

# Retinoic acid and pattern formation in the developing chick wing: SEM and quantitative studies of early effects on the apical ectodermal ridge and bud outgrowth

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## SUMMARY

When retinoic acid is locally applied to the anterior margin of developing chick wing buds on ion-exchange beads, dose-dependent changes in the skeletal pattern result. At low doses, additional digits develop. At high doses, there is thinning of the symmetrical wing. Local application of retinoic acid to the apex of the bud also leads to pattern changes, but in contrast normal wing patterns are almost always obtained following application posteriorly. These effects are manifest at 6–7 days after the operation although only a brief exposure (14–20 h) to retinoic acid is required. Therefore the morphology of wing buds was studied at shorter times after the start of treatment.

The local application of retinoic acid to the wing bud margin leads to changes in extent of the apical ridge that can be detected at 24 h after application. The behaviour of the apical ridge with varying doses and positions of retinoic acid application has been analysed quantitatively and dose response curves obtained. At low doses of retinoic acid, the length of the apical ridge increases or remains constant, but then progressively decreases with higher doses. The progressive obliteration of the ridge starts first near the bead and then involves more distant parts of the bud. Thus the region of the ridge affected depends on the position at which the retinoic acid is applied.

We propose that these effects on the apical ridge reflect dose-dependent responses to the local concentration of retinoic acid that varies with distance from the source. At high doses, the apical ridge disappears but at low doses it is maintained. Since grafts of polarizing region tissue also have a graded effect on ridge morphology, a possible interpretation of the retinoic acid effects is that tissue adjacent to the source is converted into polarizing region tissue. Alternatively, retinoic acid may act directly on the ridge cells.

The changes in the extent of the apical ridge produced by retinoic acid lead to different forms of bud outgrowth. The form of the outgrowth depends on the dose of retinoic acid, the position of application and the interaction between the effects of the local source of retinoic acid and those of the polarizing region of the host bud. These considerations give some insights into why anterior application of retinoic acid leads to the development of additional digits whereas posterior application generally gives normal wings.

## INTRODUCTION

Local application of retinoic acid to the developing wing bud brings about pattern changes (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983; Summerbell & Harvey, 1983). When ion-exchange beads (AG1–X2) are used as

*Key words:* retinoic acid, apical ectodermal ridge, limb development, chick wing, SEM, bud outgrowth.

carriers for retinoic acid (Eichele, Tickle & Alberts, 1984) and implanted beneath the apical ectodermal ridge at the anterior margin of the wing bud, there is a clear dose response. A highly reproducible series of wing patterns results with effects on pattern formation across the anteroposterior axis (Tickle, Lee & Eichele, 1985). Beads soaked in low concentrations of retinoic acid ( $0.01\text{--}0.1\text{ mg ml}^{-1}$ ) which result in 3–20 picograms of retinoic acid in the wing tissue ( $0.9\text{--}25\text{ nm}$ ) at 14 h lead to the sequential formation of additional digits (digit patterns **2234**, **32234** or **3234** and **432234** or **43234**). When beads are soaked in higher concentrations of retinoic acid ( $1\text{--}10\text{ mg ml}^{-1}$ ), giving over 100 picograms of retinoic acid in the bud tissue at 14 h, there is a progressive thinning of the symmetrical wing (digit patterns **43234**, **4334**, **434** and a single symmetrical digit **4**). Beads soaked in very high concentrations of retinoic acid ( $10\text{ mg ml}^{-1}$ ) frequently result in truncated wings with no digits at all.

When beads soaked in a low concentration of retinoic acid ( $0.01\text{ mg ml}^{-1}$ ) are implanted to the apex of the wing bud beneath the apical ridge (Tickle *et al.* 1985), about 50% of the wings develop digits **234** anterior to the implant and a symmetrical digit **4** posteriorly (digit pattern **234**, **4**), while the remainder are mostly normal or lack one or more digits. With higher concentrations of retinoic acid ( $1\text{--}10\text{ mg ml}^{-1}$ ), truncations result. In contrast to the above changes, normal digit patterns (although reduced in size) result from implantation of retinoic-acid-impregnated beads to the posterior margin of the bud except at the highest concentration when truncations result (Tickle *et al.* 1985). A striking feature of these position-dependent effects of retinoic acid treatment is that additional structures appear to be generated only from anterior tissue.

The effects of local application of low doses of retinoic acid mimic those produced by grafts from the polarizing region, which is a signalling region at the posterior of the bud (Saunders & Gasseling, 1968; Tickle, Summerbell & Wolpert, 1975). Thus the sequential formation of additional digits is obtained with anterior grafts of increasing numbers of cells from the polarizing region (Tickle, 1981), digit patterns such as **234**, **4** follow grafts of polarizing tissue to the apex of the bud and normal digit patterns result from posterior grafts of polarizing tissue (Tickle *et al.* 1975; Wolpert & Hornbruch, 1981).

The patterns described above following retinoic acid treatment are assayed at 6–7 days after application when the skeletal elements are clearly recognizable. However, we have found that exposures of 14–20 h are sufficient to bring about pattern changes (Eichele, Tickle & Alberts, 1985). Thus to understand how retinoic acid exerts its effects, events occurring at much shorter times after treatment must be followed. As a start to this analysis, bud outgrowth following implantation of beads soaked in a range of retinoic acid concentrations was examined. Since the apical ectodermal ridge, a specialized epithelium that runs anteroposteriorly along the tip of the bud has been found essential for bud outgrowth (Saunders, 1948; Summerbell, 1974), particular attention was paid to its extent and disposition.

Polarizing tissue grafts have previously been shown to lead to alterations in bud form and extent of the apical ridge before pattern changes become apparent.

For example, the formation of additional digits following anteriorly positioned polarizing region grafts is accompanied by an increase in width of the outgrowth of the developing bud across the anteroposterior axis (Tickle *et al.* 1975; Smith & Wolpert, 1981). This may reflect changes in the extent of the apical ridge. Although the apical ridge immediately adjacent to a polarizing region graft disappears, the ridge persists as a thickened epithelium over the anterior margin of the bud (Saunders & Gasseling, 1968). Irradiation of buds following anteriorly grafted polarizing tissue leads to a reduction in the width of outgrowth and wings with digit patterns similar to those produced by high concentrations of retinoic acid are obtained (Smith & Wolpert, 1981). Digit patterns such as **434** also result when polarizing region cells are grafted beneath the apical ridge over extensive distances along the anterior of the bud (Honig, 1983) and here too outgrowth is presumably affected. In principle, severe width reductions could ultimately lead to truncations.

The observations reported here reveal that shortly after implantation of retinoic-acid-impregnated beads, the shape of the bud outgrowth was affected and reflected consistent changes in the extent and disposition of the apical ridge. In contrast to the pattern changes that are position-dependent, the graded effect of retinoic acid on the ridge occurred irrespective of the position of application. This paradox can be understood in terms of the interaction between the locally applied retinoic acid and the polarizing region of the host bud that acts as a boundary.

## MATERIALS AND METHODS

### *Implanting carrier beads*

This procedure has been described previously by Tickle *et al.* (1985). Briefly, AG1-X2 beads (200  $\mu\text{m}$  diameter) were soaked in solutions of all-*trans*-retinoic acid (Sigma lot Nos. 41F. 0440 and 63F-0476) dissolved in dimethyl sulphoxide (DMSO), then rinsed in tissue culture medium and implanted beneath the apical ectodermal ridge of the right wing bud of stage-20 embryos (Hamilton-Hamburger stages). The left wing bud served as an unoperated control. Control beads were soaked in DMSO, rinsed and implanted in the same way. The main series of bead implants was made beneath the ridge at the anterior margin of buds but some beads were implanted under the ridge at the apex or at the posterior margin.

### *Grafting tissue*

The limb buds of embryos at stage 21-22 were dissected off and placed in 2% trypsin in calcium- and magnesium-free saline, for 1 h at 4°C to loosen the ectoderm from the underlying mesoderm. The buds were then transferred to tissue culture medium (MEM+10% foetal calf serum; Gibco: Biocult) at 4°C and the ectoderm removed. Next cubes of polarizing region mesenchyme, of size 200  $\mu\text{m}^3$ , were dissected out of the posterior region of the bud. For control tissue, cubes of mesenchyme were similarly dissected from the anterior of the buds. The tissue was grafted beneath the apical ridge in the same way that the bead implants were made.

### *Preparation of specimens for SEM*

At various times (4-48 h) after performing the bead implants or tissue grafts, the embryos were dissected out of the egg and fixed in half-strength Karnovsky fixative (Karnovsky, 1965) for at least 24 h at 4°C. Next, the right and left wing bud of each embryo was carefully removed and placed into a small bag made out of lens tissue (Whatman 105). Bags containing a pair of wing

buds from each embryo could then be processed further without damage to the buds. The processing involved a rinse in 0.01 M-sodium cacodylate and postfixation in 1% osmium tetroxide for 1 h. Following dehydration through a series of alcohols, the buds were transferred in trichloroethane (Arlone) to liquid CO<sub>2</sub> for critical-point drying in a Polaron critical-point dryer. The dried specimens were mounted onto aluminium stubs with double-sided tape. A conducting layer of silver paint was applied closely around the specimens and the stub surface. These specimens were then coated with a 50 nm thick layer of gold and palladium using an SEM-coating unit E5000. Prepared in this way, all specimens were viewed with a Jeol JSM-35 scanning electron microscope.

### *Measurements of bud perimeter and extent of apical ridge*

Some data were obtained by making measurements on scanning electron micrographs of buds in profile using an IBAS computer. Since the buds had shrunk considerably (up to 42%) during preparation and in addition could not always be orientated to give suitable profile views, another series of treated buds was used to provide the bulk of the data. In this series, beads soaked in a range of retinoic acid concentrations were implanted to the right wing bud as before. At 24 h or 48 h, the embryos were removed from the egg, placed in Tyrodes solution, and stained with Nile blue sulphate (0.005% in Tyrodes). Both right and left (untreated) buds were dissected out. Each wing bud was positioned in a Petri dish so that a profile view was obtained. Drawings of the bud and the extent of the apical ridge were made using a camera lucida. Measurements were made from the camera-lucida drawings using an IBAS computer as before. A minimum of four buds was usually measured for each data point.

## RESULTS

### *SEM observations*

Beads were soaked in 10, 1, 0.1 or 0.01 mg ml<sup>-1</sup> retinoic acid. The buds to which these treated beads were implanted were compared with limbs to which control beads soaked in DMSO only had been implanted and which were fixed after the same time periods. Treated buds were also compared with left unoperated buds, which served as controls. The complete series of experiments is listed in Table 1.

### *Effects of beads implanted anteriorly*

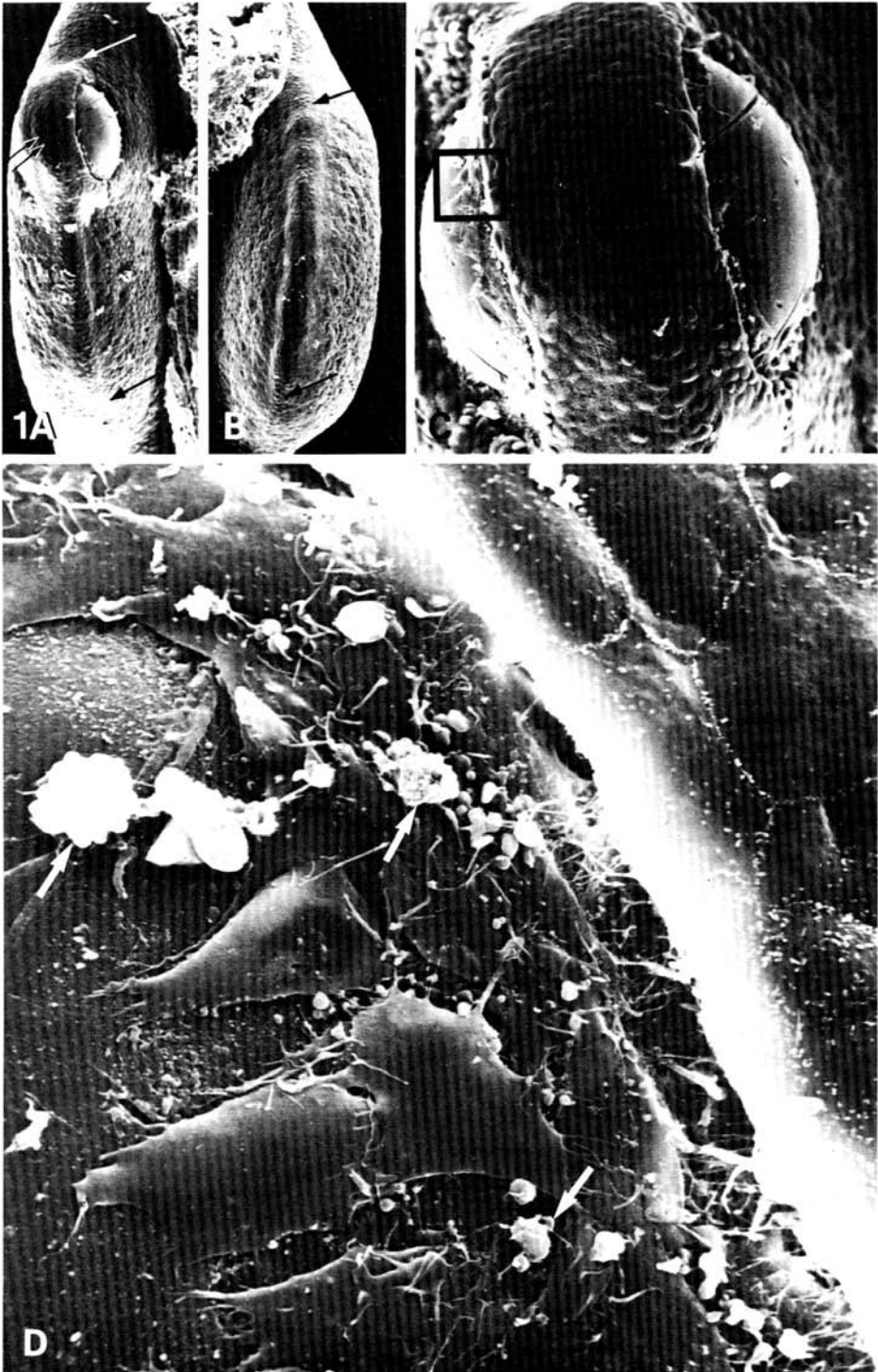
#### *4-6 h*

At this time, no differences could be detected between limb buds irrespective of their treatment. The shape of retinoic-acid-treated buds closely resembled that of their left counterparts (Fig. 1A,B), except that the bead was clearly visible bulging beneath the apical ridge at the anterior of the bud. These buds were also indistinguishable from buds which received control beads soaked in DMSO only. In buds to which beads had been implanted, the apical ridge extended over the bead and along the bud profile to its posterior margin (Fig. 1A). In the region of the bead, the ridge was stretched to accommodate the carrier and so its thickened morphology was not apparent. However, extending posteriorly from the bead, the thickened ridge was well defined and could be estimated as being about 10 cells wide. Cells at the edge of the cut tissue had begun to migrate over the surface of the implanted bead (Fig. 1C,D). This marked the first stages of the healing process during which the beads become completely embedded in the buds. The extent of cell migration onto the beads was variable and did not appear to be correlated with

Table 1. Summary table showing the total number of buds examined in each case and the digit patterns that resulted after 6 days

Conc. RA in soaking soln. (mg ml <sup>-1</sup> ) or tissue grafted	Position in wing bud to which retinoic acid is applied or tissue grafted													
	Anterior						Apex						Posterior	
	4-6 h	18-24 h	48 h	Digit patterns†	24 h	48 h	24 h	48 h	Digit patterns†	24 h	48 h	Digit patterns†	24 h	48 h
DMSO	7	5	—	234	(11)*	5	—	234	(3)	3	—	234	(2)	
10	7	7	2	truncations 34	(16) (4)	3	5	truncations†	(4)	—	—	—	—	
				434	(2)									
				4334	(3)									
				4334	(7)									
1	2	7	3	truncations	(6)	—	—	—		11	10	23	(1)	
				4	(3)							234	(8)	
				34	(1)									
				434	(2)									
				4334	(2)									
				43234	(2)									
0.1	—	2	—	4	(3)	—	—	—		5	6	234	(6)	
				434	(1)									
				4334	(4)									
				4334	(4)									
				43234	(6)									
				432234	(1)									
0.01	4	2	2	434	(1)	3	2	2		—	—	—		
				4334	(4)			34						
				4334	(1)			334						
				43234	(8)			2,4						
				432234	(3)			234,4						
				32234	(1)			234 + post elements						
				234	(1)			234						
Polarizing region	—	5	3	4334	(2)	5	4	234,4		1	3	234	(2)	
				43234	(3)			234,44						
								234,434						
								234						
Anterior mesenchyme	—	7	—	234	(2)	2	1	234		2	—	234+extra element at elbow projecting ventrally	(5)	

\* Numbers in brackets indicate number of cases. ~ Indicates fused digits. † Cumulative data from all experiments using all-trans-retinoic acid from Sigma Lot. 41F-0440 and 63F-0476. ‡ Obtained with retinoic acid Lot. No. 12F-0598.



the treatment of the bead. In addition to the migrating cells, which resembled fibroblasts, a few macrophages could be seen, particularly at the corners of the healing wound.

#### 18–24 h

Differences were now observed between buds, depending on the treatment of the implanted beads. At 24 h, the difference in shape was most marked between control buds (or those with control beads) and buds treated with the highest concentration of retinoic acid. Buds to which beads soaked in progressively lower concentrations of retinoic acid had been implanted showed a dose-dependent effect: the difference between the control and treated buds becoming progressively reduced as lower concentrations of retinoic acid were applied on the beads.

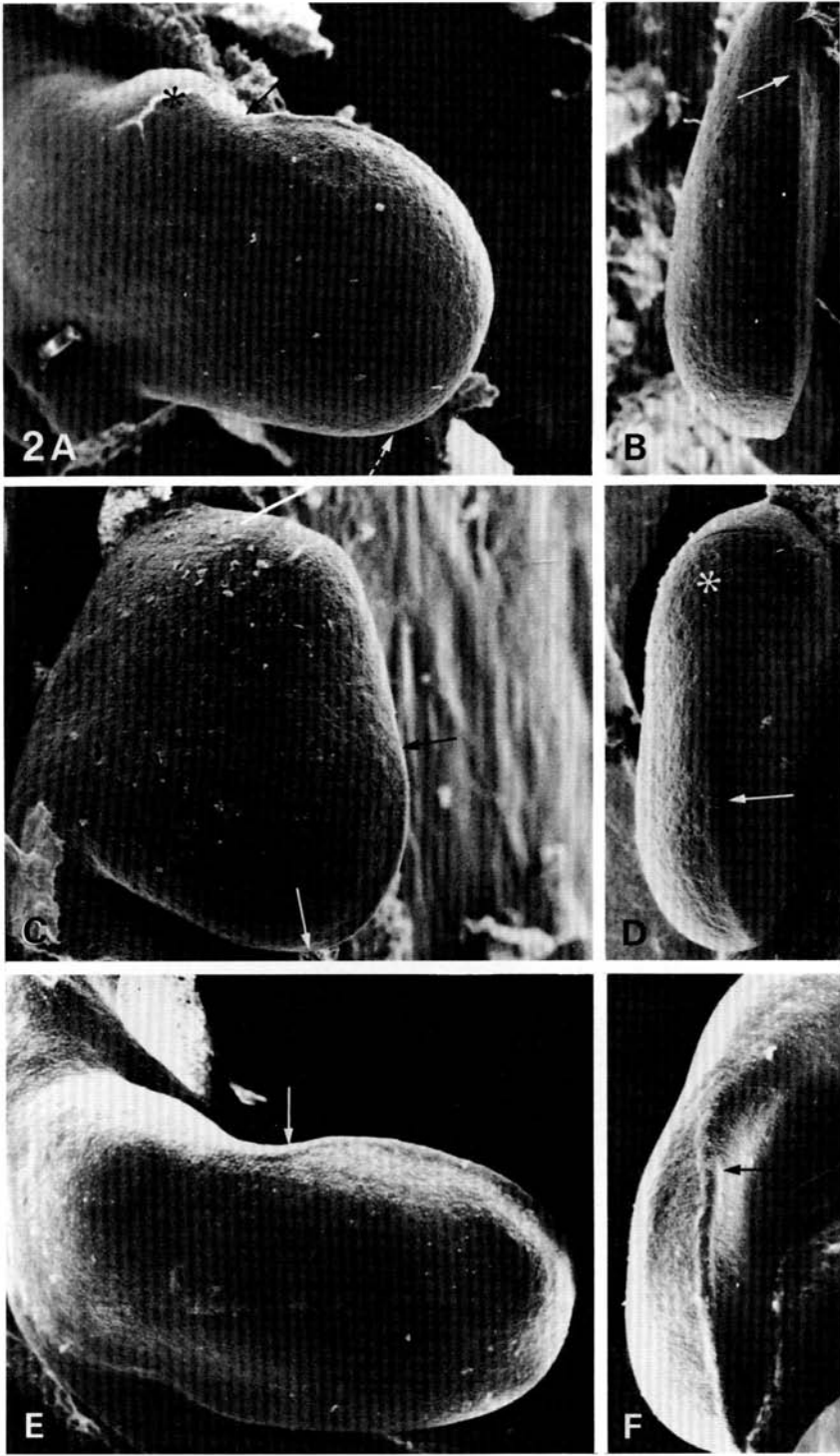
In buds to which control beads had been implanted (Fig. 2A,B), there was a slight bulge anteriorly marking the position of the bead that had become completely embedded in the wing tissue. The wing bud was now elongated. The apical ridge was well-defined and extended from beneath the embedded bead at the anterior, around the rim of the bud to the posterior. The contours of these buds containing control beads were identical to those of normal untreated left-hand buds (approximately stage 24).

In striking contrast, wing buds which had received an implant soaked in  $10 \text{ mg ml}^{-1}$  retinoic acid were considerably shortened and steeply tapered to a pointed tip at the posterior margin. The extent of the apical ridge was considerably reduced, being absent over most of the bud margin and present only over the pointed tip at the very posterior of the bud. Buds to which beads soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid had been implanted also resulted in shortened buds and the ridge was similarly present only over the posterior tip (Fig. 2C,D).

With beads soaked in lower concentrations of retinoic acid, the buds were progressively less tapered. For example, with beads soaked in  $0.1 \text{ mg ml}^{-1}$  retinoic acid, the bud profile was narrow, spadelike and the anterior edge was not as curved as that of the normal bud at this stage. The extent of the apical ridge was reduced but not as severely as those buds with beads soaked in higher concentrations of retinoic acid. Thus the apical ridge was absent anteriorly near the site of the bead but its anterior limit was closer to the bead than in the treated buds just described.

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Fig. 1. (A) Edge view of a right wing bud fixed 4–6 h after implanting a bead soaked in  $10 \text{ mg ml}^{-1}$  retinoic acid at the anterior margin. The apical ridge (arrowed) runs over the bead and extends along the bud profile to the posterior margin. Note that in the region of the bead, the ridge (double arrowed) is stretched to accommodate the carrier. Picture width (PW) = 0.37 mm. (B) Edge view of the contralateral left wing bud to that shown in A. The apical ridge can be distinctly seen extending from the anterior to the posterior margin (extent indicated between arrows). PW = 0.36 mm. (C) Enlarged view of A showing the bead positioned directly beneath the apical ridge. Flattened cells can be seen moving out over the surface of the bead from under the ridge. PW = 0.22 mm. (D) A highly magnified view of C in region indicated, showing pavement of apical ridge cells and fibroblast-like cells migrating over bead surface. A few macrophage-like cells (arrowed) are also present. PW = 0.051 mm.





Finally, with implanted beads soaked in the lowest concentration of retinoic acid ( $0.01 \text{ mg ml}^{-1}$ ), the buds appeared to be about the same width as normal buds, (Fig. 2E) but were distally more symmetrical (compare Fig. 2A,E). The apical ridge was present over the entire wing bud margin except in the region of the embedded bead (Fig. 2F).

#### 48 h

Further development had produced even greater differences in the shapes of bud outgrowth. Application of the highest concentration of retinoic acid ( $10 \text{ mg ml}^{-1}$ ), which had greatly reduced the extent of the apical ridge at 24 h, had now resulted in stunted buds with a very narrow region of outgrowth posteriorly (Fig. 3A). The apical ridge was present only over the posterior outgrowth (Fig. 3B). It should be noted that where the apical ridge had disappeared anteriorly and outgrowth ceased, the bud bulged and was not flattened dorsoventrally as normal. Beads soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid produced narrow, finger-like outgrowths with apical ridges running along their tips (Fig. 3C,D). These narrow contours were quite unlike the shape of the left-hand bud which was paddle-shaped. With beads soaked in the lowest concentrations of retinoic acid, the buds were broad with additional tissue at the anterior margin (compare Fig. 3E with contralateral normal bud in Fig. 3G). The apical ridge was present over the entire bud margin, and thus was longer than usual because it extended along the perimeter of the widened outgrowth (Fig. 3F, also see quantitative analysis).

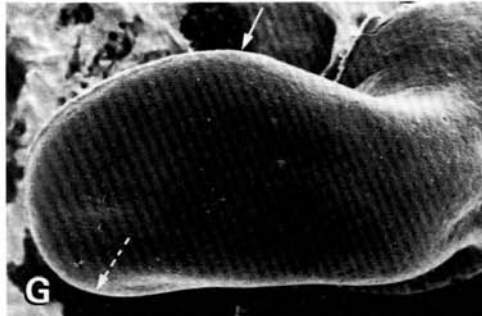
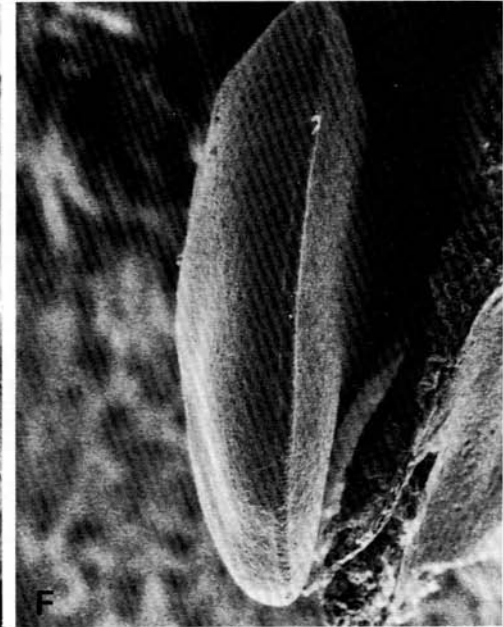
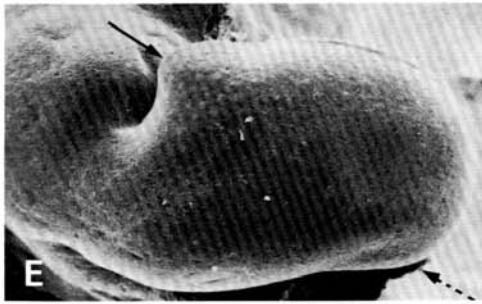
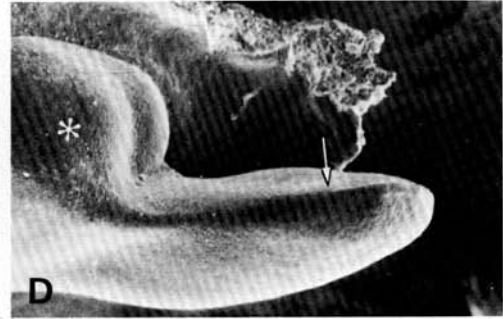
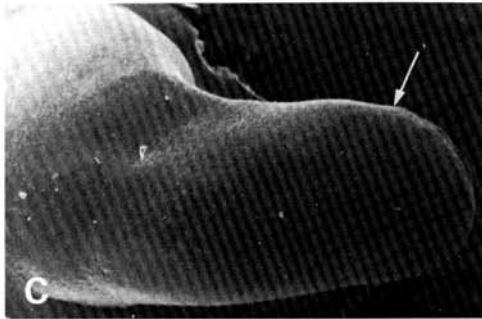
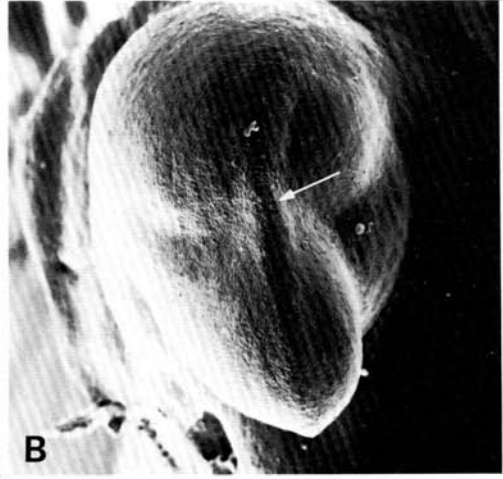
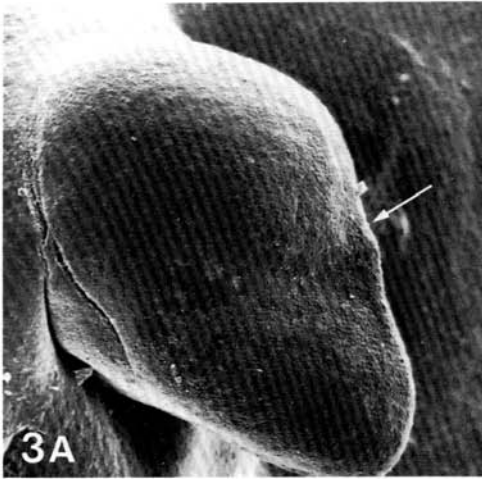
#### Effects of beads implanted at the wing bud apex

At 24 h, control beads placed at the apex of the bud had led to the development of an indentation in the margin of the bud which corresponds to the site of implantation (Fig. 4A,B). The indentation had been displaced anteriorly by greater outgrowth posteriorly. Although the ridge was perturbed in the region of the indentation, it appeared to be virtually continuous around the bud margin.

In contrast, implants of beads soaked in high concentrations of retinoic acid ( $10 \text{ mg ml}^{-1}$ ) after 24 h had produced extremely stunted wing buds in which the

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Fig. 2. Buds 24 h after implanting beads anteriorly. (A) Dorsal view. Control bead soaked in DMSO only. A bulge at the anterior margin marks the site of the embedded bead, shown by \*. Anterior limit of apical ridge indicated by arrow. Posterior limit determined by rotation of specimen, indicated by dotted arrow. PW = 1.2 mm. (B) Edge view of A. Note extent of apical ridge along the wing bud margin (anterior limit of ridge indicated by arrow). PW = 0.47 mm. (C) Dorsal view. Bead soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid. Note the bud is much shorter (compare shape of bud with that of bud in A). Extent of the apical ridge is indicated between the arrows. Note that the ridge is confined to the posterior part of the bud. PW = 1.17 mm. (D) Edge view of C. The site of the embedded bead is indicated by \*. Anterior limit of apical ridge is indicated by arrow. PW = 0.46 mm. (E) Dorsal view. Bead soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid. The shape of the bud is similar to that of the control bud except that the tip is more symmetrical (compare with A). Anterior limit of apical ridge indicated by arrow. PW = 1.46 mm. (F) Edge view of E. Anterior limit of apical ridge indicated by arrow (compare with A). PW = 0.68 mm.



apical ridge had completely disappeared (Fig. 4C,D). By 48 h, still no further outgrowth had occurred.

With beads soaked in low concentrations of retinoic acid ( $0.01 \text{ mg ml}^{-1}$ ), asymmetrically bilobed wing buds developed after 24 h. The apical ridge was clearly seen extending along each lobe. However, between the two lobes the ridge was more difficult to distinguish and was raised very little above the surrounding ectoderm. By 48 h the buds had developed into two lobes that had elongated in parallel and were separated by a narrow groove (Fig. 4E). The apical ridge could be distinctly seen extending around the apex of each elongated lobe (Fig. 4F).

#### *Effects of beads implanted at the posterior margin*

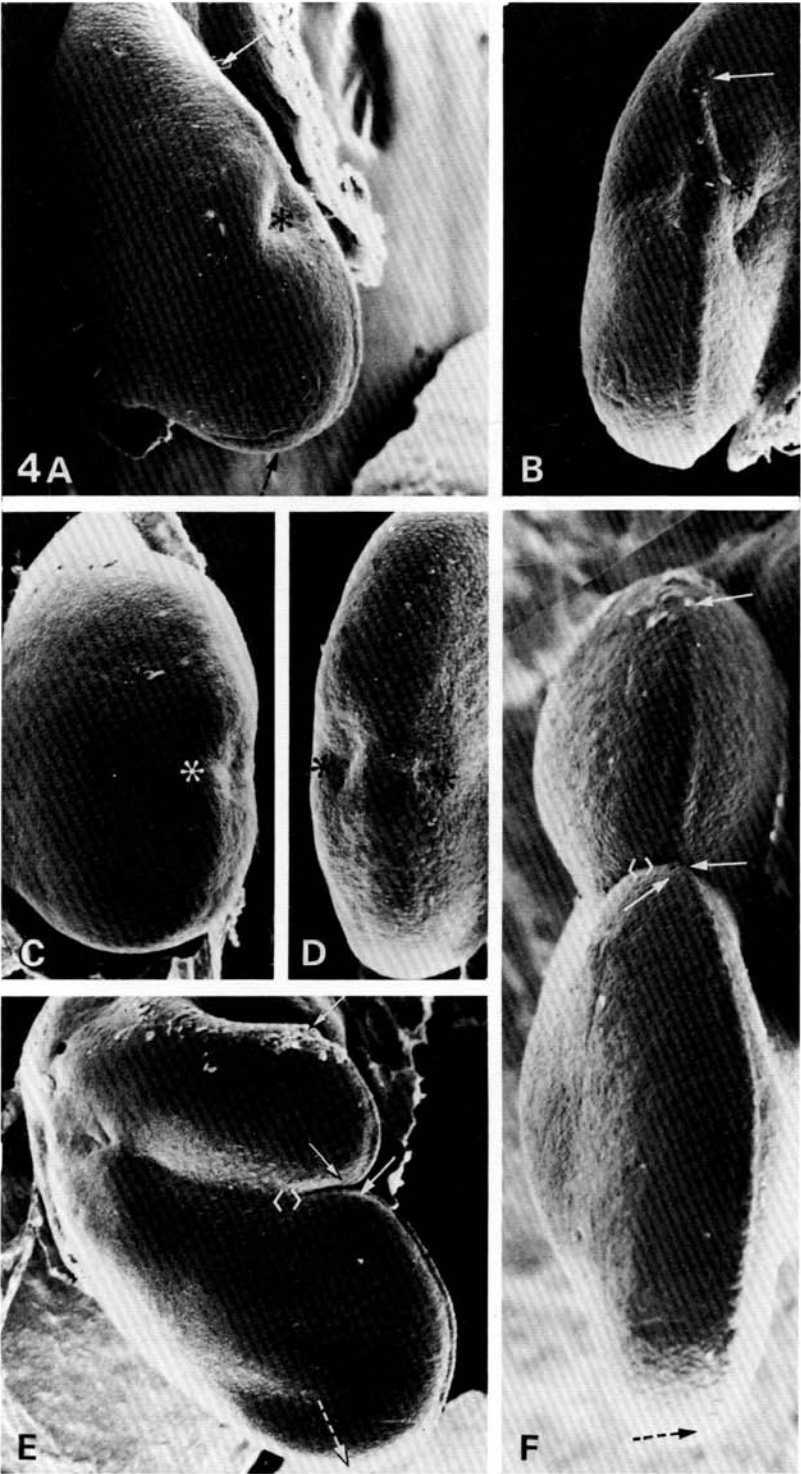
Implants of beads soaked in DMSO only, resulted in normal wing bud outgrowth with the normal extent of apical ridge at 24 h. The outline of such treated buds was slightly perturbed posteriorly by a bulge marking the position of the embedded bead.

By 24 h, beads soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid had resulted in short, rounded wing buds (Fig. 5A). Posterior outgrowth was reduced. The shape of the bud approximated to an inverted version of that which resulted from anteriorly positioned beads soaked in the same concentration of retinoic acid (compare Fig. 5A with Fig. 2C). This was because, in contrast to normal buds, the apical ridge was absent posteriorly in the region of the implanted bead but persisted over the anterior margin (Fig. 5A,B).

After 48 h, outgrowth of wing buds had resumed. However, instead of the outgrowth involving posterior tissue, outgrowth continued from the anterior part of the bud (Fig. 5C). Thus the bud was abnormally positioned. It was noticeably short and the apex narrower than control buds. The posterior limit of the apical ridge had been shifted more anteriorly (Fig. 5D). The original posterior margin of the bud over which the apical ridge had disappeared remained as a bulbous mass of tissue posteriorly.

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Fig. 3. Buds 48 h after implanting beads anteriorly. (A) Dorsal view. Bead soaked in  $10 \text{ mg ml}^{-1}$  retinoic acid. Note the stunted shape of the bud. The apical ridge (anterior limit indicated by arrow) is present over the pointed posterior part of the bud. PW = 1.26 mm. (B) Edge view of A. Anterior limit of apical ridge indicated by arrow. Note that where the apical ridge has disappeared anteriorly, the bud bulges dorsoventrally. Compare with dorsoventral flattening of posterior part of the bud where the ridge is still present. PW = 1.28 mm. (C) Dorsal view. Bead soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid. Note narrow symmetrical outgrowth. Anterior limit of apical ridge indicated by arrow. PW = 1.6 mm. (D) Anterior view of C. Bulbous mass of tissue (\*), covers bead. Anterior limit of apical ridge indicated by arrow. PW = 1.7 mm. (E) Dorsal view. Bead soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid. Note broadened bud with extensive apical ridge. Anterior limit indicated by arrow and posterior limit determined by rotation of specimen, indicated by dotted arrow. PW = 1.7 mm. (F) Edge view of anterior margin of bud shown in E with extensive apical ridge. PW = 1.0 mm. (G) Dorsal view. Contralateral unoperated bud to that shown in E for comparison with experimental buds. The shape of this bud and extent of its apical ridge (indicated between arrows as previously) are normal. PW = 1.64 mm.



Beads soaked in  $0.1 \text{ mg ml}^{-1}$  retinoic acid produced a similar but less-pronounced anterior shift in the position of outgrowth. This could be detected after 24 h but was more noticeable after 48 h. In addition, outgrowth was less retarded than when beads soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid were implanted. However, the apex of the buds was still narrower than that of control buds (Fig. 5E). The apical ridge extended from the anterior to the new posterior margin, which had been displaced anteriorly, only a short distance from its original position (Fig. 5F).

*Effects of grafting polarizing region or anterior tissue to (a) the anterior margin; (b) the apex of the wing bud*

Grafts of polarizing tissue were performed so that the effects of these could be compared with those obtained from beads soaked in retinoic acid. Grafts of anterior wing bud tissue served as controls for polarizing region grafts.

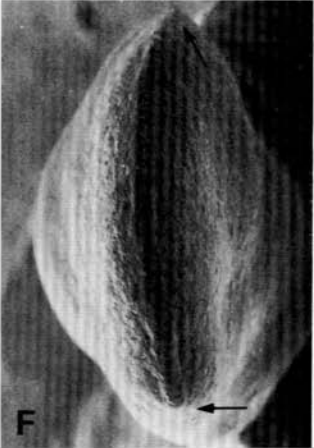
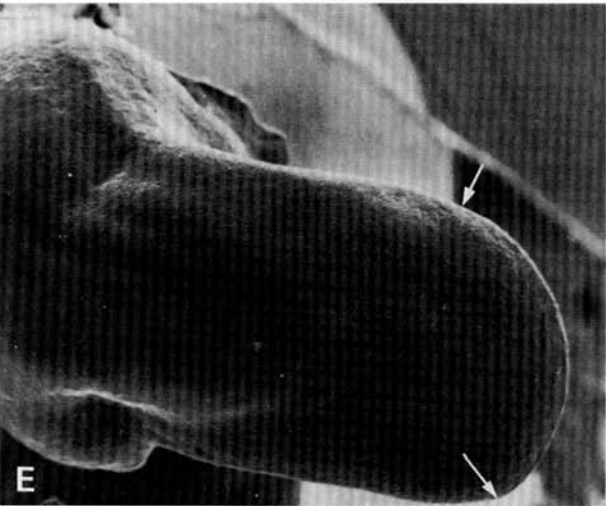
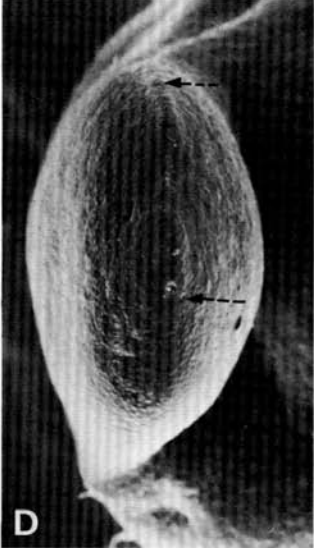
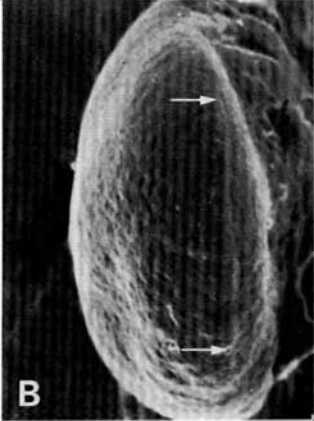
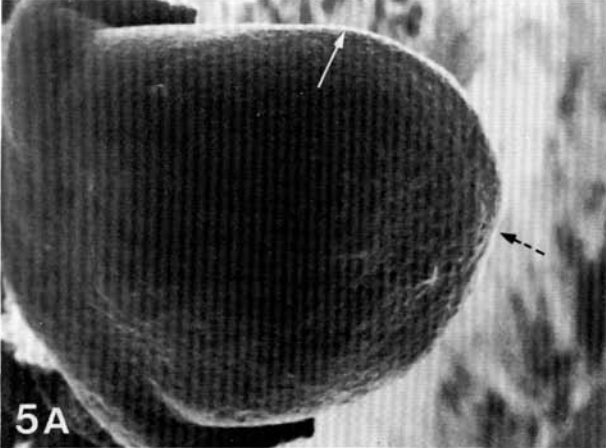
*(a) Grafts to the anterior margin*

*24 h.* At this time, no change in the gross shape of the wing bud could be detected following a polarizing region graft. The bud was a paddle-shaped outgrowth not noticeably wider than a normal bud of the same stage (Fig. 6A: compare with normal bud in Fig. 2A). However, the bud was distally more symmetrical and strikingly similar in shape to the buds to which beads soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid had been implanted anteriorly (compare with Fig. 2E). The apical ridge was absent at the graft site but extended around the remainder of the bud rim to the posterior margin (Fig. 6A,B). Wing buds which received grafts of anterior tissue developed normal outgrowths. The extent of the apical ridge was unaffected by these grafts.

*48 h.* Buds which had received grafts of polarizing region were by this time much broader than controls (Fig. 6C). This widening resulted from the outgrowth

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Fig. 4. Buds following implants of beads to the apex. (A) Dorsal view. 24 h after implanting a control bead soaked in DMSO only. A slight indentation (\*) anteriorly marks the site of the implant. Anterior limit of apical ridge indicated by arrow. Posterior limit determined by rotation of specimen, indicated by dotted arrow. PW = 1.12 mm. (B) Edge view of A. Note that the apical ridge is continuous over the site of the embedded bead (\*) and along the wing bud margin (anterior limit of ridge indicated by arrow). PW = 0.60 mm. (C) Dorsal view. 24 h after implanting a bead soaked in  $10 \text{ mg ml}^{-1}$  retinoic acid. A slight indentation (\*) at the bud apex marks the site of the embedded bead. Note the absence of apical ridge. PW = 0.75 mm. (D) Edge view of C. Shallow indentations (\*) either side of the apex mark the site of the embedded bead. The apical ridge is completely absent. PW = 0.46 mm. (E) Dorsal view. 48 h after implanting a bead soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid. A narrow groove (<>), separates the anterior outgrowth from the posterior one. Apical ridge extends along the margin of both outgrowths (as indicated between solid arrows on anterior outgrowth; on posterior outgrowth between solid and dotted arrows as used previously). PW = 0.47 mm. (F) Enlarged view of E. The narrow groove separating anterior from posterior outgrowth is indicated by (<>). Note the apical ridge extending along the margin of each separate outgrowth (as indicated between arrows, posterior limit by dotted arrow). PW = 1.51 mm.



of an extra portion of tissue from the anterior margin. A well-defined apical ridge extended along the broadened rim of the bud (Fig. 6D). These buds were similar in shape to those which developed following anteriorly implanted beads soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid (compare Figs 6C & 3E).

(b) *Grafts to the apex*

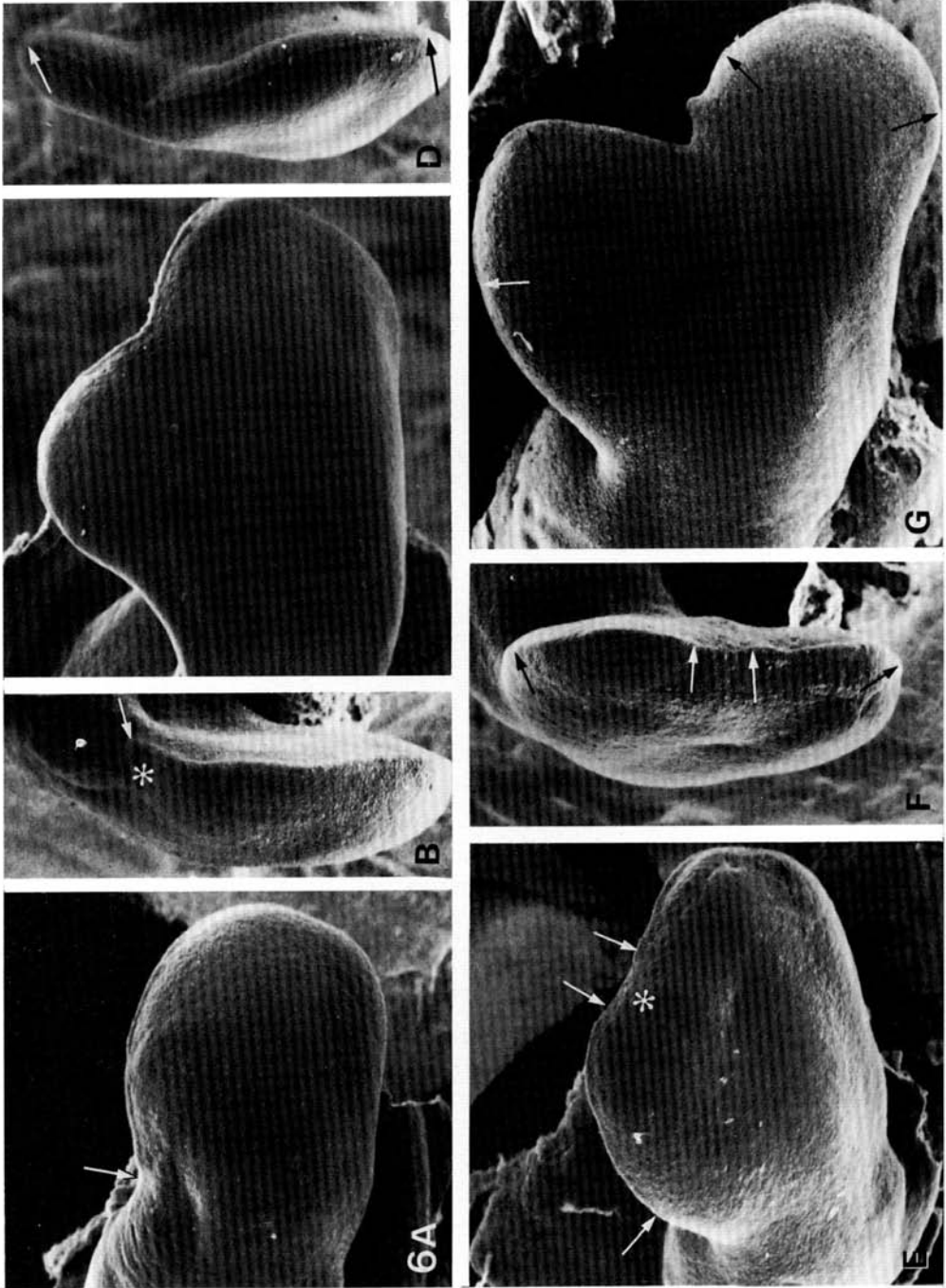
*24 h.* Buds which had received grafts of polarizing tissue at their apex were slightly bilobed (Fig. 6E). The bud margin was slightly indented in the region of graft. The apical ridge was absent at the graft site but extended on either side both posteriorly and anteriorly from the graft around the rim of the bud (Fig. 6F). Following control anterior tissue grafts, the bud had a distorted shape. The outline of the bud was indented in the region of the graft where the apical ridge had disappeared directly over it. This indentation had then been displaced anteriorly.

*48 h.* The indentation in the bud margin produced by a polarizing region graft was much more marked at this time. The bud had become distinctly bilobed (Fig. 6G). If these bud shapes are compared with those that developed following implants of beads soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid, after 24 and 48 h, it can be seen that the sequence of shape changes is superficially similar. However, buds to which retinoic acid had been applied became distinctly bilobed at 24 h, whereas such a bilobed shape was only beginning to become apparent with buds which received grafts of polarizing tissue. Furthermore, later outgrowth of the buds led to different relationships between the two lobes. With bead implants, the two lobes lay close together whereas with polarizing tissue, the two lobes were splayed out and separate.

Examination of buds with anterior tissue grafts showed that after 48 h further anterior displacement of the grafted tissue had occurred and normal bud outgrowth had been restored.

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Fig. 5. Buds following implants of beads to the posterior margin. (A) Dorsal view. 24 h after implanting a bead soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid. The shape of this bud is inverted compared to that shown in Fig. 2C. The apical ridge extends over the anterior margin only (anterior limit of ridge indicated by arrow, posterior limit determined by rotation of specimen, indicated by dotted arrow). PW =  $1.57 \text{ mm}$ . (B) Edge view of A. Note that the extent of the apical ridge has been shifted anteriorly (as indicated between arrows). PW =  $0.75 \text{ mm}$ . (C) Dorsal view. 48 h after implanting a bead soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid. The bud is shorter and the apex narrower than normal. The apical ridge runs along the anterior margin only (as indicated between arrows). PW =  $1.82 \text{ mm}$ . (D) Edge view of C. Note the complete absence of apical ridge posteriorly. Extent of apical ridge is indicated between arrows. PW =  $0.61 \text{ mm}$ . (E) Dorsal view. 48 h after implanting a bead soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid. Note that the shape changes of this bud are not as marked as those shown in C. The extent of the apical ridge is indicated between arrows. PW =  $1.86 \text{ mm}$ . (F) Edge view of E. The extent of the apical ridge (indicated between arrows) has been shifted only slightly anteriorly compared with D. PW =  $0.70 \text{ mm}$ .





*Quantitative analyses of the effects of retinoic acid application on the extent and disposition of the apical ridge*

For analysis, the perimeter of the wing bud has been divided into three zones; anteriorly, a zone extending from the body wall to the anterior limit of the ridge – the anterior margin; a zone occupied by the apical ridge; and posteriorly, a zone extending from the posterior limit of the ridge to the body wall – the posterior margin (Fig. 7).

Measurements of the length of these stretches have been plotted as a function of retinoic acid concentration in the solution in which the beads were soaked. The curves obtained illustrate quantitatively the effects seen morphologically in the scanning electron micrographs.

Fig. 8A,B shows how the perimeter of the bud and the lengths of the three zones are affected by local application of retinoic acid anteriorly at 24 h and 48 h. For comparison, measurements following anteriorly grafted polarizing region tissue and anterior mesenchyme are plotted with the data for beads soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid and for normal buds respectively (see key).

The curves at 24 h show that with low doses of retinoic acid the perimeter of the bud had increased (by 1.5 mm). With increasing doses the perimeter progressively decreased so that with beads soaked in  $1 \text{ mg ml}^{-1}$  retinoic acid or more the perimeter was shorter than that of normal buds. At  $10 \text{ mg ml}^{-1}$  the perimeter was 0.05 mm shorter than normal. These changes in the length of the bud perimeter reflect the different bud shapes just described. At low doses, the buds are more rounded distally, and at high doses bud outgrowth is stunted.

The increase in bud perimeter at low doses appears to be accompanied by a slight increase in the length of the apical ridge (Fig. 8A). At higher doses, the decrease in bud perimeter was accompanied by a marked decrease in the length of the ridge. This was due to an increase in the length of the anterior margin.

The inverse relationship between the lengths of apical ridge and anterior margin is clearly shown in curves drawn for data obtained over the whole concentration range at 48 h (Fig. 8B). As the length of the ridge first increased at low doses and

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Fig. 6. Buds following grafts of polarizing region tissue. (A) Dorsal view. 24 h after grafting tissue anteriorly. Bud is not significantly broader (compare with normal bud profile in Fig. 2A). Anterior limit of apical ridge indicated by arrow. PW = 0.95 mm. (B) Edge view of A. Site of graft (\*) is indicated by indentations on either side of bud. Anterior limit of apical ridge indicated by arrow. PW = 0.54 mm. (C) Dorsal view. 48 h after grafting tissue anteriorly. Note broadening of bud. PW = 1.37 mm. (D) Edge view of C. Continuous apical ridge extends around the broadened bud margin as indicated by arrows. PW = 0.56 mm. (E) Dorsal view. 24 h after grafting tissue at apex of bud. The bud is broad and slightly indented at the graft site (\*). The ridge has disappeared at the site of the graft but is present anteriorly and posteriorly (ridge indicated by arrows). PW = 1.34 mm. (F) Edge view of E. Indentations either side indicate graft site. Note the ridge is absent in this region of the bud margin but is present anteriorly (between arrows) and also posteriorly (similarly indicated). PW = 0.77 mm. (G) Dorsal view. 48 h after grafting tissue at the apex. Note two distinct outgrowths, the anterior outgrowth is asymmetrical whereas the posterior one is symmetrical. Apical ridge (arrowed) is present at the apex of both outgrowths. PW = 1.60 mm.

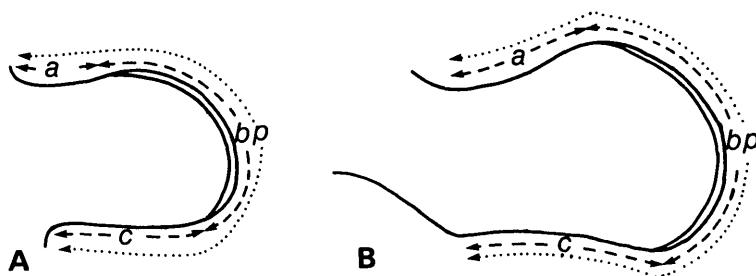


Fig. 7. Diagram to show how measurements were made for the quantitative analysis of bud outgrowth (A) normal bud at 24 h, (B) normal bud after 48 h. *p*, Perimeter; *a*, Anterior margin; *b*, Apical ridge; *c*, Posterior margin. Note that for the 48 h bud profile, measurements were made around the distal paddle-shaped part of the bud only and did not extend to the body wall. This was because the proximal part of the bud lies in a different plane and accurate measurements of its profile could not be made.

then decreased at high doses so the length of the anterior margin initially decreased then increased. In contrast to these changes in the anterior part of the bud, the length of the posterior margin remained constant.

To see how the lengths of apical ridge, anterior and posterior margin change in relation to each other, their lengths are expressed in terms of the proportion of bud perimeter they occupy (relative lengths) and plotted against the concentration of retinoic acid (Fig. 8C,D). Since proportions are now being considered, data from scanning electron micrographs can be included (see key). These data points are in close agreement with those obtained from camera-lucida drawings of unfixed buds. The curves now demonstrate clearly the inverse relationship between the relative length of the apical ridge and anterior margin at both 24 and 48 h. This verifies quantitatively the impressions gained from the morphological observations. Modulation of the anterior bud margin only is involved and the posterior margin remains constant.

A further interesting point is that the increase in the length of the ridge at 24 h with low doses of retinoic acid can be accounted for by the increase in perimeter of the bud. In fact the relative ridge length rather than increasing appears to decrease slightly. It is only at 48 h that a significant increase in the length of the ridge leads to this occupying a greater proportion of the limb bud margin.

When retinoic acid is applied to the apex of the bud, as just described morphologically, the ridge disappears or becomes flatter in the region of the bead. Thus in addition to the three regions of the bud perimeter that we have defined, there is a stretch at the apex to be considered where the ridge has disappeared. We will call this the apical zone of ridge regression. It should also be noted that the relative length of the ridge has been calculated from the sum of the lengths of ridge present on both sides of the implant.

Fig. 9A,B shows the relative lengths of the apical ridge, the anterior and posterior margins and the apical zone of ridge regression at 24 and 48 h plotted as a function of the concentration of retinoic acid.

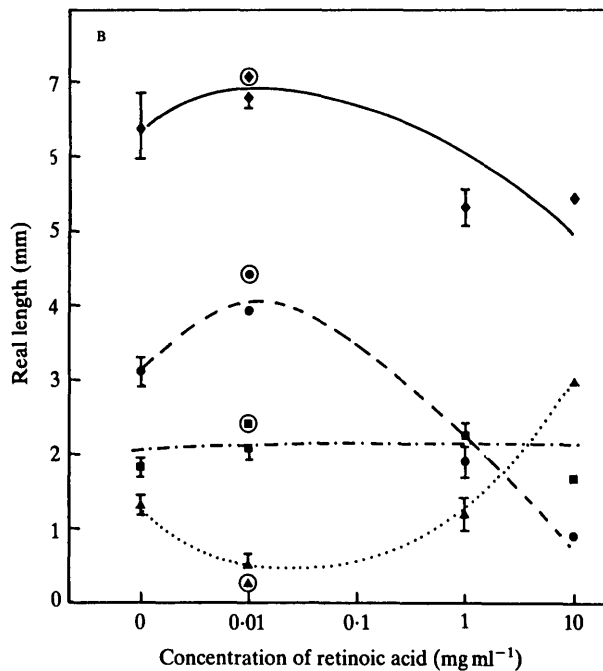
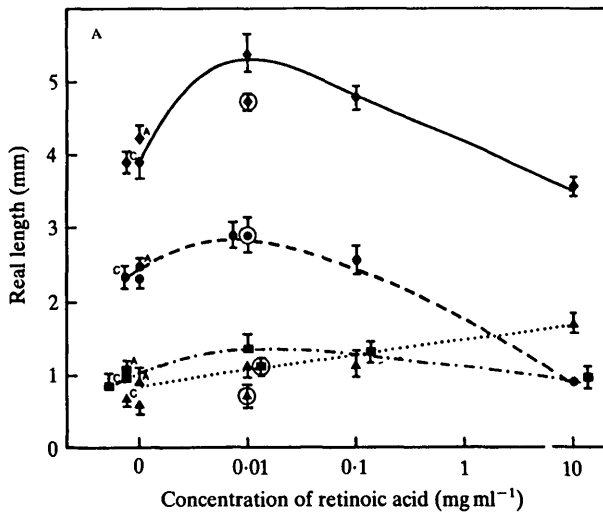
As for anterior implants, the relative length of the apical ridge remains virtually constant at low doses but then progressively decreases. The decrease in the relative length of the ridge in this case, however, is brought about by the increase in the length of the apical zone of ridge regression with increasing doses of retinoic acid. At doses above  $0.1 \text{ mg ml}^{-1}$ , the ridge is completely obliterated by 48 h. To calculate at these high doses the proportion of the perimeter occupied by the apical zone of ridge regression, the proportions of the anterior and posterior margins were subtracted from 100%. This treatment of the data is used to illustrate the inverse relationship between the length of the ridge and that of the apical zone of ridge regression over the whole concentration range of retinoic acid. In this treatment, the lengths of anterior and posterior margins remain constant.

Fig. 10A,B shows the relative lengths of apical ridge, anterior and posterior margins plotted as a function of retinoic acid concentration applied posteriorly after 24 and 48 h respectively. At 24 h with low doses the relative length of the apical ridge is unchanged but then progressively decreases as higher concentrations of retinoic acid are applied. This is a similar dose response to that observed when retinoic acid is applied anteriorly or to the apex. The decrease in the relative length of the ridge is due to an increase of the posterior margin. This increase in the length of the posterior margin is particularly marked at high concentrations. This is consistent with the impressions gained from scanning electron microscope observations, that the ridge was shifted to more anterior positions with increasing doses of retinoic acid.

When implants are made at the anterior or apex of the bud, the same relationship between the relative ridge length and the region of ridge regression in the vicinity of the bead is found after 48 h as at 24 h. However, the relative length curves for the parameters of bud outgrowth at 48 h after posterior application are quite different from those at 24 h. The decrease in the relative length of the ridge seen at high concentrations of retinoic acid at 24 h is not continued. Even though the perimeter of the bud steadily decreases with higher doses, the length of the ridge only decreases slightly. Thus the relative length of the ridge actually appears to increase slightly. This maintenance of a more normally proportioned ridge involves changes in both the anterior and posterior margins. The anterior decreases while the posterior increases, reflecting that bud outgrowth is now from more anterior parts of the bud. At 48 h, the differences between the curves showing the effects of applying retinoic acid posteriorly and to other positions reflect the re-establishment of a correctly proportioned ridge rather than an amplification of the changes that had taken place at 24 h.

## DISCUSSION

When all-*trans*-retinoic acid is locally applied to chick wing buds on AG1-X2 beads, there are dose-dependent effects on the pattern of structures that develop across the anteroposterior axis (Tickle *et al.* 1985). These studies have shown that there are reproducible, dose-dependent changes in the form of bud outgrowth



Key to symbols used in Figs 8, 9 & 10. Closed symbols represent data from camera-lucida drawings, while open symbols represent data from SEM pictures. (n = the number of buds measured with the treatment specified.)

Perimeter of buds with:

◆, ◇ retinoic-acid-treated beads

◆<sub>C</sub>, ◇<sub>C</sub> DMSO-treated beads

⊙, ⊙ polarizing region grafts

◆<sub>A</sub>, ◇<sub>A</sub> anterior mesenchyme grafts

Ridge length (real or relative) of buds with:

●, ○ retinoic-acid-treated beads

●<sub>C</sub>, ○<sub>C</sub> DMSO-treated beads

⊙, ⊙ polarizing region grafts

●<sub>A</sub>, ○<sub>A</sub> anterior mesenchyme grafts

Length of posterior margin (real or relative) of buds with:

■, □ retinoic-acid-treated beads

■<sub>C</sub>, □<sub>C</sub> DMSO-treated beads

⊙, ⊙ polarizing region grafts

■<sub>A</sub>, □<sub>A</sub> anterior mesenchyme grafts

Length of anterior margin (real or relative) of buds with:

▲, △ retinoic-acid-treated beads

▲<sub>C</sub>, △<sub>C</sub> DMSO-treated beads

⊙, ⊙ polarizing region grafts

▲<sub>A</sub>, △<sub>A</sub> anterior mesenchyme grafts

Apical zone of ridge regression of buds with:

▼, ▽ retinoic-acid-treated beads

▼<sub>C</sub>, ▽<sub>C</sub> DMSO-treated beads

⊙, ⊙ polarizing region grafts

▼<sub>A</sub>, ▽<sub>A</sub> anterior mesenchyme grafts

*Effects of retinoic acid on wing bud outgrowth*

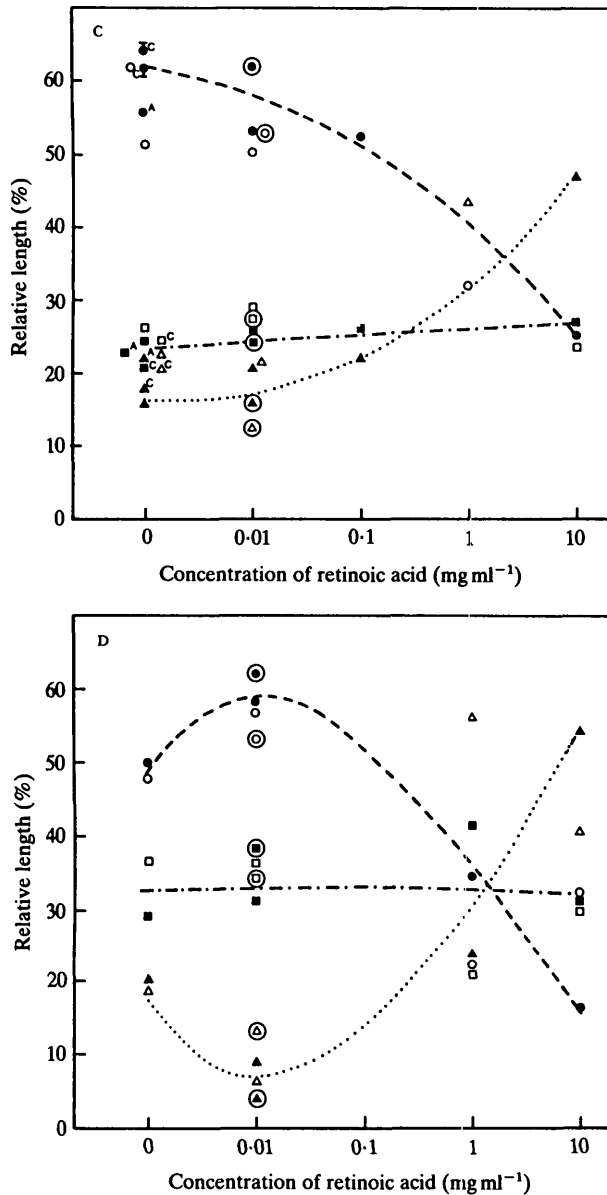


Fig. 8. (A) Beads soaked in a range of retinoic acid concentrations were implanted at the anterior of the bud. Lengths of bud perimeter apical ridge, anterior and posterior margin after 24 h were plotted as a function of retinoic acid concentration. Curves represent as follows: perimeter (—), apical ridge (---), anterior margin (.....), posterior margin (-·-·-). Normal bud, n = 7; 0.01 mg ml<sup>-1</sup> retinoic acid, n = 3; 0.1, n = 4; 10, n = 4; DMSO, n = 5; polarizing region, n = 5; anterior mesenchyme, n = 5. (B) Parameters of bud outgrowth (as in Fig. 8A) after 48 h as a function of retinoic acid concentration. Normal bud, n = 4; 0.01, n = 3; 1, n = 3; 10, n = 1; polarizing region, n = 1. (C) Changes in the real length of apical ridge, anterior and posterior margin shown in Fig. 8A expressed as a proportion of bud perimeter (relative length) and plotted as a function of retinoic acid concentration. (D) Changes in the parameters of bud outgrowth (as in Fig. 8B) after 48 h expressed as relative lengths and plotted as a function of retinoic acid concentration.

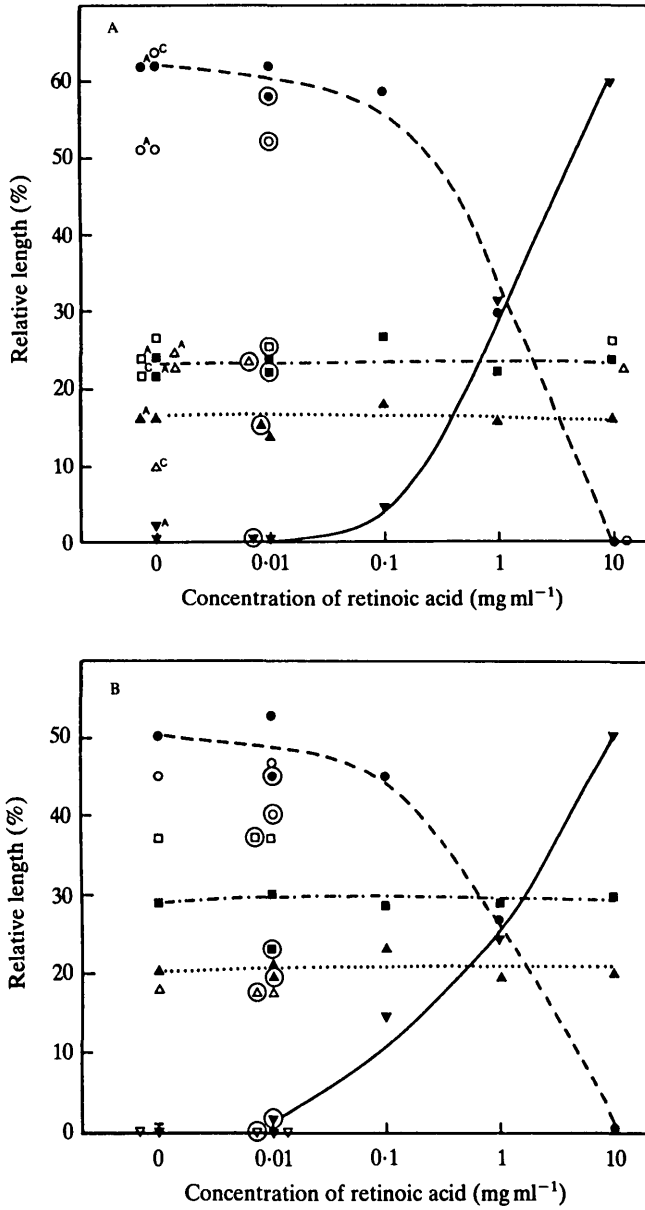


Fig. 9. (A) Beads soaked in a range of retinoic acid concentrations were implanted at the apex of the bud. Relative lengths of apical ridge, apical zone of ridge regression and anterior and posterior margin after 24 h were plotted as a function of retinoic acid concentration. Since a discrete portion of ridge disappeared at the apex, its length was considered separately from that of both the anterior and posterior margins. Curves represent apical ridge (---), anterior margin (.....), posterior margin (---), apical zone of ridge regression (—). Normal bud, n = 7; 0.01 mg ml<sup>-1</sup> retinoic acid, n = 4; 0.1, n = 4; 1, n = 3; 10, n = 4; polarizing region, n = 3; anterior mesenchyme, n = 5. (B) Parameters of bud outgrowth (as in Fig. 9A) after 48 h plotted as a function of retinoic acid concentration. Normal bud, n = 4; 0.01, n = 4; 0.1, n = 2; 1, n = 3; 10, n = 3; polarizing region, n = 4.

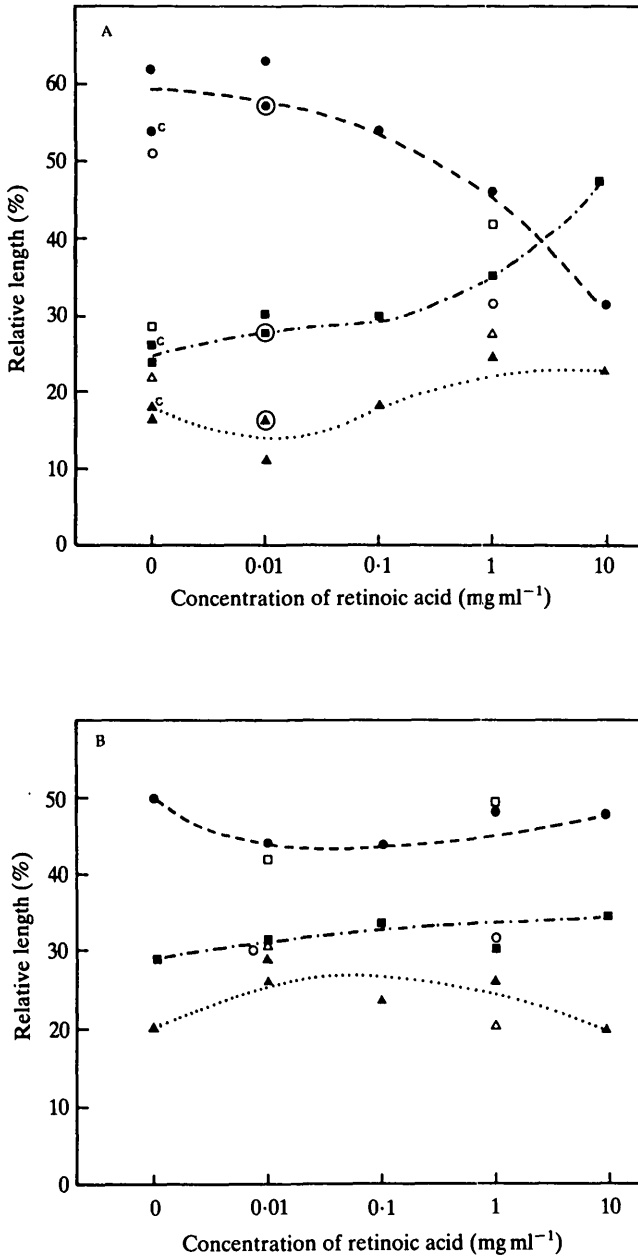


Fig. 10. (A) Beads soaked in a range of retinoic acid concentrations were implanted at the posterior of the bud. Relative lengths of apical ridge anterior and posterior margin after 24 h were plotted as a function of retinoic acid concentration. Curves represent apical ridge (---), anterior margin (.....), posterior margin (-.-.-). Normal bud, n = 7; 0.01 mg ml<sup>-1</sup> retinoic acid, n = 3; 0.1, n = 4; 1, n = 5; 10, n = 5; DMSO, n = 4; polarizing region, n = 4; anterior mesenchyme, n = 3. (B) Parameters of bud outgrowth (as in Fig. 10A) after 48 h plotted as a function of retinoic acid concentration. Normal bud, n = 4; 0.01, n = 5; 0.1, n = 5; 1, n = 6; 10, n = 7.

following retinoic acid application. The changes in bud form are brought about by alterations in the extent of the apical ridge and its position around the bud perimeter. These alterations are detected at 24 h of treatment. They are subsequently modified and growth takes place so that by 48 h clearly different bud forms result. The changes in the extent of the apical ridge have been documented quantitatively and dose-response curves obtained.

To summarize, we will consider first the effects of application of retinoic acid to the anterior of the bud. With low doses, the length of the ridge increases. However, since at 24 h the perimeter of the bud has also increased, the proportion of the perimeter occupied by the ridge is not significantly changed. By 48 h, the length of the ridge has increased still further and now occupies a larger proportion of the bud perimeter. The increase in ridge length is due to its extension along the anterior margin of the bud. These changes lead to additional outgrowth anteriorly. With application of higher doses of retinoic acid, the length of the ridge, instead of increasing, becomes restricted to more posterior parts of the bud. Outgrowth is then confined to these narrow posterior portions.

When retinoic acid is applied at the apex or to the posterior margin, similar dose-dependent changes in the extent of the apical ridge are seen at 24 h but the region affected depends on the position of application. With application of low doses of retinoic acid at the apex, the proportion of the bud perimeter occupied by the ridge is unchanged. The ridge, although not significantly longer as with anterior application, similarly tends to persist over anterior parts of the bud. This leads by 48 h to additional outgrowth anteriorly and a bilobed bud. With application of high doses, the length of the ridge decreases as it does following anterior application. However, since the ridge is obliterated, first near the implant and then at progressively greater distances away, the ridge first disappears at the apex and then the ridge both anterior and posterior to the implant is progressively affected. The total obliteration of the ridge that soon results halts outgrowth.

When low doses of retinoic acid are applied posteriorly, the ridge becomes shorter and confined to more anterior parts of the bud. This progressive restriction of the ridge is, at 24 h, a mirror image of that following anterior application. At 48 h, an almost normally proportioned ridge has been re-established. The anterior margin is progressively reduced as the ridge is shifted more anteriorly.

### *The apical ridge and bud shape*

One interesting observation is relevant to the possible role of the apical ridge in shaping the contours of the bud. It was seen that where the apical ridge had disappeared, the bud bulged instead of being dorsoventrally flattened. Although the overall shape of the bud will be the net result of forces generated by both the mesenchyme and the ectoderm, this observation suggests that the ridge acts as a seam to maintain the shape of the ectodermal casing of the bud (Hornbruch & Wolpert, 1970). This is in contrast to the view that the mesoderm is the most important determinant of bud shape (Ede, 1971).



*A model to account for the effects of retinoic acid on the extent of the ridge*

Changes in the extent and disposition of the ridge can be interpreted in terms of a graded effect of retinoic acid. We have already shown that when a bead soaked in retinoic acid is implanted at the anterior margin of the bud, a concentration gradient is soon established across the bud tissue (Tickle *et al.* 1985). A similar gradient is also established when the bead is implanted posteriorly. Thus, we propose that the effects on the apical ridge reflect dose-dependent responses to the local concentration of retinoic acid that varies with distance from the source. At high concentrations, above a certain threshold concentration, the ridge would disappear but at lower concentrations of retinoic acid the ridge would persist. At extremely low concentrations, the effects on the ridge are negligible and it is not maintained. The model is shown in Fig. 11 and can account for all the main effects of retinoic acid found here. These include the apparently paradoxical effect of first the increase and then the gradual decrease in ridge length found when beads soaked in progressively higher concentrations of retinoic acid are implanted anteriorly. Furthermore, the model illustrates why the ridge is confined to different regions of the bud perimeter when the position of application is varied.

The changes in the extent of apical ridge give rise to the different forms of bud outgrowth. An increase in extent of the apical ridge leads to a broadened outgrowth and a decrease to a narrower one. Complete absence of the apical ridge would lead to truncations. In this respect, it is interesting that the apical ridge of limb buds of mouse embryos was reported to disappear when the embryos were systematically treated with vitamin A at doses which lead to truncations (Yasuda & Nakamura, 1983).

*A direct or indirect effect of retinoic acid on the ridge?*

The model outlined above proposes that the local concentration of retinoic acid determines ridge morphology. One possibility is that there is a direct effect on the epithelial cells. Indeed there are many precedents for retinoids affecting epithelia (reviewed Sporn & Roberts, 1983). A second possibility is that the effects of retinoic acid on the ridge are indirect and mediated by the underlying mesenchyme. Thus the local concentration of retinoic acid could produce alterations in the mesenchyme that then secondarily lead to changes in the ridge. The time course of changes makes this possibility more likely because the apical ridge may not show full alterations in its extent until 48 h after treatment. This is particularly clearly seen when retinoic acid is applied to the posterior of the bud. The shortening of the apical ridge seen 24 h after treatment is not maintained at 48 h and the normal proportion of perimeter occupied by ridge is re-established.

Of particular relevance to the role of the mesenchyme is the similarity between the effects on the ridge of grafting polarizing region tissue and implanting beads soaked in  $0.01 \text{ mg ml}^{-1}$  retinoic acid. In both cases, the ridge immediately adjacent to the implant disappears but persists further away. Thus the effects of retinoic acid could involve converting cells adjacent to the bead into polarizing region

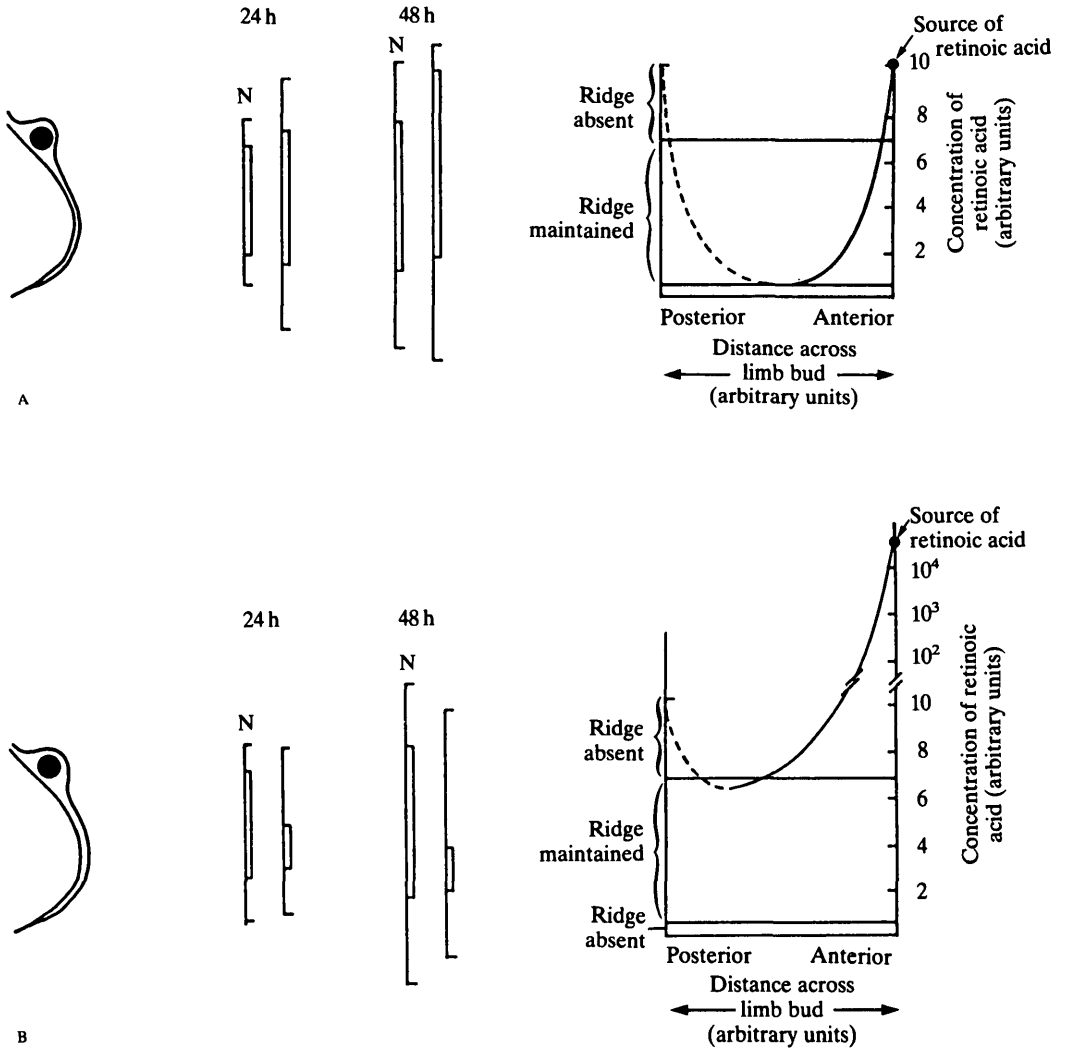
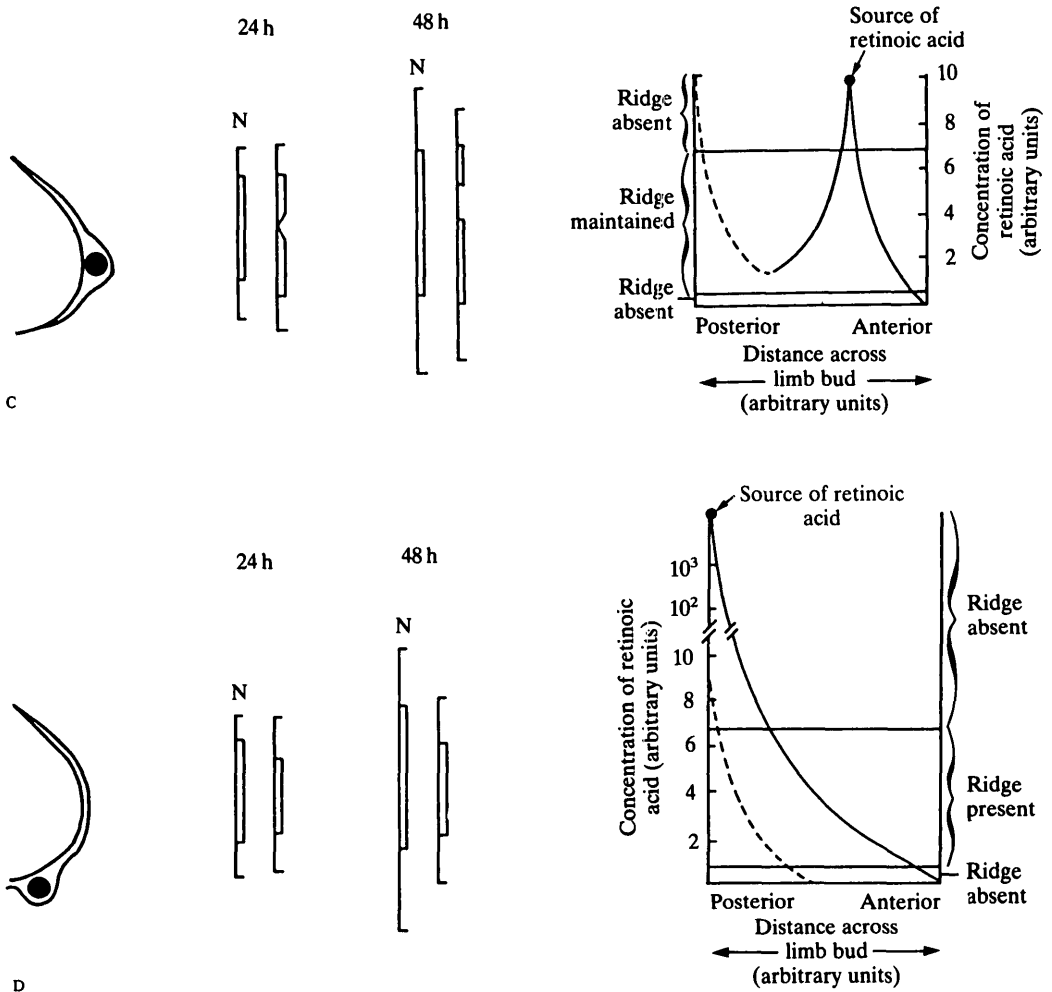


Fig. 11. Diagrams to illustrate the effects of retinoic acid on the extent and position of the apical ridge and a model that accounts for these changes. On the left is depicted the position of the bead immediately after implantation. Next to this are linear representations of the bud perimeter drawn to scale (1:10) showing the extent and position of the apical ridge (indicated by boxed section) at the times shown. For comparison similar representations of normal buds (N) are shown alongside. On the right is shown diagrammatically the gradient of retinoic acid (solid line) that is established across the bud. The horizontal lines represent the thresholds for the limits of the ridge. The dotted curve represents the gradient of the hypothetical morphogen from the polarizing region. (A) Anterior application of low concentrations of retinoic acid. (B) Anterior application of high concentrations of retinoic acid. (C) Apical application of low concentrations of retinoic acid. (D) Posterior application of high concentrations of retinoic acid. Note that the linear diagrams are aligned by their midpoints and this makes the anterior shift of the ridge less obvious.



tissue that then leads to changes in bud morphology. Thus the region of the bud over which the ridge disappeared would reflect the distribution of polarizing tissue. On this interpretation, increasing doses of retinoic acid would convert progressively extensive regions of the bud into polarizing tissue.

Effects of polarizing tissue on the morphology of the ridge have previously been reported by Saunders & Gasseling (1968). They noted that the ridge disappeared in the immediate vicinity of polarizing region grafts. This effect on the ridge also appears to underlie the failure of development of limbs reconstructed from cell suspensions containing a high proportion of polarizing region cells (Crosby & Fallon, 1975; Frederick & Fallon, 1982). However, for many years more attention has been given to the activity of posterior bud tissue that leads to persistence of the ridge at some distance away. It has been postulated that the tissue from this part of

the bud produces a diffusible substance that has been called the apical ridge maintenance factor (Zwilling, 1956; Saunders & Gasseling, 1968). More recently, studies in culture have demonstrated a long-range effect of polarizing tissue on ridge morphology. When anterior tips of wing buds are cultured in contact with polarizing tissue, the apical ridge over the anterior part of the bud maintains its thickened morphology (MacCabe & Parker, 1975). Furthermore, this effect on the ridge was produced even when the polarizing tissue was not in direct contact with the responding tissue. Some progress has been made in purifying the factor necessary to maintain the ridge (MacCabe, Leal & Leal, 1983).

The experimental effects of polarizing tissue on the apical ectodermal ridge draw attention to the relationship between the two in the normal bud. The apical ridge is absent in the immediate vicinity of the polarizing region. Thus the polarizing region in the normal bud appears to define the posterior margin of outgrowth. The apical ridge is thickened over the posterior part of the bud adjacent to the polarizing region. Furthermore, there is some evidence for a graded effect on ridge morphology over the posterior part of the bud; the thickness of the ridge varies with distance from the polarizing region (Todt & Fallon, 1984). However, the ridge is not maintained over the anterior part of the bud. We suggest that this is due to the tissue being out of range of influence of the polarizing region rather than the presence of an anterior boundary region.

*Interaction between signal generated by retinoic acid application and signal from host polarizing region*

To appreciate the effects of retinoic acid application on bud outgrowth, interaction with the host polarizing region must be considered (see Fig. 11). Thus, the effects of retinoic acid that lead to ridge persistence are always seen at the anterior of the bud since the ridge here is not maintained by the host polarizing region. For example, with anterior application of low doses of retinoic acid, the total length of the ridge can increase because of summation of the length maintained as a result of retinoic acid release from the bead and that maintained by host polarizing tissue. A second example concerns the effect of retinoic acid when applied posteriorly over a fairly wide range of concentrations; again the ridge persists over the anterior parts of the bud. In this case, however, the total length of the ridge does not increase because the high concentrations posteriorly apparently override the maintenance effect of the host polarizing region and lead to disappearance of the ridge posteriorly. The displacement of the ridge anteriorly is allowed because the anterior limit of the ridge is not fixed by a boundary region. The ridge is therefore free to extend along the anterior margin as the posterior boundary is shifted anteriorly. These considerations provide some insights into why anterior application of retinoic acid can lead to the development of additional digits whereas posterior application almost always gives normal wings.

The phenomenon of high concentrations of retinoic acid overriding the maintenance activity of the host polarizing region is also seen when high

concentrations are applied anteriorly or at the apex. In these cases displacement of the ridge posteriorly cannot occur because of the boundary specified by the polarizing region. The apparent synergism between the effects of the local source of retinoic acid and the polarizing region suggest that the same signals are involved: either the polarizing region produces retinoic acid (or a related retinoid) or retinoic acid converts cells adjacent to the source into polarizing tissue that then produces the appropriate signal.

#### *Establishment of a posterior boundary for limb outgrowth*

The changes in the extent of the apical ridge induced by local application of retinoic acid appear to do more than just mark the limits of bud outgrowth. In some way, new posterior boundaries are defined near the retinoic acid implant, just as in the normal bud the polarizing region is sited at its posterior margin. An attractive hypothesis is that the same signalling mechanism controls both the extent of the ridge and pattern formation across the anteroposterior axis. Thus the apical ridge maintenance factor might be equivalent to the hypothetical morphogen produced by the polarizing region (reviewed Tickle, 1980). In this context, the ideas of Meinhardt (1984) are relevant. He has proposed that boundaries act as organizing regions for pattern formation. At the cellular level, it may be significant that the cells of the apical ridge are extensively linked by gap junctions (Kelley & Fallon, 1976; Fallon & Kelley, 1978). Thus cell-cell communication along the anteroposterior axis might be mediated via the ridge cells. When the ridge disappears or becomes thinner, as, for example, at the apex of the bud as a result of grafting polarizing tissue here or implanting beads soaked in retinoic acid, this pathway of communication could be interrupted. One can speculate that this might be an important factor in establishing a boundary region.

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