

Control of maintenance and anteroposterior skeletal differentiation of the anterior mesenchyme of the chick wing bud by its posterior margin (the ZPA)

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SUMMARY

If the posterior half of the chick wing bud (stages 17–22) is excised, the anterior half which normally forms humerus (part), radius and digit 2, forms only a single skeletal element, either humerus or humerus fused with reduced radius. Beginning at 18 h after operation, and continuing to 48 h the anterior and distal mesenchyme in such anterior halves becomes necrotic and the AER regresses. By contrast, if the anterior half of the chick wing bud (stages 17–22) is excised, the posterior half develops as in the normal bud, and forms humerus (part), ulna and digits 3, 4 and 5. Such posterior halves develop no more mesenchymal necrosis than the normal contralateral wing buds and the AER remains healthy. Further, if the excision of the posterior part is made in such a way as to leave in place a part of the zone of polarising activity (ZPA), a normal wing with complete skeleton is formed. Thus in order to survive and differentiate, the anterior part of the wing bud needs a factor supplied by the posterior part containing the ZPA. These results support the view that the ZPA plays a role in controlling the anteroposterior differentiation of the *normal* wing bud.

INTRODUCTION

The first experimental studies (Murray, 1926; Huxley & de Beer, 1934) on the chick embryonic limb raised questions – such as whether its development was ‘mosaic’ or ‘regulation’ – which are still actively discussed today (Kieny & Pautou, 1976; Wolpert, 1978; review Hinchliffe & Johnson, 1980). The question of the mosaic development or regulation of experimental excesses or deficiencies has been studied in detail along the proximodistal axis. The possibility of regulative development along the anteroposterior (A-p) axis has,

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however, been relatively neglected since the early work of Murray (1926), Warren (1934) and Wolff & Kahn (1947*a, b*). In one of these studies, barriers inserted along the A-p axis showed the limb bud lost its regulative capacity along this axis at stage 18 (Wolff & Kahn, 1947*a, b*). The detailed study of Warren (1934) who also inserted barriers along the A-p axis of the wing bud, and studied skeletal development on either side of the barrier, led him to the conclusion that development along this axis was mosaic, though there was some evidence that the anterior part in this experiment developed less than its prospective fate.

In recent years, analysis of control of the A-p axis has focused on the ZPA (zone of polarising activity) an area of post-axial mesoderm which, in one of the most striking of recent embryological experiments (Saunders & Gasseling, 1968; Saunders, 1972; Summerbell & Tickle, 1977), has been shown to have the capacity for causing limb duplication when transplanted to the preaxial margin of a wing bud. Following such a graft, the preaxial apical ridge (AER) lengthened, and a preaxial mesenchymal excess appeared which differentiated into the supernumerary limb whose digit 4 was always adjacent to the ZPA graft. MacCabe, Gasseling & Saunders (1973) mapped out the duplicating capacity of the different areas of the wing bud, identifying a high activity area (the ZPA, producing 50% or more duplications), an adjacent intermediate area (generally below 50% duplications) and an area without activity.

Impressed by such duplication properties, Saunders initially considered the ZPA probably also controlled the normal A-p axis of amniote limb development. Increasingly, however, doubts have arisen as to whether the ZPA duplicating capacity implies that it controls the A-p differentiation of the *normal* wing bud. Fallon & Crosby (1975), found that, following ZPA removal, a wing bud developed normally, though digit 4 was sometimes affected. Moreover, ZPA properties were not restricted to the ZPA itself, since they were also found in considerable areas of non-limb mesoderm (Saunders, 1977). These considerations led Saunders (1977) to conclude that the ZPA is not a factor in normal limb morphogenesis, a view which however is still disputed by Smith (1979), Wolpert (1978), Summerbell & Tickle (1977).

The experiments reported here were designed to resolve this controversy concerning ZPA action, and comprise amputations planned to totally excise regions of both higher and intermediate ZPA activity or to include part of the ZPA, rather than simply to delete the zone of higher activity (hereafter called the ZPA).

When it became clear that removal of the areas of high and intermediate ZPA activity resulted in failure of anterior and distal prospective areas to differentiate their normal skeletal parts, studies of the pattern of cell death resulting from the operation were carried out. Cell death is an important feature of normal wing development (Saunders, Gasseling & Saunders, 1962), and variation in its extent in different mutants (e.g. wingless, talpid) is causally

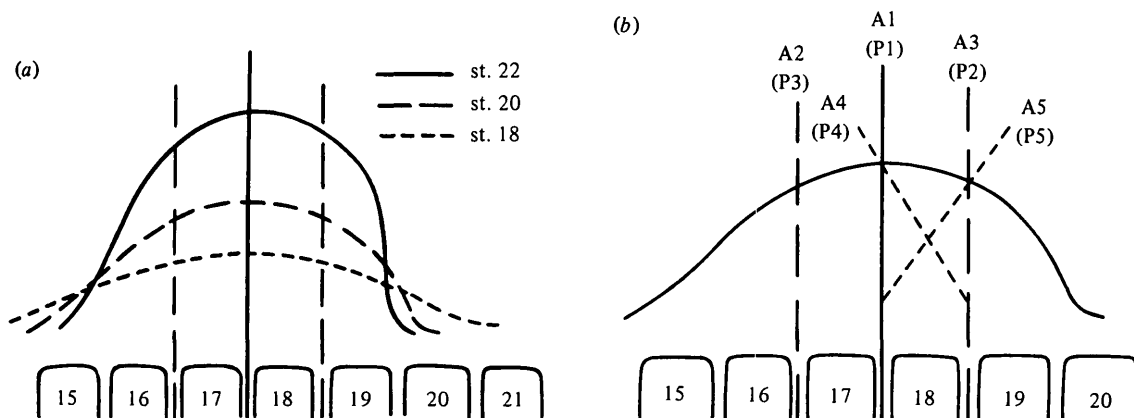


Fig. 1. (a) The chick wing bud at stages 18, 20 and 22 shown in relation to the somites which are used to mark the midline (17/8) and one third (16/7) and two thirds (18/9) lines in the experiments.

(b) Diagram of chick wing bud showing the amputations carried out. The A1 (P1) line indicates either the amputation of an anterior half (A1) or posterior half (P1). See text for details.

related to the characteristic pattern of wing abnormality (reviewed, Hinchliffe, 1981*a, b*).










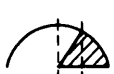


MATERIAL AND METHODS

Chick embryos of stages 17–22 (Hamburger & Hamilton, 1951) of White Leghorn stock were operated on, following windowing on the third day of development. The position of the wing bud was determined in relation to the somites. The dividing line between anterior and posterior half lies between somite 17 and 18 with little individual variation. The width of the wing-bud base narrows as development proceeds, and the bud-somite relationship at the different stages is summarized in Fig. 1*a*, which is based on examination of 86 embryos. The convention is adopted that the one-third division line lies between somites 16 and 17, and the two-thirds division line between 18 and 19 (Fig. 1*b*).

Using Pascheff–Wolff microscissors (Moria), anterior or posterior parts were removed. Initially half wing buds were removed, later one third and two thirds (anterior and posterior) were deleted, while finally cuts were made at different angles (Fig. 1*b* – see results). Embryos were resealed and allowed to develop for a further 3 or 4 days for histology, or 4 or 5 days for skeleton clearance preparations. Limbs were cut off, fixed in Carnoy's, and either blocked, sectioned and stained with haematoxylin and eosin, or made into skeleton clearance preparations by the method of Lundvall (1927).

For another group of embryos, studies were made of the pattern of cell death in the wing buds at between 5 h and 2½ days following deletions of the

Table 1. *Amputations – summary table*

Type	Total number of cases	Macroscopic examination (no fixation)	Skeleton examination			
			Histology	Skeleton stained (Lundvall)	Total	
Anterior amputations						
$\frac{1}{2}$ 	A ₁	24	4	5	15	20
$\frac{1}{3}$ 	A ₂	13	5	2	6	8
$\frac{2}{3}$ 	A ₃	26	6	—	20	20
	A ₄	29	7	7	15	22
	A ₅	19	2	2	15	17
Posterior amputations						
$\frac{1}{2}$ 	P ₁	28	2	9	17	26
$\frac{1}{3}$ 	P ₂	41	4	12	23	35
$\frac{2}{3}$ 	P ₃	11	4	—	7	7
	P ₄	12	—	2	8	10
	P ₅	21	—	—	18	18
	P ₆	16	3	1	12	13
	P ₇	15	3	1	11	12

Numbers of anterior (A1–5) and posterior (P1–7) amputation experiments carried out (see text). The diagram under ‘type’ shows the amputation (anterior to left: the dotted midline passes between somites 17/18, the dotted line to the right between somites 18/19 – see Fig. 1*b*).

Table 2

Experiment	Total no. of cases	Stylopod H	Zeugopod		Autopod		
			R + U	U	d2	d3	d4
A1	20	20	1	19	7	20	20
A4	22	21	1	20	6	21	22

Analysis of the wing skeleton which results from anterior amputations of type A1 and A4. See text for further detail (H, humerus; R, radius; U, ulna; d2-4, digits 2-4).

types previously described. The number of embryos examined was as follows: anterior deletions (A1-A3) 22, posterior deletions (P1-P3) 144. The cell death pattern in the more numerous posterior deletion group was examined mainly by vital staining at 12, 18, 24, 36 and 48 h after operation (P1-P3, 84 embryos). Some of the wing buds were fixed, sectioned and stained with haematoxylin and eosin. Vital staining *in ovo* was carried out using neutral red which becomes concentrated in the dying cells and the macrophages. Many of these wing buds were made into permanent preparations following fixation with formol-calcium (which preserves the neutral red) according to the method described by Hinchliffe & Ede (1973).

RESULTS

The different types of excision are summarized in Table 1. Two hundred and fifty-five embryos were operated on, of which 208 embryos were fixed after 3-5 days development for examination of the skeleton.

(A) Anterior amputations

(a) Amputation of anterior half (A1)

Twenty cases were studied for skeletal development (Table 2). The most frequent result (Fig. 2B, 3A, 4A) was humerus (H), ulna (U), digits 3 and 4 (d3, d4). The anterior elements radius (R) and digit 2 are usually missing. The humerus is reduced in size, the ulna is normal. 'Digit 2' develops in 7 cases out of 20, and these results are from operations at earlier stages, since the average stage of operation is 19 where d2 forms, but is 20.4 where d2 is absent. Such 'digits 2' are frequently small and composed of a single element.

(b) Amputation of anterior third (A2)

In all the 8 cases, the skeleton was complete (Fig. 3B). However, there was clearly an anterior deficiency in soft tissue at the level of the base, while the humerus was shortened (Ratio of lengths of experimental H/control H:0.8).

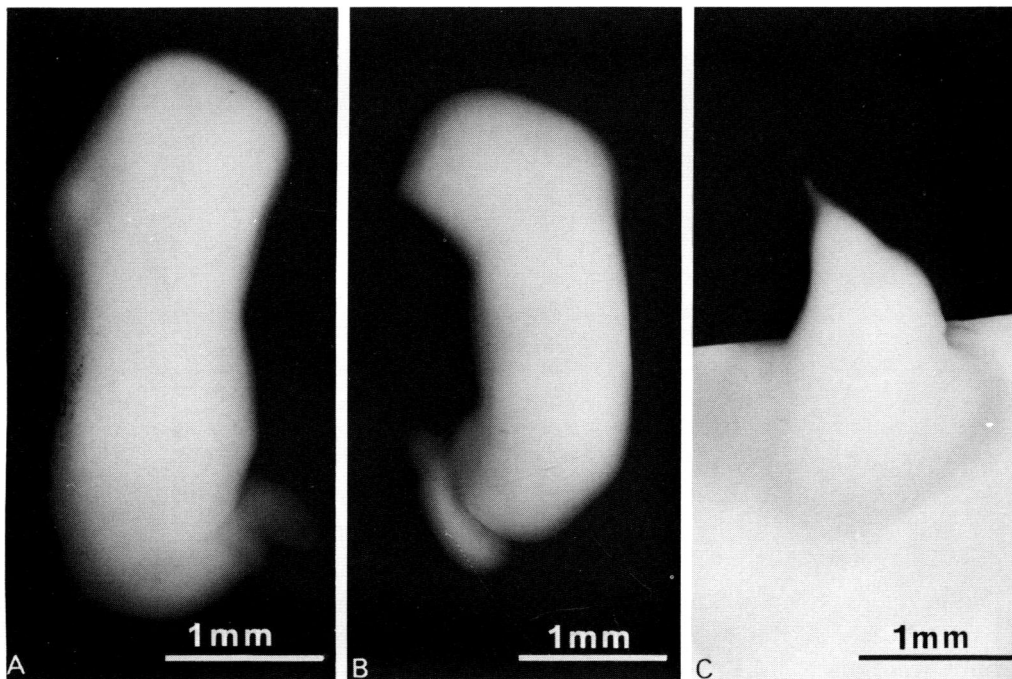


Fig. 2. External appearance of wings 3 days after anterior or posterior half amputation. (A) control (stage 20 plus 3 days) (B) amputation of anterior half (A1) at stage 21; (C) amputation of posterior half (P1) at stage 21; note the subsequent regression.

(c) *Amputation of anterior two-thirds (A3)*

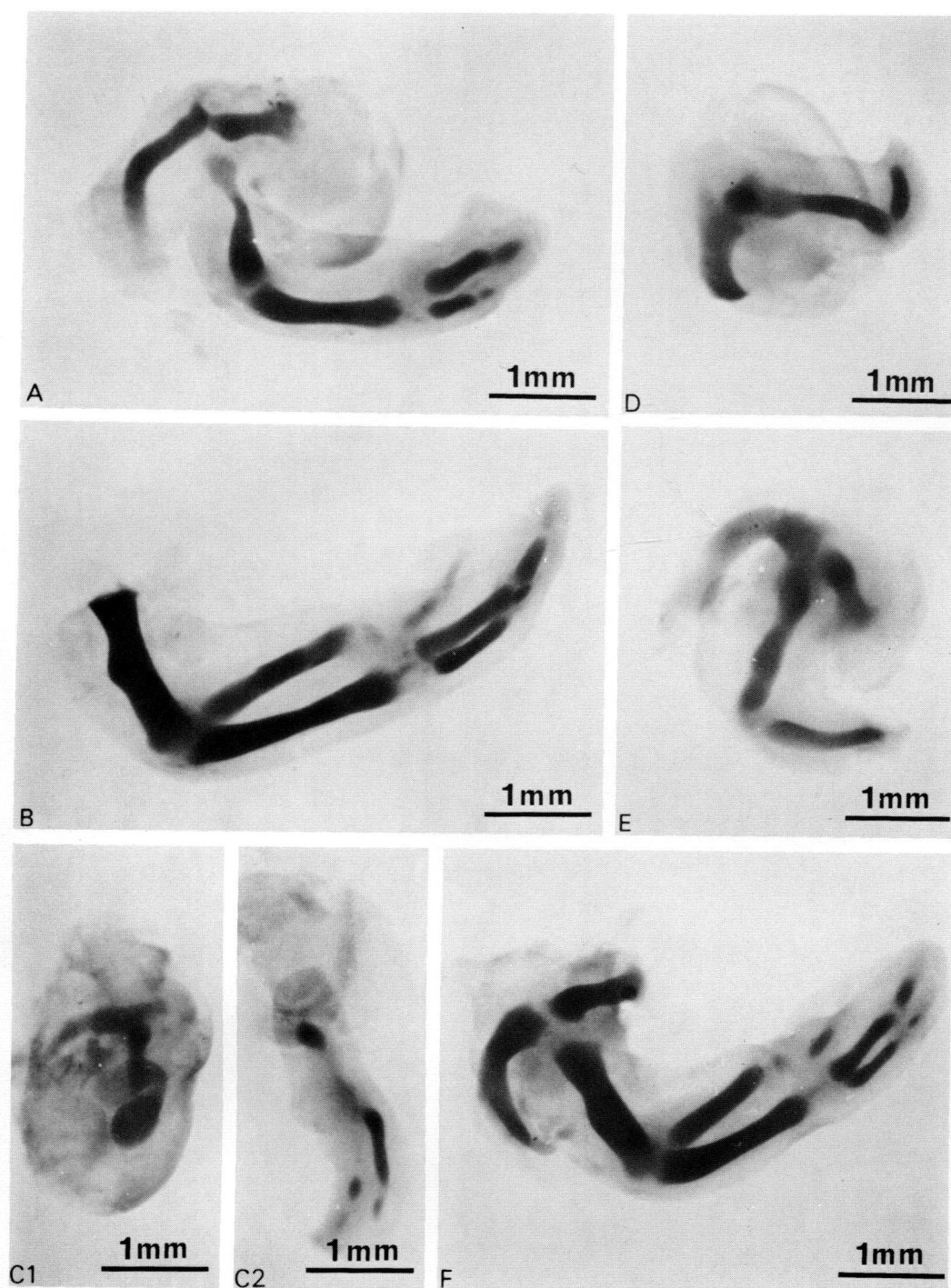
In all 20 cases studied, a reduced limb, much wider at the distal extremity than at the base, is formed (Fig. 3C). In nine cases, there is no skeletal development within the outgrowth (Fig. 3C₁). In six cases, there are three morphologically abnormal skeletal elements aligned along the proximodistal axis. In five cases, the two most distal elements are doubled, and these are considered to represent abnormal digits of the autopod, while the proximal elements represent what is left of the stylopod and zeugopod (Z) (Fig. 3C₂).

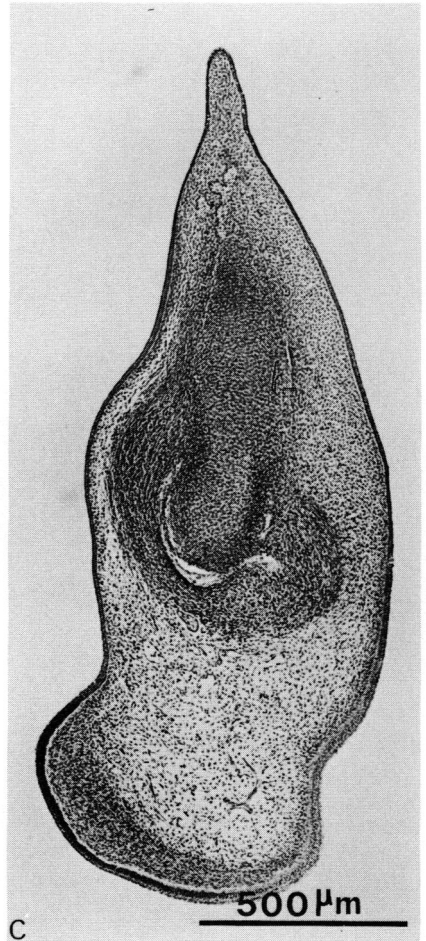
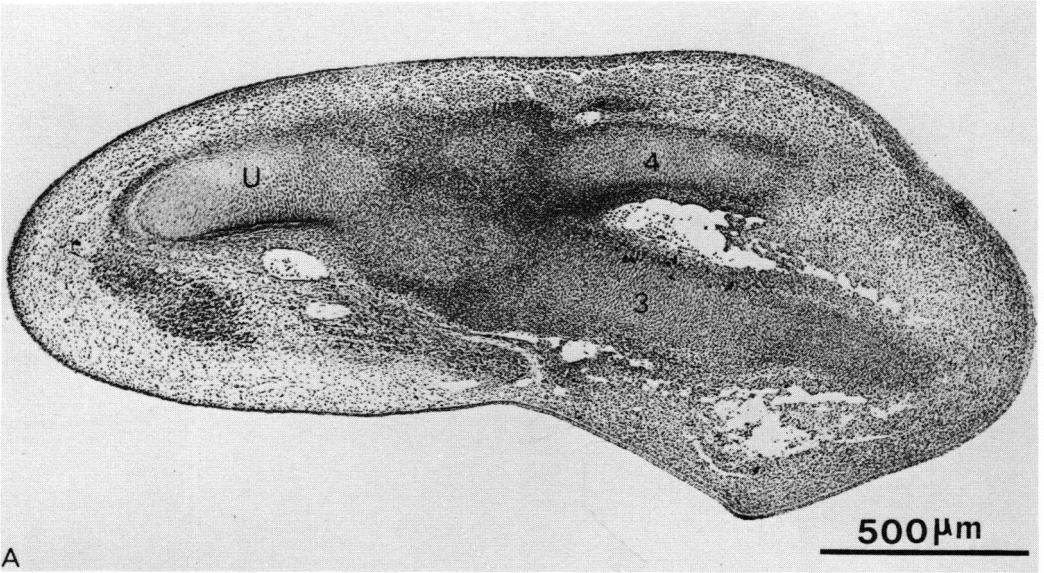
(d) *Amputation of anterior part (A4)* (Fig. 1b)

Twenty-nine cases were studied (Table 2), of which seven were examined macroscopically and were not fixed (omitted from table). The angle of cut was

FIGURE 3

Clearance preparations of wing skeletons 4 days (except B) after anterior or posterior amputation. (A) anterior half amputation (A1) at stage 20; (B) anterior one third amputation (A2) at stage 20 (5 days after operation); (C₁₊₂) anterior two thirds amputation (A3) at stages 19 (C1) and 21 (C2); (D) posterior half amputation (P1) at stage 20; (E) posterior one third amputation (P2) at stage 20; (F) control, stage 20 plus 4 days. Note the severe anterior regression which follows the amputation of posterior parts.





varied slightly in some cases, sometimes including distal material just posterior and sometimes just anterior to the line A4 as drawn in Fig. 1*b*. When more anterior-distal material was included, the second digit was frequently present, but when the cut was more posterior, digit 2 was almost always absent. Apart from this, the variation in angle did not significantly affect the ensuing skeletal pattern, so that Table 2 does not distinguish between these variations.

The typical result is: H (very reduced), U, d3 and d4. In the histological preparations, the humerus appears to fuse with the ulna as a single element, while in the clearance preparations, the humerus is always very small. The fact that a reduced humerus develops at all suggests that the prospective humerus is very extensive, extending posteriorly into material at the level of somite 19.

(e) *Amputation of anterior part (A5)* (Fig. 1*b*)

The 17 cases studied produced results similar to those in A3, except that the proximal elements were slightly better formed. Thus the typical result (seven cases) is a single row of elements aligned along the proximodistal axis, while in some six further cases the most distal elements are doubled.

Thus, with anterior deletions, skeletal deficiency is proportional to the quantity of wing bud removed. As reported by Warren (1934) and Wolff & Kahn (1947*a, b*), the wing bud does not regenerate or regulate in any major way for deficiency along the anteroposterior axis. Removal of anterior wing-bud parts does not inhibit development of the remaining posterior wingbud. Anterior deletions give quite different results from posterior amputations, in which there is no quantitative relation between quantity of wing bud removed and proportion of skeleton developing. Posterior amputations are described next.

(B) *Posterior amputations*

These amputations are particularly interesting (Fig. 1*b*, Fig. 5) because they are designed to show the effects of excluding from the wing bud the ZPA, or including a ZPA portion (according to the ZPA maps by MacCabe *et al.* 1973).

(a) *Amputation of posterior half (P1)*

The limb which develops is very reduced, tapering to a point (Fig. 2C). Twenty-six cases have been examined for skeletal development which can be classified as follows:

2 cases: no skeleton – average stage of amputation 17.5

17 cases: a single elongated skeletal element, tapering distally, and representing either H or fused H-R (Fig. 4C) – average stage of amputation 19.6

FIGURE 4

Histology of wings 3 days after anterior or posterior amputation. (A) anterior half amputation (A1) at stage 21 (same limb as Fig. 1*b*); (B) posterior half amputation (P1) at stage 21 (same limb as Fig. 2C); (C) posterior half amputation (P1) at stage 19 (H, humerus; R, radius; U, ulna; 3, 4 digits).

Table 3 (P2)

Total no. of cases	Stylopod H	Zeugopod (No. of elements)			Autopod (No. of digits)			
		0	1	2	0	1	2	3
35	35	2	31	2	21	11	2	1

Analysis of the wing skeleton which results from posterior amputation of type P2. See text for further detail.

5 cases: two distinct skeletal elements, H and R, (Fig. 3D, 4B) – average stage of amputation 21.3

3 cases: three aligned skeletal elements.

The total length of the limb is related to the stage of operation: the limb is longer when the operation is at the later stages.

(b) Amputation of posterior one third (P2)

As with P1, the limb is shortened and pointed (Fig. 3E). Shortening is very severe: the average length is only approximately one third that of the control limbs. The typical skeleton consists of a humerus, one zeugopod element and no digits (Table 3). The reduction in skeleton is very severe distally and is more marked the earlier the amputation stage i.e. (i) limb with one element (H or H-Z fused): 2 cases, average stage of amputation 18, (ii) limb with two distinct parts (H+Z): 19 cases, average stage of amputation 19.3 (Fig. 3E), (iii) limb with three distinct parts (H+Z+d): 14 cases, average stage of amputation 20.

The humerus is always present and has its normal form (apart from reduced diameter) and length. The single zeugopod element is difficult to identify as either radius or ulna. The autopod skeleton is usually absent but even when present it is often (11 cases out of 14) reduced to a single element.

(c) Amputation of posterior two thirds (P3)

In all 11 cases, the limb is reduced to a small rounded protuberance terminating in a point: skeletal elements are completely lacking.

(d) Amputation of posterior part (P4) (Fig. 1b)

The results are essentially similar in overall form and skeletal development to those obtained with posterior half amputation (P1). Of the ten cases studied for skeletal development, one has no skeletal elements, three have a single element (H or H-R fused), and six have two skeletal elements (H plus one zeugopod element).

(e) *Amputations of posterior part (P5–P7, Fig. 1b)*

These form a group of angled cuts designed to either exclude ZPA, or include a ZPA fragment in the remaining wing bud (Fig. 5). In P5, the cut goes from the apex at somite 18/19 level, to the base at somite 17/18 level, so that ZPA material is excluded. In P7, the cut begins in the apex a little more posteriorly, so that ZPA material is included.

Amputation P5. The limb forms a short spike, similar to those in P1. Of the 17 cases studied, in 16 there is only a humerus and one zeugopod element (Fig. 5B), in the remaining one there is in addition a digital element.

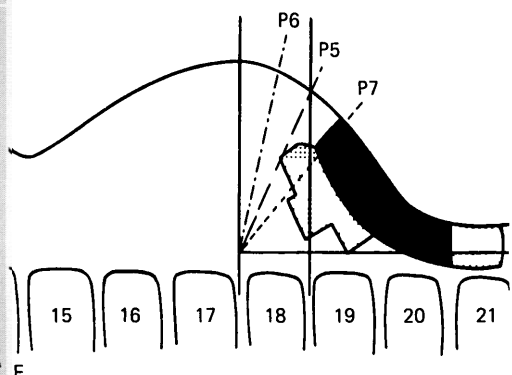
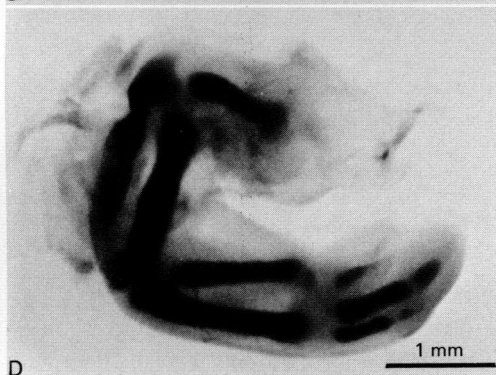
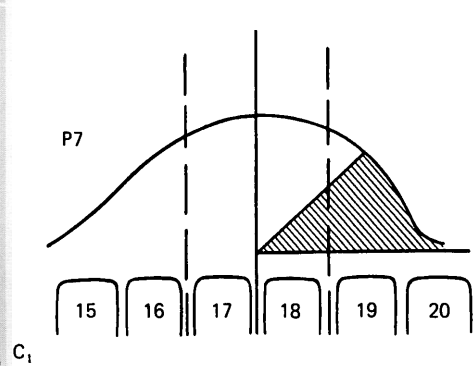
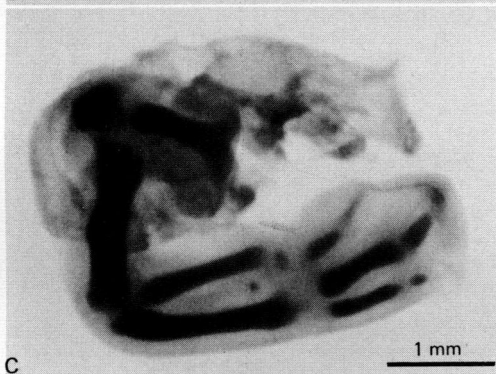
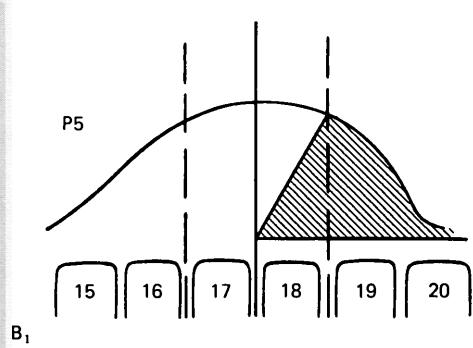
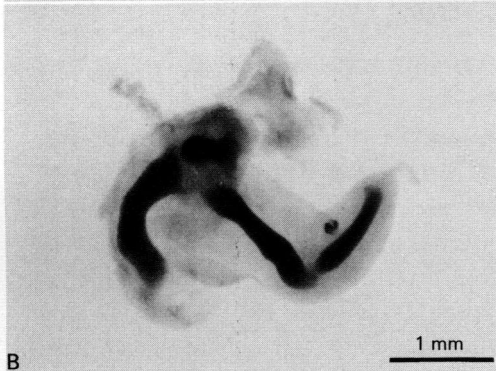
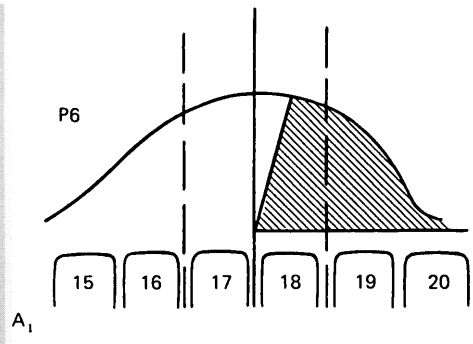
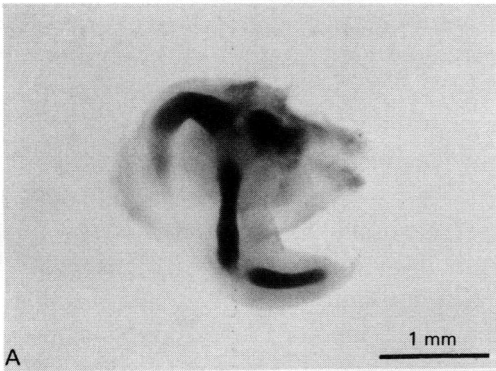
Amputation P6. With a more severe amputation than P5, the reduction in the limb (which again resembles those in P1) is also more severe. Of the 14 cases studied, in 11 (78%) there is only H+1Z (Fig. 5A), while in the other three there is only one skeletal element (H or H-1Z fused). No digits are formed.

Amputation P7. With a less severe amputation than P5, the form of the limb is markedly different, since it develops in most cases into a normal wing. Of the 12 cases studied, in 7 (58%) the skeleton is complete (H+2Z+3d) (Fig. 5C), in 3 there are H+1Z+2 or 3 digits, in one H+1Z+1 digit, and in one a single skeletal element (H or H-1Z fused). Thus a small change in angle, between P5 and P7 produces dramatically different results. The extent of the regression in P5 is clear: digits 2 and 3 and ulna all fail to form. In experiment P7, by contrast, not only are digits 2, 3, and 4 usually formed, but there appears to be some deficiency regulation, since in spite of the loss of much of the proximal posterior mesenchyme a normal-sized ulna is usually formed. The explanation is surely that part of the ZPA (as mapped by MacCabe *et al.* 1973) is left in all P7 experiments carried out on stages 19–22, while ZPA is always excised in P5 and P6 experiments at the same stages (Fig. 5E).

(C) *Pattern of cell death following amputations*

Anterior amputation. There is no increased mesenchymal cell death at either 24 or 48 h following amputation of the anterior half (A1) from wing buds of stages 18/9 to 22/3 (Fig. 6B, C). There is no anterior necrosis and the posterior necrotic zone (PNZ) which normally appears at stage 24 occupies its normal area. Such limb buds are narrow at the base, while distally the wider apex is covered by a healthy AER. Similarly, amputations of anterior one third and two third (A2, A3, Fig. 6D) do not cause any increase in mesenchymal cell death, and the AER remains healthy.

Posterior amputation. Vital staining shows that while at 12 h after amputations of the posterior half or one third (P1, P2) in wing buds of stages 18–22 there is little or no change in the cell death pattern, beginning at about 18 h after operation, there is increased mesenchymal cell death. In typical examples, following operation at stage 20, after 18–24 h there is a precocious and enlarged ANZ (the ANZ is just starting to appear in the stage-23 contralateral wing



bud), but cell death (at a lower frequency) is in addition found in the mesenchyme adjacent to the AER, which is also beginning regressive changes indicated by increased cell death (Fig. 6E, G, compare with control 6A). The enlarged ANZ contains macrophages, but in more distal mesenchyme, the neutral-red-stained particles are smaller and there are no macrophages, indicating that the cell death process is at an earlier stage. For this reason, the increase in cell death is interpreted as first enlarging the ANZ, and only later spreading in a posterodistal direction (Fig. 6G). By 24–36 h after operation, there is massive cell death marked by macrophage presence throughout the distal mesenchyme and there is marked regression of the AER which is necrotic, or has virtually disappeared (Fig. 6F, H). Massive distal cell death continues at 48 h after operation, though where this was carried out on later wing buds (stages 22 and 23) a part of the base remains healthy, cell death being restricted to the tip of the ‘spike’. Histological studies confirm that AER regression and sub-jacent mesenchymal cell death are associated, but do not indicate which event occurs first (Fig. 6J compare with 6I). The AER regression involves flattening and death of many of the ridge cells, which are phagocytosed by neighbouring ectoderm cells (Fig. 6J). Cell death is also found in the normal ridge (Jurand, 1965) especially preaxially at stages 24 and 25, but on a much smaller scale. Ectodermal cell death is restricted to the ridge. Histology also shows that the cell death process in the mesenchyme is similar to that previously described in the normal areas of cell death (Dawd & Hinchliffe, 1971; Hurlé & Hinchliffe, 1978). Isolated fragmenting dead cells appear among viable neighbours, some of which phagocytose them and become transformed into macrophages.

DISCUSSION

(1) *The role of the ZPA in normal wing morphogenesis*

In essence, these experiments show that the anterior part of the wing bud depends for its survival and differentiation on the presence of the posterior part. In relation to the controversy over the role of the ZPA in normal wing morphogenesis (Saunders, 1977; Fallon & Crosby, 1975; Summerbell, 1979), this work supports the interpretation that the differentiation of all the distal mesenchyme anterior to the ZPA depends on ZPA presence.

This conclusion is based on the spectacular reduction in wing skeleton

FIGURE 5

Skeleton clearance preparations showing effect after 4 days of posterior deletions which exclude (P5–6) or include (P7) ZPA material. (A) and (B), P6 and P5 amputations: note severe deficiencies in distal skeleton. (C) P7 amputation results in a normal wing skeleton. In (A)–(C) the amputation is shown diagrammatically on the right. (D), control. Diagram E (after MacCabe *et al.* 1973) shows ZPA material in relation to amputations P5–P7. Black represents ZPA, stippled mesenchyme of intermediate activity.

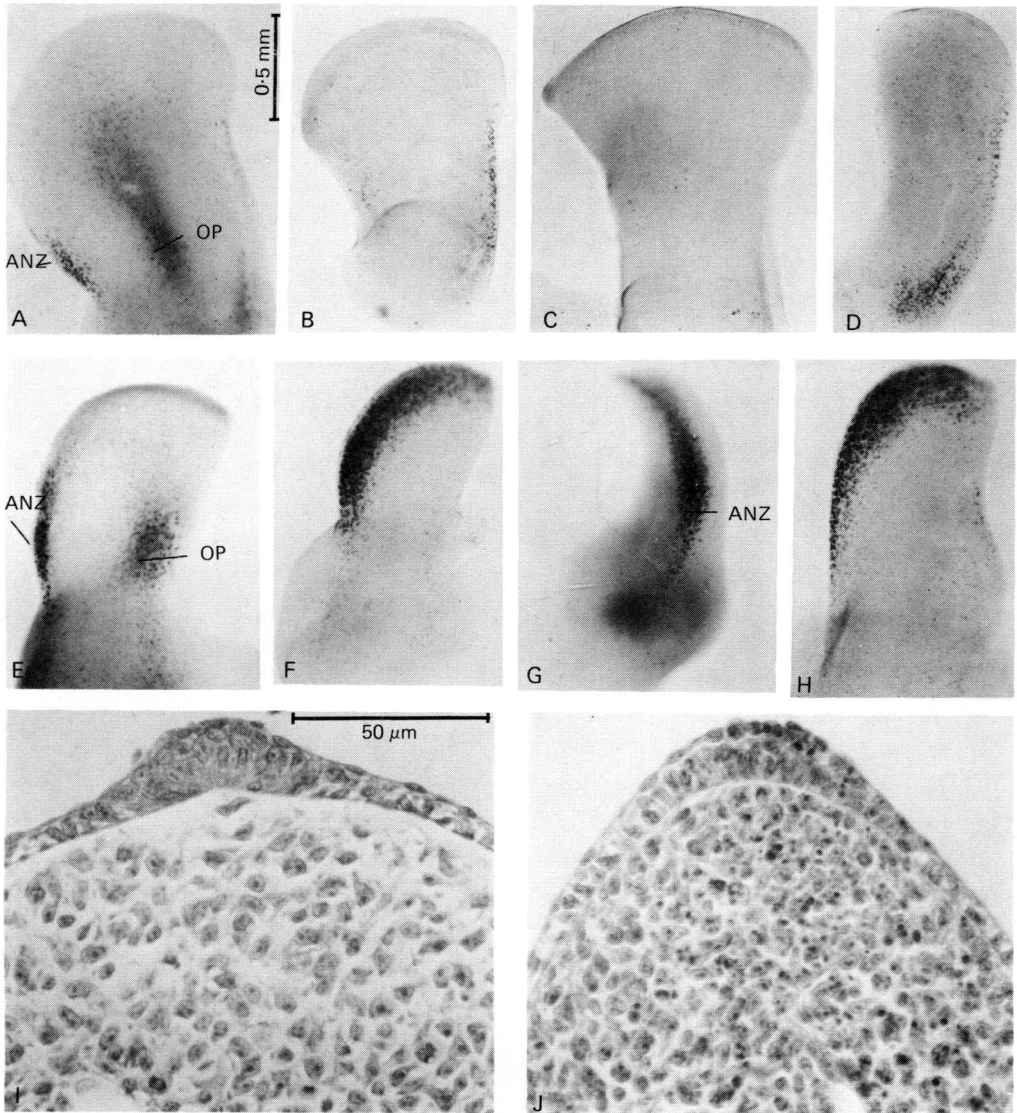


FIGURE 6

The pattern of cell death 1 day after amputations shown by vital staining (A-H). (A) control, stage 26. (B, C) Anterior half amputations (A1) at stages 17/8 and 22/3. (D) Anterior two-thirds amputation (A3) at stage 20. Note there is no increase in cell death above its normal level in the PNZ. (E-H) Posterior amputations. (E) posterior one-third amputation (P2) at stage 21. (F) posterior half amputation (P1) at stage 17/8. (G) posterior half amputation (P1) at stage 22/3, seen from anterior. (H) posterior one-third amputation (P2) at stage 21, 36 h later. Note the increased size of the ANZ (E, G) and its extension into the apical mesoderm (F, H). (I, J) Histology, (I) control, (J) posterior half (P1) amputation at stage 17/8 after 20 h. Note cell death in regressing AER, and in apical mesoderm. (ANZ, anterior necrotic zone; OP, opaque patch).

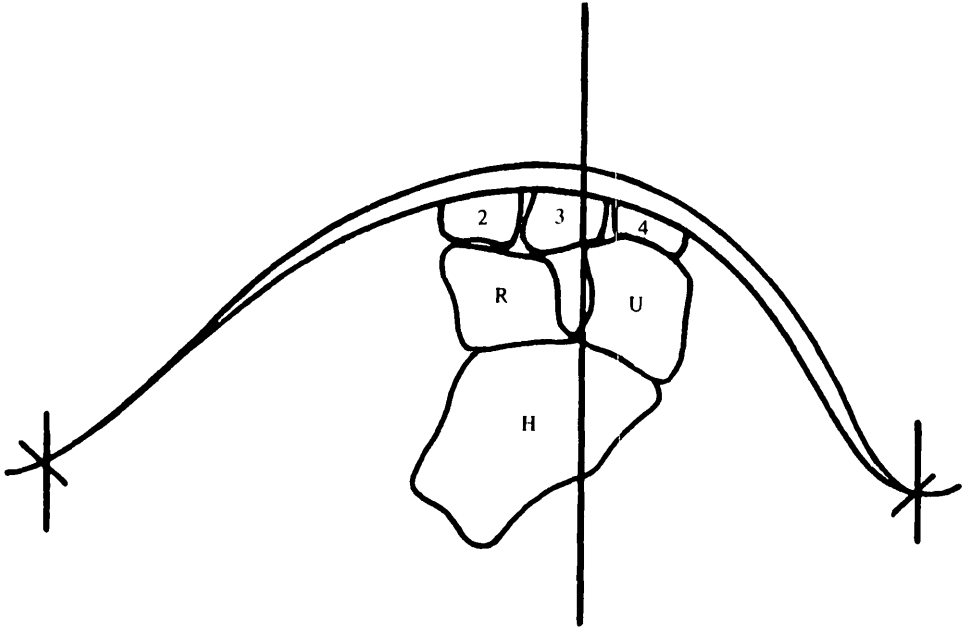


Fig. 7. The prospective areas of the stage-21 wing bud as mapped by Stark & Searls (1973), with the midline added. H, humerus; R, radius; U, ulna; 2, 3, 4, digits.

which follows posterior amputations (P1–6). For interpretation of these experiments, reference must be made to the Stark and Searls' map (1973) of the prospective areas of the wing, which was established by implanting small labelled mesoderm fragments into the wing bud. Experiment P2 is particularly interesting as according to the Stark-Searls' map (Fig. 7) all the skeletal presumptive areas are left in place in this experiment, since the one third removed which includes the ZPA makes no contribution to the skeleton. Yet with all prospective areas present only a humerus and stunted zeugopod element are actually formed. Decisive proof of the ZPA role is provided by the last experimental series (P5–7) in which ZPA is either partially or totally eliminated.

Following amputations of type P5 which extirpate ZPA, all digits and one zeugopod element are lacking, but after type P7 amputations in which virtually the same area of wing-bud mesenchyme is left, but this time including part of the ZPA, a normal wing skeleton is formed. The role of the ectoderm can be discounted since it is only the ZPA mesenchyme which has duplicating properties (Saunders & Gasseling, 1968).

These results appear to conflict with those of Fallon & Crosby (1975), who excised the ZPA (as mapped by MacCabe) and found the wing developed normally (apart from minor deficiencies in digit 4). Our experiments differ in that in posterior excisions (P1–6), resulting in anterior regression, the ZPA and the area of intermediate activity (producing generally below 50% dupli-

cations when grafted preaxially) were both removed. A possible explanation of Fallon & Crosby's results (1975) is that the areas of intermediate ZPA activity left in position may well be sufficient to 'polarize' the limb buds and permit anterior differentiation, since our own results show that only a fraction of the ZPA is required and since experimental duplication may be obtained with only a small part of the ZPA.

Recent experiments by Summerbell (1979) also support the interpretation of a normal role of the ZPA. Instead of amputating wing-bud parts, Summerbell inserted an impermeable barrier in various positions along the anteroposterior axis. When the barrier was placed at the level of intersomite 17/18, the anterior half produced no skeletal elements (as in P1) while the posterior half formed ulna and digits 3 and 4 (as in A1). Preliminary experiments carried out by Clare Gribbin at Aberystwyth suggest that the regression anterior to such a barrier is due to substantial cell death at 24 h after operation, as in our posterior amputations.

(2) *ZPA mode of action*

Models of the ZPA mode of action are currently under discussion. According to Tickle, Summerbell & Wolpert (1975) and Summerbell (1979) the ZPA acts as the source of a hypothetical morphogen whose level declines anteriorly in the wing bud. Different morphogen levels specify differentiation of digits 2-4. One difficulty for this theory raised by our observations is that only a part of the ZPA is required for normal development. Other experiments also show that experimental duplication requires only a fraction of the ZPA. One possibility that remains to be investigated is that a whole ZPA can be regenerated from a surviving fragment.

Another model is that of Fallon & Crosby (1977) who, regarding the ZPA as dispensable for later wing morphogenesis, suggest that the ZPA effect is exercised only during initial limb determination. According to Smith's (1979) interpretation of duplication experiments the wing bud can 'remember' the former presence of the ZPA. By contrast our own results, which show that anterior defects follow posterior amputations at stage 22, suggest that differentiation of the distal mesoderm depends on the continuous supply of a ZPA factor until at least stage 22.

(3) *Control of cell death by the posterior border of the wing bud*

These studies show clearly that the developmental failure of the anterior half in experiments P1 and P2 is due to cell death removing prospective skeletal areas throughout almost all the anterior and distal mesenchyme, beginning some 18 h after operation. The reciprocal experiment (A1) shows that the posterior and distal mesenchyme and its AER remain healthy. At present it is not clear whether the mesenchyme cell death proceeds AER collapse, or whether the two events are simultaneous.

It is reasonable to suppose that the increased anterior cell death is due, directly or indirectly, to ZPA removal. Cell death occurs normally in a restricted anterior necrotic zone (ANZ) which first appears at stage 22/3. Such zones of cell death are a feature of normal limb development (reviewed, Saunders, 1966; Hinchliffe, 1981*a*), but apart from the interdigital zones their role is still unclear. Saunders *et al.* (1962) and Saunders & Fallon (1966) investigated the control of the posterior zone (PNZ). They considered cell death was a normal end point of differentiation, and that prospective PNZ became programmed for cell death, one factor being its position within the limb bud, since close contact with central limb mesoderm could 'switch off' the death programme. There is also direct or indirect genetic control of these areas, which may be contracted, as in talpid (Hinchliffe & Ede, 1967) or expanded, as in wingless (Hinchliffe & Ede, 1973; Hinchliffe, 1976), causing respectively polydactyly or reduction in digit number. Indeed, wingless wing buds develop in a way strikingly similar to the experiments (P2, P5) in which ZPA material is removed: in both cases there is precocious appearance of an expanded ANZ which then extends posterodistally. The similarity is underlined by MacCabe's findings (Saunders, 1972) that the American wingless mutant (Zwilling, 1956, 1974) lacks a ZPA.

Further support for the hypothesis of ZPA control of the cell death pattern comes from MacCabe & Parker's *in vitro* studies (1975). If a small section of the preaxial border of the wing bud is cultured alone, or with non-limb or anterior limb-bud mesenchyme, the AER flattens and macrophages appear in the underlying mesoderm. By contrast, preaxial border combined with ZPA tissue continues as healthy mesoderm complete with AER.

A formal model for ZPA control of cell death in the ANZ* (modified from Ede, 1976) may be proposed, based on the view of Tickle *et al.* (1975) that the ZPA acts as the source of a diffusible morphogen whose different levels along the A-p axis specify the differentiation of digits 2-4. In our model, limb mesenchyme dies below a certain (low) threshold of morphogen. Normally only the extreme anterior mesenchyme (the ANZ) falls below this threshold but when posterior wing bud including the ZPA is removed, the level anteriorly falls so that the remaining anterior and distal mesenchyme is below the critical level and thus dies. This scheme has the advantage that it accommodates both the amputation and wingless observations. Present experiments (with D. Wilson) are testing the model by grafting quail ZPA material into the posterior border of early wingless wing buds.

Finally, it should be made clear that the changed pattern of cell death which follows ZPA removal is quite different from that in the distal mesenchyme resulting from AER removal (Amprino & Camosso, 1958; Cairns, 1975).

* The PNZ is considered less important since it appears only briefly in the chick and is absent from other avian species (Hinchliffe, 1981*a*).

Saunders (1977) claims that such cell death is not in the exposed sub-ridge mesoderm, but below the surface, and that it does not account for the distal skeletal deficiencies which follow AER removal. In similar experiments Kaprio and Tähkä (1978) have shown a lack of correlation between such mesenchymal cell death and skeletal deficiencies, since removal of anterior AER is followed by mesenchymal cell death but not skeletal deficiencies, while removal of central AER was followed by very little cell death and resulted in distal skeletal deficiencies.

(4) *Maps of presumptive areas*

Several studies carried out by barrier insertion or amputation since Warren's (1934) initial studies serve to underline the absence of regulation along the A-p axis of the avian wing bud. Even though the presumptive area of the wing bud is capable of regulation at very early stages, once the definitive wing bud is formed, regulation can no longer be obtained (Wolff & Kahn, 1947*a, b*). Thus the skeletal structures which develop in isolated posterior regions should correspond with their presumptive areas present in this part of the wing bud, and this is reasonably close to what we find.

One of the few accurate maps which attempts to locate prospective areas along the anteroposterior axis is that of Stark and Searls (1973) (Fig. 7), though here the somitic level is not clearly defined. Assuming mosaic development of posterior parts, our results are in broad agreement with this map but differ in the following points of detail:

Humerus: the presumptive area is wider along the A-p axis, extending from near intersomite 16/17 (A2) to 18/19 (A4).

Autopod: according to the map, digits 2 and most of 3 should not form following amputation of the anterior half (A1), and no skeletal elements should form after the amputation of the anterior two thirds (A3). But in our experiment (A1), digit 2 is formed in one third of cases, especially following early amputations, and digit 3 always forms, while in A3, skeletal elements form in the majority of experiments.

These differences at autopod level suggest the possibility that the posterior part of the limb, when it possesses ZPA, is capable of a limited degree of reprogramming. Such reprogramming appears greater distally (since it is particularly digits which appear when their prospective area is absent) and is more likely to affect the earlier wing buds. The possibility of reprogramming can only be tested after establishing a more precise presumptive map, since because of the possibility of this limited regulation the amputation experiments just described can not be used as the basis of such a map. We have now attempted by chimeric quail grafts (Gumpel-Pinot & Hinchliffe, in preparation) to construct a presumptive map which also enables us to check the accuracy along the A-p axis of the Stark-Searls map as well as the possibility of limited regulation along this axis.

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