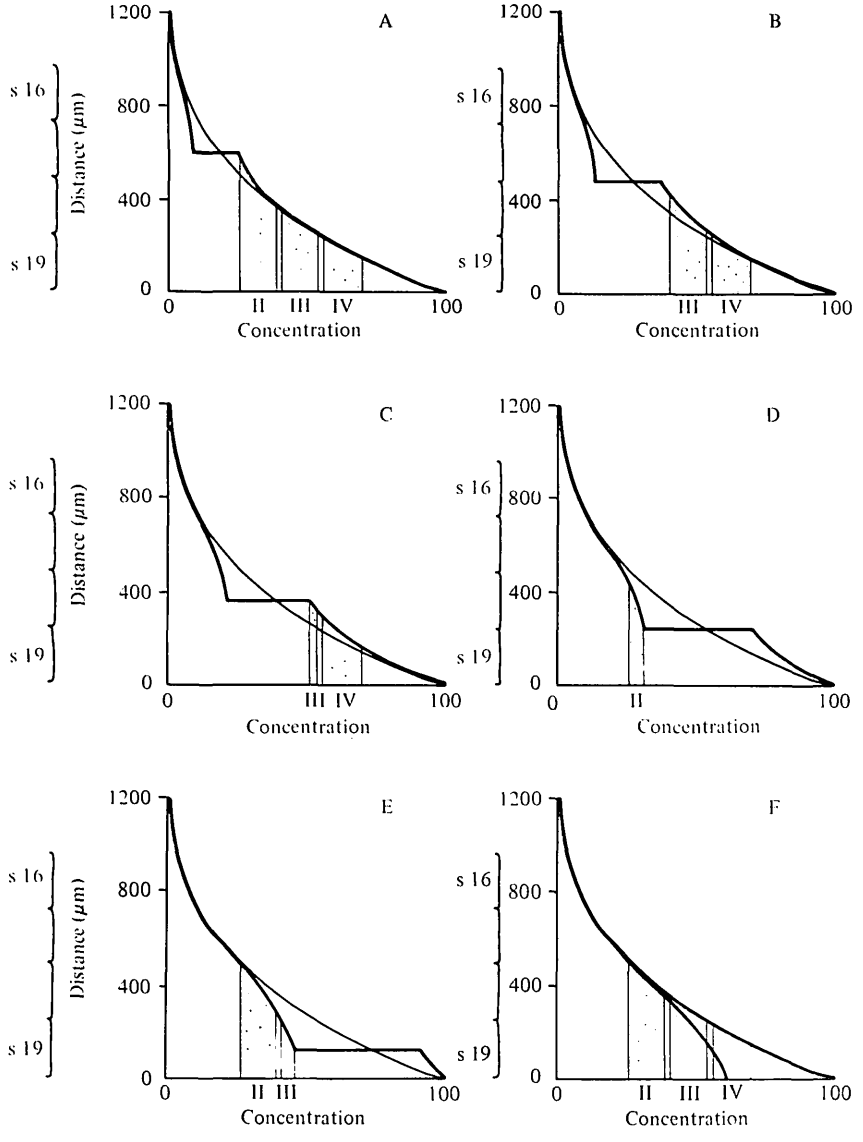

Fig. 6. Concentration profiles at stage 25–26 following barrier insertion at stage 21. The discontinuity in the heavy line shows the position of the barrier. The light line shows the profile for a normal limb without barrier at the same stage. Stipple shows the concentration range specifying a skeletal element. Figure 6F has two heavy lines. The upper is the profile when some ZPA lies anterior to the barrier. The lower shows the profile when the barrier just misses the ZPA.

intracellular diffusion via gap junctions of a small molecule of a few hundred daltons (see Crick, 1970; Wolpert, 1978). The start of the simulation (time = 0) was chosen to coincide with the time that the cranio-caudal axis first becomes autonomous (Saunders & Reuss, 1974). Barriers were inserted at appropriate times and the concentration profiles determined separately for zeugopod and autopod from the times at which they seem likely to be first specified (Summerbell, Lewis & Wolpert, 1973; Summerbell, 1974*a*, *b*; Summerbell & Lewis, 1975). Concentration profiles for the three main sets of data using tantalum foil are shown in Fig. 4–6. When the barrier is inserted at stage 21–22 the ulna and radius should already be specified and therefore both elements should always be present. This was found to be the case in 30 out of 34 embryos. Details of the simulation are to be found in the Appendix.

Following insertion of the barrier the concentration of morphogen anterior to the barrier (cut off from the ZPA) falls, while the concentration posterior to the barrier rises. This results in a discontinuity in the concentration profile. If the discontinuity is sufficiently large then entire skeletal elements are lost. The longer the time given the greater the discontinuity. If sufficient time is given the concentration anterior to the barrier drops to effective zero and specifies nothing, and the concentration posterior to the barrier rises to a level which again specifies nothing. If the barrier is placed so that it bisects the ZPA then a normal concentration profile develops anterior to the barrier and a normal limb results. The predicted outcome for each barrier position is shown by the stippled areas. The predictions provide a close fit to the observed data.

When a Millipore filter is used instead of tantalum foil the barrier is imperfect and does not stop all diffusion. It seems likely that the barrier causes a reduction in cell contact and in the intracellular flux rather than directly affecting extracellular diffusion (see review: Saxén, 1977). In other words, the barrier leaks. This means that over the same time period the discontinuity is less and a more complete set of skeletal elements can be formed. This is amply illustrated in Table 3 where at the zeugopod level 17 cases developed a bone on each side of the barrier compared with 16 deficient limbs. With the tantalum barrier the homologous figures were 3 complete and 41 deficient. At the level of the autopod the difference is less dramatic for the concentration profile has had time to move close to equilibrium conditions with both normal and leaky barriers. The results (Table 3) show increased variability when compared with the comparable tantalum foil experiment (Table 1).

Fate maps

By combining the three sets of concentration profiles it is possible to estimate the comparative concentration ranges specifying each skeletal level. One can then use the profile from a normal limb to estimate a fate map for the anterior-posterior axis (Fig. 7). This fate map correlates tolerably well with the fate map of Stark & Searls (1973). It is worth comparing these views on the Stark &

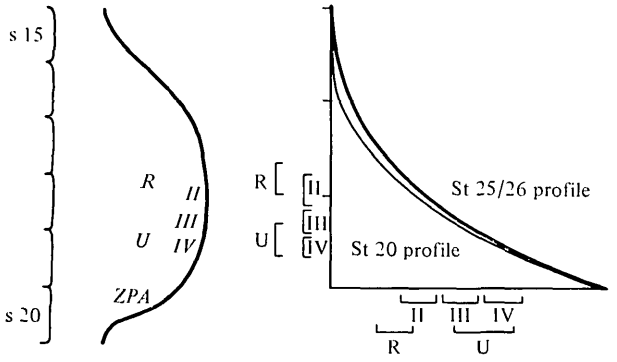

Fig. 7. Concentration profiles at stages 20 (light) and 25–26 (heavy). The horizontal axis shows the concentration ranges specifying each skeletal element and the vertical axis shows the positions in which the elements are specified. This is interpreted in a presumptive fate map.

Searls paper with those of Amprino (1976, 1977), who produced good evidence of development of skeletal elements from tissue outside the fate map. It is possible that Amprino's experiments could be explained equally well by this diffusion model, reconciling these two important and apparently contradictory works.

CONCLUSION

The predictions made by the proposed model fit the observed data better than predictions made by a purely mosaic model. This suggests that the ZPA may well play a role in normal morphogenesis.

I stated at the start of this paper that the key issue is the behaviour of the limb-bud following removal of the ZPA. MacCabe *et al.* (1973), Fallon & Crosby (1975*a*), and Tickle *et al.* (1975) have all reported obtaining normal or near normal limbs after removing the zone. I will now re-examine the data in the context of this model. Figures 4F, 5F and 6F illustrate an operation in which a barrier is placed at the anterior edge of the ZPA. In each case two concentration profiles are shown. In one the barrier just excludes ZPA from the rest of the limb, and in the other it just includes some of the ZPA. In the latter case the resulting limb is normal. In the former case the resulting limb is deficient. When the barrier is inserted at stages 16/19 a limb with normal ulna, radius, digit II and digit III but with no digit IV results. When the barrier is inserted at stages 20–22 most of the limb is again normal but only part of the field for digit IV is present. Digit IV should therefore be represented but in an abnormal or reduced form. MacCabe *et al.* (1973) never obtained a digit IV when they operated at stage 17–18 and often obtained considerably less. When they operated at stages 19–24 they obtained more complete limbs with all skeletal elements represented in 8 out of 15 cases. Fallon & Crosby (1975*a*) performed 47 experiments in the stage 19–24 group. They reported 70% deficient limbs

(usually involving the ulna and digit IV) and 30% normal limbs including digit IV. The model therefore also provides a better fit to this data than a purely mosaic model.

The results also provide good support for the experiments and conclusions of MacCabe & Parker (1976) and MacCabe, Calandra & Parker (1977). The morphogen that they assay clearly shares many of the properties of the ZPA morphogen and I concur with MacCabe & Parker in seeing little reason for not accepting them as being two expressions of the same phenomenon. The concept of apical ectodermal ridge maintenance factor is misleading and unhelpful.

While these results do not support the idea of a strong positional memory they do provide evidence for a short-term memory provided by the diffusible signal. One should bear in mind that once the concentration profile nears equilibrium even a very much reduced activity by the ZPA is sufficient to maintain the concentration range necessary to specify the entire skeleton. Such a low activity ZPA could, on grafting to the anterior edge, be too weak to produce any additional digits (cf. attenuated signals from the ZPA, Smith *et al.* (1978)), and would therefore be very difficult to detect.

This work was inspired by the publication of the Third Symposium of the British Society for Developmental Biology: 'Vertebrate Limb and Somite Morphogenesis'. I am indebted to all of the participants and wish that I could have been there.

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Appendix

For the simulation of diffusion the limb is treated as a one-dimensional line of cells along the anterior-posterior axis. Using the parameters chosen the line is effectively semi-infinite as the concentration does not rise appreciably at the anterior end during the period studied. The concentration profile is calculated by the Schmidt–Binder method (see references), in which the new concentration ($C_{i(t+\delta t)}$) is given by the local average concentration ($C_{(t)}$) at time (t). Thus:

$$C_{i(t+\delta t)} = F_0 (C_{i-1(t)} + C_{i+1(t)}) + (1 - 2F_0) C_{i(t)}. \quad (1)$$

Where

$$F_0 = \frac{a.\delta t}{(\delta s)^2}. \quad (2)$$

Where a is the diffusion coefficient ($cm^2\ sec^{-1}$), δt is the time interval (sec), and δs is the distance between points (cm). In practice F_0 must be less than or equal to $\frac{1}{2}$. For the simulation a, δt, and δs are chose so that F_0 is exactly one half so that:

$$C_{i(t+\delta t)} = \tfrac{1}{2}(C_{i-1(t)} + C_{i+1(t)}). \quad (3)$$

This gives a very rough estimate of the real concentration profile and in practice the simulation is improved if $C_{1(t)}$ is given half its normal value for the first round of diffusion. I have followed this convention.

In the example illustrated the start time for the simulation is stage 11. This is chosen to conform to the time when asymmetry along the anterior-posterior axis of the presumptive limb rudiment becomes determined (Saunders & Reuss, 1974). The simulation then runs for 80 h (or 10 time periods) until stage 25–26. The concentration of morphogen is held constant at 100 units at the ZPA but it is degraded elsewhere. I have used a value for degradation similar to that used in Tickle, Summerbell & Wolpert (1973) so that at equilibrium the concentration 1 mm away from the ZPA will be about 10 units. Provided that degradation is a power function (so that the morphogen is destroyed relatively rapidly at high concentrations and relatively slowly at low concentrations) the simulation is insensitive to variation in the rate of degradation.

During early stages of the simulation it is necessary to take into account expansion of the anterior-posterior axis. Working from the data of Herrmann, Schneider, Neukom & Moore (1951) I estimate that the length of the limb region approximately doubles from stage 11 to 17. From stage 17 on the axis maintains constant length.

The simulation is relatively sensitive. Fine tuning is possible by modifying the diffusion constant, the compartment size and the time interval (these three are inter-related in equation (2)). It is very sensitive to the relative timing of events and to the position of presumptive fate maps. If these two were very

different from the estimates used then it would be difficult to fit a simulation of this type. This is very satisfactory for these two are of course determined by observation.

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