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The effect of cell killing by X-irradiation on pattern formation in the chick limb

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SUMMARY

It has been suggested that positional information along the proximo-distal axis of the limb-bud is specified by time spent in the progress zone. Mesenchyme cells have been killed by X-irradiation, reducing the rate cells leave the zone. The time spent there by some cells is thus increased. When limbs, stage 18/19, stage 21, or tips of stage 24, are treated with increasing doses of X-irradiation, from 1000 rads to 2500 rads proximal structures are progressively lost, whereas distal ones – the digits – are relatively unaffected. There was no evidence for intercalation of missing parts. These effects are due to killing or damage of mesenchyme cells: the ectoderm is not affected at these doses. The results are consistent with a quantitative analysis based on the progress zone model, in which viable cells repopulate the progress zone and gradually restore it to normal as non-dividing cells are diluted out. It is suggested that any treatment causing damage to the mesenchyme at early stages will give similar results.

The mesenchyme cells appear to be surprisingly resistant to radiation damage. The form of the limb-bud is not altered by damaging the mesenchyme. Differences in the development of structures at similar proximo-distal levels, following irradiation, is considered in terms of the requirement of a threshold number of cells.

INTRODUCTION

A model for pattern formation in the development of the chick wing suggests that cells are assigned positional values in a co-ordinate system and that the cells interpret this positional information by appropriate cytodifferentiation (Wolpert, Lewis & Summerbell, 1975). The pattern of the limb is laid down in a proximo-distal sequence during development; first, proximal structures such as humerus, while the distal structures, the digits, are formed last of all. A theory has been put forward for the way in which positional value could be assigned along the proximo-distal axis of the limb (Summerbell, Lewis & Wolpert, 1973; Summerbell & Lewis, 1975). This suggests that the positional value of cell changes autonomously with time, in a special region at the tip of the limb-bud – the progress zone. The extent of the progress zone is controlled by the apical ectodermal ridge (AER). Since all the cells in the progress zone

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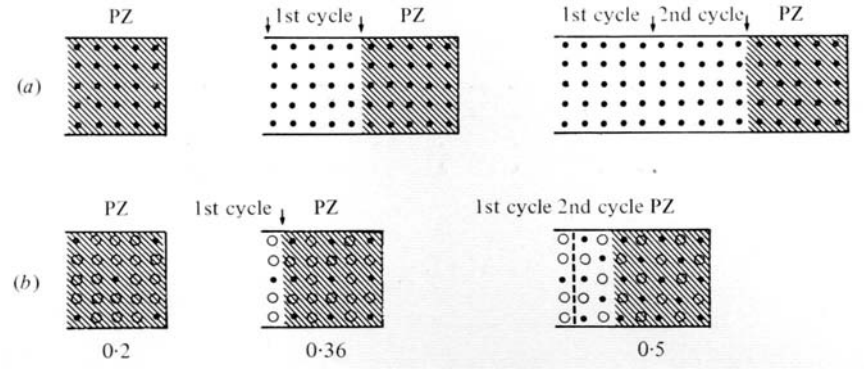

Fig. 1. A simplified model to show the effect of X-irradiation on the progress zone. Two discrete cells cycles in the outgrowth of an idealized limb are shown. In the control (*a*) 25 cells leave the progress zone PZ at each cell cycle and these are assumed to form a limb rudiment such as a humerus, or radius and ulna. In the irradiated case (*b*) the open circles represent cells no longer capable of dividing and the fraction of dividing cells is 0·2. That is 80% of the cells have been inactivated. At the first cell cycle only five cells come out of the progress zone and only one of these is normal. However, at the next cell cycle ten cells will emerge, of which five are normal. Note that the fraction of normal cells in the progress zone is now 0·5.

are dividing (Summerbell & Lewis, 1975), cells are continually overflowing and leaving the zone. As soon as cells leave the zone their positional value ceases to change. The cells thus can measure how long they have been in the zone and this provides them with positional information. The cells that leave the progress zone early give rise to humerus, those leaving a little later give rise to radius and ulna and those which leave last are those which form the digits. A possible mechanism for measuring time spent in the progress zone would be by counting the number of cell divisions a cell undergoes while in the zone (Lewis, 1975).

This model can account for a variety of experimental data obtained on chick limb development but it has not been easy to devise further tests for it. Grafts of the tip regions between limbs at different stages of development have, on the whole, supported the idea that the cells in the tip develop autonomously and there is no influence from proximal tissue (Summerbell & Lewis, 1975). Somewhat different results have been obtained by Kieny (1977) who has evidence for interaction and regulation at early stages in development. Ideally what one would like to test is whether the development of a group of cells is simply determined by the length of time the cells spend in the progress zone or whether other influences are important, such as signalling from more proximal or distal mesenchyme cells. One approach is to change the length of time cells spend in the progress zone. We have been able to do this by killing many of the cells in the progress zone. As some of the cells in the tip are now incapable of dividing, the rate at which cells leave the progress zone will be reduced. This means that cells will spend more time in the tip than they would normally.

Again, if some cells are killed and disappear, cells may divide in order to repopulate the progress zone. According to the progress zone model we would expect that the resultant limbs would lose or have reduced proximal structures but that distal ones would be relatively unaffected (Fig. 1).

It is clear from the results of Goff (1962) and Summerbell (1978) that the skeletal anomaly seen in a limb which had been treated with doses of X-irradiation up to 1000 rads depends on the stage at which the embryo was irradiated. The defects are localized; at early stages the proximal parts tend to be shortened, while at later stages distal parts are affected. The structures which are most affected are those which are just about to be laid down. If cells just leaving the progress zone are incapable of dividing that portion of the limb to which they normally give rise will be reduced (see Appendix). If cells are killed and removed, then cells must remain longer in the zone until it is repopulated. Again the structure just to be laid down is reduced. This assumes that there is no compensatory growth control mechanism along the proximo-distal axis (Summerbell, 1977).

The defects produced by X-irradiating young limb-buds with doses of 1000 rads are relatively small. With higher doses of X-irradiation the embryos die before one can recognize what structures have been produced. We have overcome this difficulty by grafting irradiated limb-buds to unirradiated chick hosts to continue development. We have thus been able to look at the effects of higher doses of X-irradiation than possible before, and to examine the effects of increasing doses of X-irradiation on limb-buds given at the same stage of development. However, unknown to us, Pinot (1970) had already carried out a very similar study.

MATERIALS AND METHODS

The major series of experiments was to treat chick embryos at stages 20/21 (Hamilton–Hamburger stages) with increasing doses of X-irradiation. The eggs were windowed on the third day of incubation and the embryos staged. The eggs were then returned to the incubator until the desired stage of development. The embryos were then irradiated *in ovo*, after cutting a hole in the sellotape used to seal the eggs, with doses of X-irradiation between 1000 and 3000 rads using a Marconi X-ray machine at 230 kV and a dose rate of 1000 rads/min at a height of 14 cm. The right wing was removed from the irradiated embryos and grafted into a host chick embryo, stage 24–25. The irradiated embryos, from which the right wings had been removed, were reincubated and in all cases died. The site for the graft was prepared by removing the ectoderm over the anterior margin of the wing of the host embryo together with a little of the underlying mesenchyme. The irradiated limb-buds were pinned to the anterior margin with two pins made out of platinum wire, 25 μm in diameter. The grafted limb-bud was positioned so that its dorsal surface was uppermost, its posterior edge was towards the distal end of the host wing and its anterior edge

at the proximal part of the host wing. The pins were usually removed 12 h after the graft was carried out. Control grafts were carried out by pinning unirradiated stage-20/21 right wing-buds to the anterior margins of host wing-buds. Seven days following the operation the host embryos were removed from their shells and the wings bearing the grafts cut off and placed in 5% trichloroacetic acid. The limbs were stained with Alcian green to show the cartilage pattern. After clearing in methyl salicylate the limbs were examined and the lengths of the ulna and digit *3* were measured.

A similar series of experiments were carried out using embryos at stage 18/19. The embryos were treated with doses of X-irradiation between 1000 and 3000 rads. The limbs were then grafted to the anterior margin of limb-buds of unirradiated hosts. In addition, we looked at the effect of high doses of X-irradiation on older limb-buds. For this we used the tips of stage-24 limb-buds as these were easier to graft than the whole limb at this stage. Grafts were carried out following treatment with the same range of X-irradiation doses.

As controls for the irradiated wing-buds to check that the grafts did indeed develop autonomously and cells were not contributed by the host wing, we carried out two different types of experiments. We grafted quail wing-buds; quail cells can be recognized histologically in sections stained with Feulgen as they have a Feulgen-positive nucleolus (Le Douarin, 1973). We have looked at the type of cells in quail limbs grafted to the anterior margin of host chick wing-buds (see later for details of Methods). As a more gross control we grafted leg buds that had been treated with high doses of X-irradiation of 1500 and 2000 rads. We made whole mounts of the skeleton of the limbs that developed from the grafts. We could then tell from the character of the structures formed whether the limb developed from graft or host.

In one series we followed the development of stage 20/21 irradiated buds after grafting, by making camera lucida drawings of the outline of the developing limbs which were fixed at 12 h, 24 h and 48 h, after grafting. This enabled us to determine how much outgrowth had taken place. We also examined the limbs histologically to see what effects X-irradiation had on the morphology and mitotic index of the limb mesenchyme cells. The limbs which were irradiated with 1000 or 2000 rads were fixed 12 h, 24 h and 48 h after grafting in half-strength Karnovsky's fixative (Karnovsky, 1965). They were then dehydrated through a series of ethanols, cleared in epoxypropane and then embedded in araldite. Semi-thin sections (1–1½ μm thick) were cut on a Cambridge ultramicrotome and stained with 0·1% toluidine blue in borax (Trump, Smuckler & Benditt, 1961). In cases where quail limbs were grafted, alternate sections were placed on a second batch of slides, which were then stained with a modified Feulgen technique.

In order to find out which component of the early limb-bud, the ectoderm or the mesenchyme was affected by the X-irradiation treatment or whether both were equally affected, we separated the ectoderm and the mesenchyme of limb-

Table 1. *Effect of increasing doses of radiation on the length of the ulna and digit 3*

Stage	Dose (rads)	No.	Length of ulna in mm (S.D.)	Length of digit *3* in mm (S.D.)	Remarks
18/19	Control	4	3·3 (0·6)	4·35 (0·3)	—
	1000	4	1·8 (0·2)	4·1 (0·1)	Fused at elbow
	1200	5	1·6 (0·4)	4·0 (0·3)	Fused at elbow. Radius digit *2* and *4* may be absent
	1400	6	0·9 (0·7)	2·7 (1·0)	
	1500	8	0·5 (0·7)	3·1 (0·7)	
	1700	5	0·5 (0·5)	3·1 (0·3)	
	2000	5	—	—	Small piece of cartilage
20/21	Control	10	3·6 (0·3)	4·4 (0·3)	—
	1000	9	1·9 (0·4)	4·1 (0·4)	Fused at elbow
	1500	8	1·7 (0·3)	3·7 (0·5)	Fused at elbow
	2000	11	—	2·6 (0·9)	Digit *2* may be missing
	2500	7	—	—	Small pieces of cartilage
24 (Tip only)	Control	12	—	4·6 (0·3)	Wrist present
	1000	2	—	4·9	Wrist present
	1500	3	—	3·2	Digit *2* missing
	2000	4	—	2·4 (0·2)	Digit *2* missing, *4* absent or reduced
	2500	3	—	1·7	Digits *2* and *4* absent

buds X-irradiated with 2500 rads, and recombined them with normal mesenchyme and normal ectoderm. Following treatment for 1–2 h with 2% trypsin (Difco 1:150) at 4 °C (Szabo, 1955) the limbs were placed in medium containing serum and the ectoderm hull removed from the mesemchyme core. Recombinants were then made and left at room temperature and then at 37 °C for at least an hour prior to grafting to the anterior margin of host embryo wings. In most cases leg ectoderms were used. No leg structures formed in these cases, showing that the separation of ectoderm from mesenchyme was clean.

A small number of embryos were irradiated at 1000 rads at different stages of development. The right wing only was irradiated and the rest of the embryo was shielded with tantalum. In this case the X-ray machine was used at 50 kV, with a dose rate of around 87·5 rads/min. This was to confirm the results of Goff (1962) and Summerbell (1978) and resulted in a high number of survivors presumably due to the shielding.

RESULTS

Grafts of irradiated buds

Grafts of normal stage-20/21 limb-buds to the anterior margin of host wing-buds gave normal wings (Fig. 2). Not only did the wings appear to be morphologically normal but also they were quantitatively normal as judged by

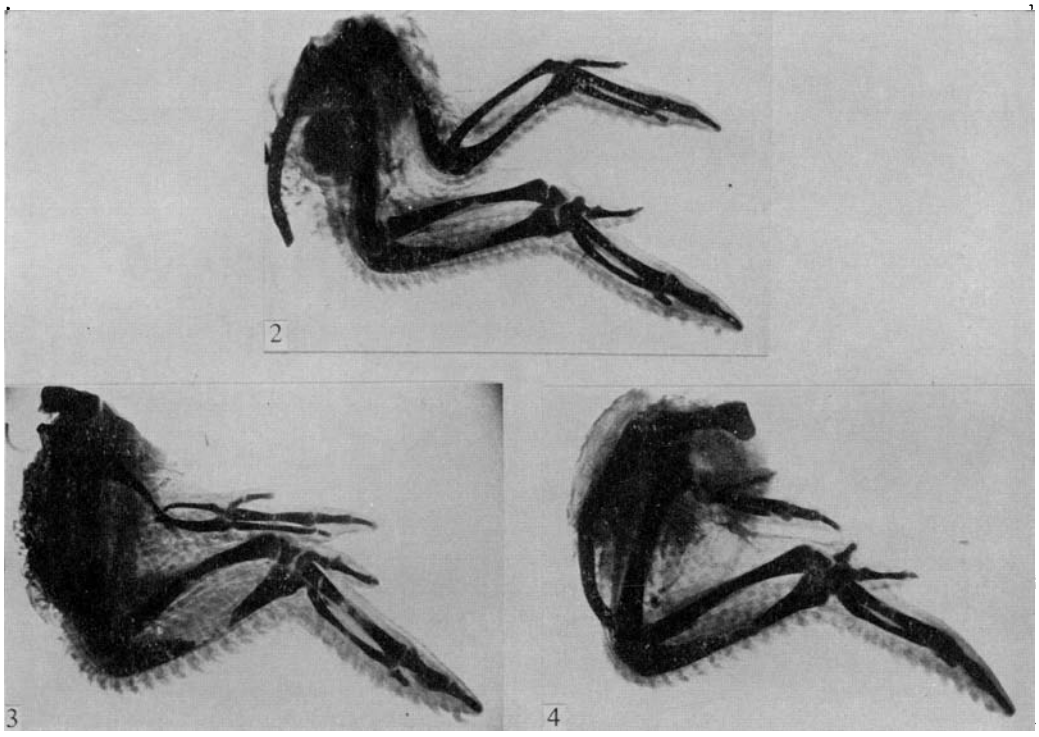

Fig. 2. Whole mount of host wing (lower) and wing (upper) that developed from a graft of a normal stage 20/21 right wing-bud. Note that the skeletal pattern of the grafted wing is the same as that of the host wing, although the grafted wing is smaller.

Fig. 3. Whole mount of host wing (lower) and wing (upper) that developed from a graft of a stage 20/21 wing-bud irradiated with 1500 rads. Note that the radius and ulna are shortened, only the distal parts being present. The proximal part of the humerus is normal, the distal part appears missing and fusion has occurred between humerus and ulna. The digits look more or less normal.

Fig. 4. Whole mount of host wing (lower) and wing (upper) that developed from a graft of a stage 20/21 wing-bud irradiated with 2000 rads. Reasonably normal digits have developed. The radius and ulna are missing and so, too, is the humerus.

the proportion of the length of the ulna to that of digit *3* (see Table 1). This was within the range of that obtained for unoperated wings by Summerbell (1976, 1978).

With increasing doses of radiation to stage-20/21 limb-buds there was a progressive increase in damage and loss of proximal parts of the wing skeleton, the distal parts being least affected. The length of the ulna and digit *3* is given in Table 1.

Fusion at the elbow was a consistent feature of doses of 1000 rads and above (see also later for shielded embryos). At 1500 rads both radius and ulna were noticeably reduced, the radius being more affected than the ulna. However, it does appear that it is the proximal parts of these forearm elements that are

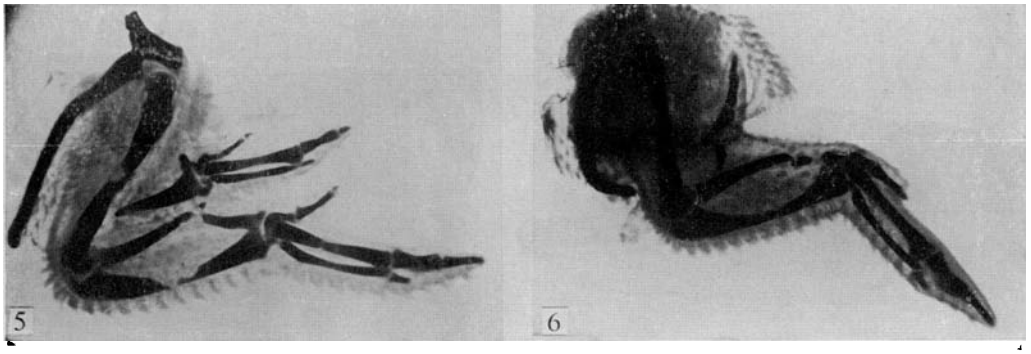

Fig. 5. Whole mount of host wing (lower) and wing (upper) that developed from a graft of a tip of a normal stage-24 wing-bud. Part of the ulna is present together with wrist and normal digits.

Fig. 6. Whole mount of host wing (lower) and wing (upper) that developed from a tip of a stage-24 wing-bud irradiated with 2500 rads. The distal phalanges of digit *3*, the only digit to form, are well developed. The proximal metacarpal of digit *3* is shortened and distorted.

missing (Fig. 3). On occasion digit *2* was missing. Notice however that the proximal part of the humerus was still present. When the limb-buds are treated with 2000 rads the humerus is absent but traces of the radius and ulna are present and digits may be well developed (Fig. 4). Digit *3* is about 65 % of the control length of digit *3*. After treatment at this dose of X-irradiation the digits were sometimes grossly abnormal. Digit *2* was often missing and occasionally digit *4* lacked the distal phalange. In one case, however, digit *4* had an extra phalange. Following 2500 rads only a few fragments of cartilage developed from the grafted bud and after 3000 rads there was no trace of the graft.

Increasing doses of radiation to stage-18/19 wing-buds gave substantially a similar pattern. Again, it is the distal parts that are least affected. With increasing doses of radiation the length of both ulna and digit *3* are decreased (see Table 1). However, whereas the ulna is very small or absent at 1500 rads and 1700 rads, digit *3* is still 75 % of its control length. At 2000 rads only small unidentifiable pieces of cartilage develop and at 2500 rads nothing at all can be found. As found with stage-20/21 limb-buds we do not always lose all the structures across the limb as we lose proximal parts. At 1400 rads the radius and digits *2* and *4* may be missing.

Grafts were made of the tips of stage-24 buds whose size corresponded more or less to that of a whole stage-21 wing-bud. The controls usually gave digits and a small piece of the distal part of the ulna (Fig. 5) since the more proximal parts of the wing were not included in the graft. With increasing doses of radiation, parts of the pattern were lost. At 1000 rads digit *2* was absent, but a fragment of the ulna still remained. Increasing the dose of radiation still further, resulted in more structures being lost; the ulna disappeared, also digit *4*. The metacarpals of the digits, that is the most proximal parts, were affected

first. Figure 6 shows the structures that developed following a dose of 2500 rads. The distal phalanges of digit *3*, the only digit to develop, are clearly recognizable, whereas the metacarpal is shortened and distorted. It should be noted that even at these doses of 2500 rads, parts of the wing pattern still developed (see Table 1, and compare with the effect of the same dose at stage 18/19 and stage 20/21).

All the grafts developed autonomously. There was no indication, from histological sections, that cells moved into the grafted limb from the host limb at early stages following grafting. There appeared to be a clear demarcation between the host and the grafted tissue. This impression was confirmed in sections of grafts of quail limb tips which had been on host limbs for 1 or 2 days. We wanted to make sure, however, that when irradiated limb-buds were used there was no contribution to the developing graft at later stages. When we grafted stage-20/21 leg buds which had been irradiated with 1500 rads and 2000 rads to the anterior margin of host wing-buds in the normal manner, typical leg structures developed. Toes, for instance, are readily distinguished from wing digits.

Grafts of recombined ectoderm and mesoderm

Recombinations were made between unirradiated and irradiated ectoderm hulls and mesenchyme cores. Ectoderms irradiated with 2500 rads combined with unirradiated stage-20/21 wing mesenchyme cores gave in the best cases (2 out of 15) limbs that looked almost normal. In the other cases well-formed digits usually resulted, although digit *2* was sometimes missing. The humerus was usually normal and the radius and ulna were most affected. The limbs we obtained with control combinations of unirradiated ectoderm and mesenchyme were essentially similar although the results were rather variable. The reverse combination however, using unirradiated ectoderm with mesenchyme cores that had been treated with 2500 rads, gave no growth at all. From normal

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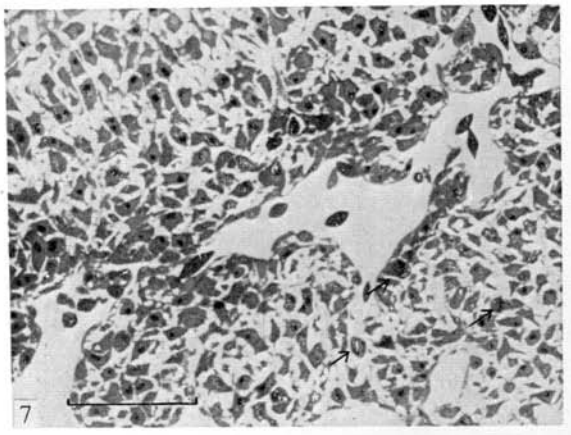
Fig. 7. Mesenchyme of a normal limb-bud grafted at stage 21 to the anterior margin of a host limb-bud. Graft fixed after 12 h. Note blood vessels and several cells in mitosis (arrowed). Scale bar is 50 μm.

Fig. 8. Tip of the same limb-bud shown in Fig. 7. Shows appearance of apical ectodermal ridge. Note some cells containing debris (arrowed) at tip of AER. Scale bar is 50 μm.

Fig. 9. Section of a limb-bud which at stage 21 was irradiated with 2000 rads, now 24 h after grafting. The mesenchyme appears extensively damaged and there are also dead cells in the ectoderm. Scale bar is 100 μm.

Fig. 10. High power of mesenchyme of same limb as in Fig. 9. Note the large macrophages and also several cells in mitosis (arrowed). Scale is 50 μm.

Fig. 11. High power of apical ectodermal ridge of same limb as in Fig. 9. The morphology looks normal and note cell in mitosis in ridge. Also, there is a cell in mitosis in underlying mesenchyme. Scale bar is 25 μm.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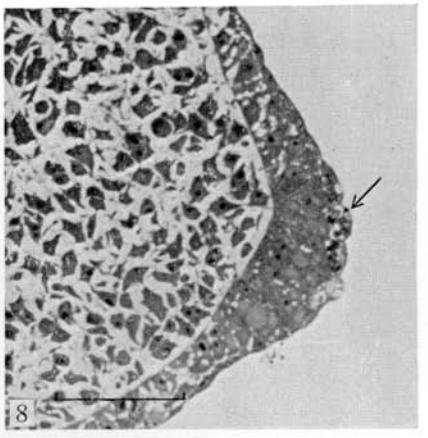
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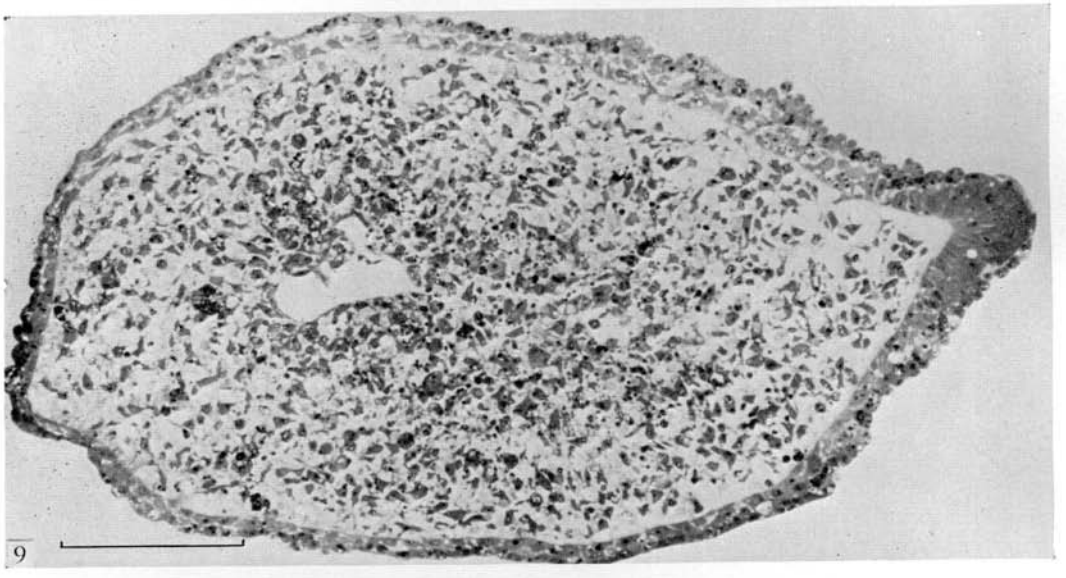
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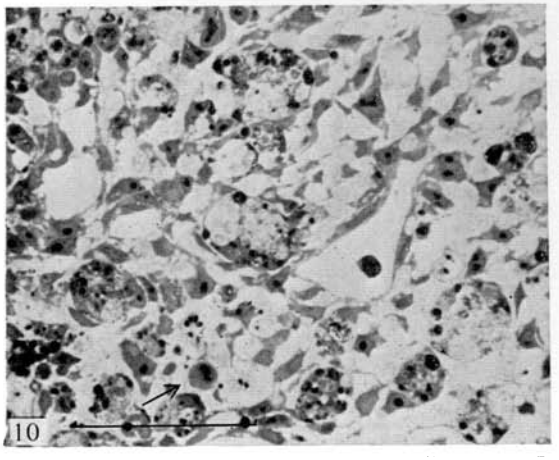
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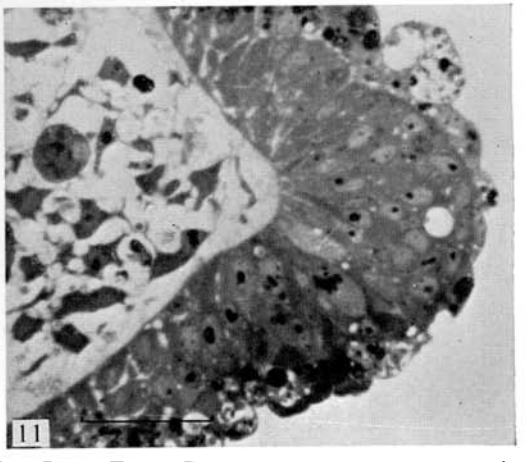
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mesenchyme cores combined with ectodermal hulls from limbs irradiated with a much higher dose, 6000 rads, we obtained truncated limbs and while humerus and sometimes part of radius and ulna developed, no digits were formed in seven experiments.

Growth of grafted buds

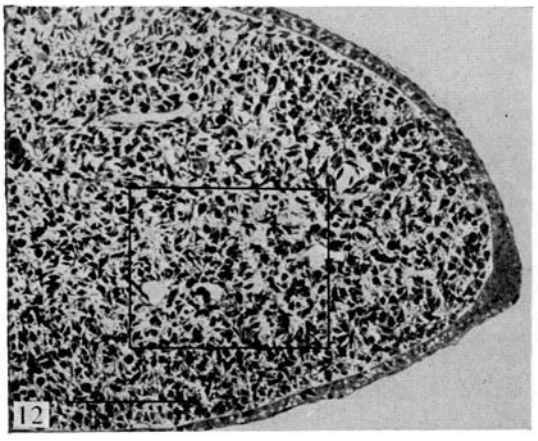
Camera lucida drawings of the outlines of limb-buds after grafting showed that limbs irradiated with 2000 rads did not grow as much as control limbs. We measured the length of the limb outgrowth at different times following grafting (see Table 2). At 12 h following grafting the irradiated limbs had grown almost as much as the control limbs, but when normal and irradiated limbs were compared at 24 h the irradiated limbs were shorter. In fact if we compare the lengths of the limbs drawn at 12 h and 24 h, the irradiated limbs did not appear to have grown very much at all. However, the length of the irradiated limbs at 48 h shows that outgrowth has been resumed but is still retarded compared with that of control limbs. If however we compare the lengths of the developing hand plates they are almost the same.

Histology

In sections of limbs, irradiated with 2000 rads and fixed 12 h after grafting, there were signs of damage. This appeared to be more extensive in the dorsal half of the mesenchyme than the ventral half. Some small macrophages were present and also small fragments of cells. However, some of the mesenchyme cells were in mitosis, and the mitotic index, about 2%, was almost the same as in a control limb, which was fixed 12 h after grafting. The population density of cells in the limb tip however was reduced by about half.

Twenty-four hours following grafting, limbs that had been irradiated with 2000 rads showed extensive damage in the mesenchyme; compare Figs. 7 and 8 with Figs. 9–11. There was debris, including small pieces of cells, and many large macrophages were present. There were also large spaces within the mesenchyme. Often there appeared to be gaps between the mesenchyme and ectoderm and also breaks in blood vessel walls. Many of the mesenchyme cells which did not appear to be dead, were scalloped in outline. Surprisingly despite the general appearance of devastation there were many cells in mitosis and the mitotic index was again about 2%. The density of the cells however was still reduced. In contrast the ectoderm looked healthy, in particular the morphology of the AER looked fairly normal (compare Fig. 8 with Fig. 11). The debris, presumably from cell death in the ectoderm, was localized in the periderm cells, which even in normal limbs sometimes contain phagocytosed material (Fig. 8). Cells in mitosis were observed both in the ectoderm and in the apical ridge itself (Fig. 11).

We also looked at limb-buds which had been irradiated with the same dose of X-irradiation, 2000 rads, at 48 h following grafting. Now, the limb looked almost normal (Fig. 12). Only traces of the damage remained in the mesenchyme



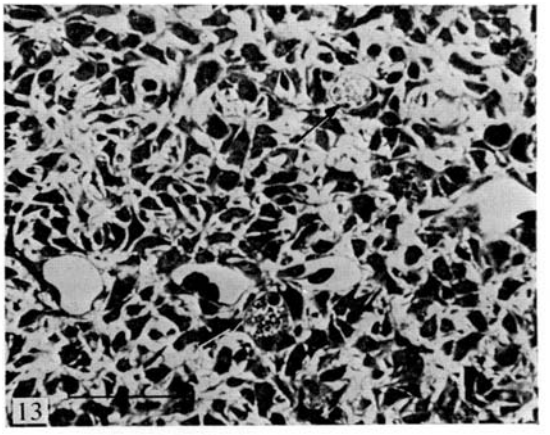


Fig. 12. General view of limb-bud which was irradiated with 2000 rads at stage 21. This graft was fixed after 48 h. The mesenchyme looks more or less normal and so does the apical ridge. Scale bar is 100 μm.

Fig. 13. High power of region of mesenchyme within box on Fig. 12. Remnants of damage are visible. Note two macrophages with vacuoles containing almost fully digested contents (arrowed). Scale bar is 50 μm.

as circular sacs of debris enclosed by a thin rim of cytoplasm (Fig. 13). These may represent the last stages in the disappearance of macrophages from the limb mesenchyme.

Irradiation of shielded embryos

Using a dose of 1000 rads, the right wings of embryos were irradiated using tantalum shielding in an attempt to protect the contralateral limb and the rest of the embryo. The effect on the length of the ulna and digit *3* is given in Table 3. It can be seen that the nature, and location of the defects caused, change with the stage of irradiation. At stage 17–18 the elbow was fused and there was significant shortening of the ulna but the digits were normal. A similar result was obtained at stage 20/21; the elbow was fused, the ulna shorter and again the digits normal. The proximal part of the humerus looked normal too. We noticed in some limbs formed from buds irradiated at both these stages a short rounded bulge of cartilage protruded posteriorly from the region of the fused elbow joint. This appeared in most cases to be part of the humerus but in one case was definitely projecting from the ulna. It was present in about 25% of the limbs which developed from irradiated buds of stage 18/19 and stage 20/21. This anomaly was also reported by Goff (1962) at about the same frequency after irradiation of around 800 rads. Limbs that developed from buds irradiated at stage 24 showed in addition to fusion of the elbow that part or all of the radius was missing. Again the ulna was shorter but the humerus looked to be fairly normal except for the fusion at its distal end. When limb-buds were irradiated at stage 26, a quite different pattern emerged. The elbow was no

longer fused and the humerus was normal. The radius and ulna were now fused in the wrist region and, curiously, in two out of eight limbs, the mid-part of the radius was absent. There was little reduction in length of the ulna but digit *3* was shortened. It was the digits that were most significantly affected and the proximal elements of digit *2* were missing. There was also fusion of elements in both digit *3* and digit *4*.

DISCUSSION

The main result was that with increasing doses of X-irradiation to early limb-buds, there was extensive damage to the mesenchyme and the limbs that developed showed a progressive loss of proximal regions, the distal structures – the digits – being least affected. Such limbs are termed phocomelic. Pinot (1970) obtained very similar results. At the higher doses of irradiation even the digits were abnormal, and digit *2* was often absent. Above 2500 rads no structures at all developed. There was no evidence for intercalation of missing parts. These results essentially support the prediction made in the introduction that killing off a significant fraction of the cells in the progress zone should lead to the loss of proximal structures, and some of the cells and their descendants, which would have made proximal structures, now give rise to distal ones. We will try to show how this can be analysed quantitatively.

The results obtained using 1000 rads on embryos at different stages confirm the observations of Goff (1962) and Summerbell (1978) that the effects upon the limb are stage specific. At early stages it is proximal structures that are most affected, whereas at later stages, it is distal structures.

A number of other interesting points emerge which will be considered separately: irradiation has little effect on the ectoderm up to 2500 rads and even the mesenchyme of the early limb-bud seem to show a surprising resistance to radiation; different elements at the same level along the proximal distal axis are affected to different extents; the overall form of the limb is unaffected by irradiation.

Analysis of the effect of irradiation on pattern formation

Treating a stage-18/19 limb-bud with increasing doses of X-irradiation causes progressive shortening of both the ulna and digit *3* (Table 1). The shortening of digit *3* is less than that of the ulna, for example, at 1500 rads the length of the ulna is 15% of the control whereas that of digit *3* is 70%. We can use these results in conjunction with the analysis in the Appendix and so obtain an estimate of the number of damaged cells. Figure 15 (Appendix) shows that digit *3* is represented by the metacarpals and 1st and 2nd phalanges, and the ulna by the forearm. It can be seen that a reduction in length of the ulna to 15% corresponds to the $(\frac{1}{5})$ curve, that is 80% of the cells are damaged. Using this curve, digit *3* should be about 80% its normal length, which corresponds quite well with the observed value.

For a lower dose of irradiation – 1000 rads – the length of the ulna is 55% and that of digit *3*, 93% of the control, and this corresponds well with the values from the ($\frac{1}{2}$) curve; that is 50% cells damaged.

Estimates can be made for stage 20/21 (Fig. 16, Appendix). For 1500 rads the length of the ulna is 47% and digit *3*, 84% of the control. This corresponds reasonably well to the ($\frac{1}{2}$) curve, that is, 50% damage, and it thus seems that for this later stage a smaller fraction of cells is damaged by radiation than at early stages. No explanation can be offered for this, but the trend is confirmed by the results from stage-24 wings. Only the tip of the wing was grafted and parts of digits developed even after 2500 rads; with stage-18/19 wings, no development at all takes place at 2000 rads.

If a stage-20/21 wing-bud is irradiated with 2000 rads, no proximal structures develop and digit *3* is 60% the length of the control. The length of digit *3* corresponds to the ($\frac{1}{5}$) curve, but this predicts that the ulna will be 20% of the control length, yet no ulna develops. At stage 20/21 the upper arm and forearm have already left the progress zone and thus the effect of irradiation is assumed to be on their growth rather than their initial specification. The absence of any ulna after 2000 rads suggests that there is a threshold number of cells required for the development of a cartilaginous structure in the limb and that this value is about 15–20%. If the number of cells in a cartilaginous element is reduced below 15–20% then it will not develop. This is reasonably consistent with our observations.

Unfortunately, it is not easy to determine directly the numbers of cells killed or blocked from further division in the progress zone following the various doses of X-irradiation in order to compare them with the values obtained from the quantitative analysis. There is no reliable way to recognize 'dead' cells and also the mesenchyme cells once dead tend to fragment as described by Hurle & Hinchliffe (1978) for cells in the posterior necrotic zone of the chick wing-bud. Thus mitotic indices must be treated with great caution since many of the cells counted may be dead or dying. The dead cells and fragments are ingested by macrophages which seem to develop from adjacent normal mesenchyme cells (see Ballard & Holt, 1968, in the foetal rat foot, and Dawd & Hinchliffe, 1971, in the chick limb). However, it is by no means clear what happens to these macrophages (see Saunders, 1966).

At 12 h following X-irradiation with 2000 rads, the population density of mesenchyme cells in the limb-bud tip was about half that of control grafted buds, showing that at least 50% of the mesenchyme cells have been killed and it is certain that some of these remaining cells will die since at 24 h there is still evidence of considerable cellular damage and dying cells, and macrophages are much in evidence. Thus the estimate (see above) that 80% of the cells are damaged following 2000 rads at stage 20/21 seems to be not inconsistent with the histology.

On a more gross level, we have looked at the linear outgrowth of limb-buds,

Table 2. *Outgrowth of buds irradiated with 2000 rads at stage 20/21*

	Time (h)	No.	Initial length (μm)	Length (μm) at time indicated	Increase (μm)
Control	12	4	780	1150	400
Experiment		4	760	1025	300
Control	24	4	810	1400	600
Experiment		3	800	1150	350
Control	48	2	750	2600	1900
Experiment		3	780	1700	1100

irradiated with 2000 rads (Table 2). This showed that at 12 h the buds have increased their length by 75 % of the controls and at 24 h there appears to have been no further increase in length. At 48 h the increase in length of the treated limbs is 57 % of the controls, and recovery has begun. This cessation of linear outgrowth is during the first 24 h. It correlates with the extensive cell damage seen at this time. This length of time represents normally about two to three cell division cycles (Summerbell & Lewis, 1975) which would restore the progress zone back to its normal size if 80 % of its cells were destroyed.

The effect of radiation has thus far been assumed to irreversibly damage cells but no account has been taken of the effect of such damaged cells being rapidly removed and thus reducing cell density in the progress zone. We should thus consider the situation in which, following radiation, cells are killed and rapidly removed and the remaining cells divide to repopulate the progress zone. Only when the progress zone is back to its initial size and density would cells begin to leave it. If 75 % of the cells are killed and removed then two cell cycles are required to repopulate the progress zone, if 87·5 %, three cell cycles. It is clear from Table 1 that these cell divisions are those normally associated with the specification of the distal radius and ulna and we can thus expect these proximal structures to be missing. Analysis along these lines suggests that the shortening should only affect the proximal structures and distal structures should be of normal length. Since we find that distal elements are shortened, the true situation is that damaged cells are gradually removed.

We have assumed that cells are damaged randomly in the bud. Could it not be that distal cells are less sensitive than proximal ones? There is no evidence from the histology to justify this and it is hard to believe that sensitivity to such high doses of radiation could vary significantly over such small distances ($\sim$ 300 μm) within the same tissue. Further, it would seem that, if anything, proximal regions should be less sensitive to radiation. At low doses, structures already laid down are little affected. Again, cartilage growth is largely due to cell enlargement rather than cell division (Holder & Wolpert, 1978) and is very resistant to radiation even up to 4000 rads (Biggers & Gwatkin, 1964).

Table 3. *The effect of 1000 rads on the pattern of limbs that developed from shielded embryos treated at different stages*

Stage		No.	Length of ulna in mm (S.D.)	Length of digit *3* in mm (S.D.)	Remarks
17/18	Controls	6	3·7 (0·7)	4·7 (0·5)	—
17/18	Irradiated	6	2·6 (0·4)	4·4 (0·6)	Fused elbow
20/21	Controls	9	3·9 (0·6)	5·0 (0·3)	—
20/21	Irradiated	9	2·4 (0·3)	4·8 (0·2)	Fused elbow
24	Controls	9	2·8 (1·2)	4·4 (0·5)	—
24	Irradiated	9	2·1 (0·6)	4·1 (0·4)	Fused elbow and part of radius may be missing
26	Controls	8	4·1 (0·3)	4·2 (0·2)	—
26	Irradiated	8	3·8 (0·3)	3·4 (1·0)	Fusion at wrist and digits

While the above analysis provides quite good support for the progress zone model it may be asked whether other models could not account for the results equally well. Unfortunately, no other models for pattern formation in the chick limb-bud have been put forward in sufficient detail to enable predictions of the effect of cell damage to be predicted. However, if, following the fate maps of Stark & Searls (1973), all the cells contributing to development are there from the earliest stages, it might be argued that those that differentiate last have the longest to recover and therefore produce the most normal looking elements. This is a very understandable approach to the problem. However, it confuses a fate map with determination map and specification of the pattern of cartilaginous elements. While it is indisputable that the cells that are going to give rise to the digits are present from the earliest stage, it is equally clear that the digits are not specified at early stages as shown by their failure to develop when the apical ectodermal ridge is removed (Saunders, 1948; Summerbell, 1974). The presumptive fate map tells us nothing about specification of fate. Thus when cells are killed we are left with the problem of why it is the proximal structures are lost. It could, for example, be argued that the cells remaining in the progress zone would all be used in making the next structure to be specified and thus distal structures should be missing. This is what might be expected from a model in which proximal structures specified distal structures. In this connexion it should be emphasized that in our results we have found no evidence for intercalation of missing elements or regulation of the length of elements.

Threshold for development of structures

Those structures that have already been specified, while little affected by lower doses of radiation, are affected by increasing doses. For instance, only the proximal part of the humerus develops from stage-20/21 buds irradiated with

1500 rads, whereas at 2000 rads no humerus formed at all. The longer an element has been specified, the more resistant it is to radiation. The simplest way to interpret this result is that if an element at any level has too few cells, that part of the element does not differentiate. From Fig. 16 (Appendix) we would have to say that if an element contains less than about 20 % of its normal population in cross-section it will not develop.

We can now consider the different sensitivities of the cartilage elements at the same proximo-distal level of the limb to X-irradiation; the radius being absent more frequently than the ulna, and in the hand, digit *2* being most sensitive, digit *4* of moderate sensitivity, and digit *3* being least affected. The size of the rudiment seems to be important: the elements that are smaller in width are more sensitive. This means that fewer cells will have to be killed for the threshold number of cells to be reached. This could occur both at the differentiation and specification stage; the radius contains fewer cells at both stages (Lewis, 1977). It seems possible that similar arguments could be put forward to explain the different sensitivities of the digits. Wolff & Kieny (1962) found in the leg that the fibula, which is much thinner than the tibia, was much the more sensitive to X-irradiation. It is not necessary to assume competition. The idea of a threshold number and/or density of cells for an organ to develop is an old idea. It also may account for the increased resistance of the tip to radiation with time. At stage 18/19 no development occurs with 2000 rads, whereas distal parts of digits still develop from a stage-24 tip after 2500 rads.

Fusion of the joints

The cells that are destined to be elbow-joint appear to be specified in the same way as the long bones of the limb (Holder, 1977) and in addition divide little (Lewis, 1977). If this inability to divide is an intrinsic property of elbow-joint cells this could account for the fact that the elbow joint is often missing, since if these cells were destroyed the cartilaginous rudiments are no longer kept separate.

Resistance of cells to radiation

The mesenchyme cells seem to be remarkably resistant to radiation. Doses of 2000 rads on mammalian cells in culture would not be expected to leave a surviving fraction greater than 0·01 % (Hall, 1973). Our results show that at the very least 10 % survive and continue to divide. This is similar to the observations of Haynie & Bryant (1977) who found that at 2000 rads, 30 % of insect wing imaginal disc cells survived and produced a normal wing. We cannot account for this apparent radiation resistance. Anoxia seems an obvious, but unlikely, explanation. It is thus of great interest that Ohnuma, Orano, Koske & Terasima (1978) have found mouse embryo cells to be very radiation resistant.

Ectoderm irradiated with 2500 rads can still support normal development when combined with unirradiated mesoderm. At doses of X-irradiation of

6000 rads, the apical ridge no longer permits limb outgrowth. Thus the apical ridge has to be active throughout limb development, unlike the zone of polarizing activity (ZPA). This group of mesenchyme cells can affect the pattern across antero-posterior axis of the limb after being irradiated with 10000 rads or more (Smith, Tickle & Wolpert, 1978). A brief exposure to the signal from ZPA can affect the pattern of the limb (Smith, 1979).

Overall form of irradiated buds

With 2000 rads the mesoderm is severely damaged and at least half the cells are killed. Nevertheless the overall form of the limb-bud remains more or less normal. The apical ridge, for example, is intact. This suggests to us that the overall form of the bud is determined by the ectoderm, the mesenchyme merely being a loose packing.

Origin of limb abnormalities such as phocomelia

If our view of the way in which X-irradiation produces abnormalities is correct, any treatment that killed off mesenchymal cells at an early stage in development should result in phocomelia of the type we have obtained. There is quite good evidence that this is the case. Kochar, Penner & McDay (1978) found that cytosine arabinoside causes cell death when applied to early mouse embryos and when applied on day 11 which corresponds approximately to stage 20 (Kochar & Agnish, 1977) causes phocomelia. Salzgeber (1966, 1968) found that nitrogen mustard caused cell death in chick limb-buds treated at stages 18–21 and obtained a significant number of phocomelic limbs. In addition, ectromelias were obtained. She also (1968) looked at the effect on mesoderm and ectoderm separately and found that the ectoderm was much less affected. If the ectoderm was affected however, which was the case if higher doses of nitrogen mustard were used, distal deformities occurred. The effects of thalidomide which often causes phocomelia (Smithells, 1973) may be interpreted as due to damage to the mesoderm, possibly due to damage to the vascular system (Poswillo, personal communication). In general, we suggest that agents which damage the mesoderm at early stages will lead to phocomelia, whereas those that affect the ectoderm will lead to ectromelia, the limb being truncated.

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Appendix

By J. H. LEWIS

A calculation of the expected effects of X-irradiation

In normal development, the rudiment of any given segment of the proximo-distal axis of the limb – for example, the forearm – consists of those cells which emerged from the progress zone during a certain period in the development of the embryo – say between age τ_1 and age τ_2. In our theory, it is indeed the age at which the rudiment emerges from the progress zone which determines what structure it shall form. X-irradiation upsets growth, and so upsets the normal relation between the age of the rudiment and its distance from other structures. Here we calculate the pattern to be expected after X-irradiation on our theory, where age is the determining factor.

We have to start by making some simplifying assumptions:

(1) A cell that has been exposed to X-rays may behave in one of two ways: it may either remain normal, and carry on dividing at the normal rate; or it may cease dividing, but nevertheless persist in the limb, moribund, for quite a long time, including the period in which the rudiments are being laid down, only to disappear subsequently during the time when those rudiments differentiate and grow.

(2) There is no compensatory regulation of the growth of abnormally small rudiments: if the rudiment of some part of a structure is reduced in length, then the final developed part will be reduced likewise.

(3) The number of cells in the progress zone remains constant and normal while the rudiments of the limb are being laid down.

Thus while the rudiments of the limb are being laid down, no cells disappear,

and the number of cells that must overflow from the progress zone in any age interval equals the number that are born there. Thanks to the X-irradiation, the birth rate in the progress zone is reduced, so that fewer cells emerge in any given age interval to form the rudiment of the structure corresponding to that age of origin. That eventual structure will therefore be reduced in size. The reduction will be all the more severe, because a certain proportion of the cells that do emerge to form the rudiment have been smitten by X-rays, and will later die and disappear. As time goes by, however, the population of healthy cells in the progress zone will grow and multiply out of proportion to the sickly cells. Long after the X-irradiation, by the time the last, most distal rudiments are laid down, the proportion of sickly cells in the progress zone may be so small that the most distal structures are practically normal, even though the more proximal structures have been severely reduced.

This can all be put in quantitative terms.

Let τ be the age of the limb-bud, measured in cell division cycles; that is, τ increases by one unit during the time it takes for a normal cell in the progress zone to perform one average division cycle; or in other words, τ increases by one unit in the time it takes for a normal healthy population of such cells to double its numbers. In an age interval $\Delta\tau$, on this definition, a population of such cells will grow by a factor $2^{\Delta\tau} = e^{\Delta\tau \ln 2}$; in a small age interval $d\tau$ the number of additional cells born per member of the population will be $d\tau \ln 2$. Let us consider first the rudiments laid down after X-irradiation; that is, the rudiments emerging from the progress zone at ages $\tau > \tau_x$ where τ_x is the age of the bud at the time of irradiation. Let N be the total number of cells in the progress zone. Let $f(\tau)$ be the fraction of the cells in the progress zone at age τ that are healthy and proliferating. The number of healthy cells in the progress zone will thus be $Nf(\tau)$. The number of additional cells born from these in the age interval $d\tau$ will be

$$Nf(\tau)\, d\tau \ln 2.$$

This must equal the number of cells overflowing from the progress zone to form the rudiment of the structure corresponding to the range of ages from τ to $\tau + d\tau$. Let us call this structure $S(\tau, \tau + d\tau)$, or simply S for short. Now of these cells in the initial rudiment of S, only a fraction $f(\tau)$ will be healthy and survive in the long term to form part of S. Thus the number of healthy cells constituting the initial rudiment of S will be

$$N[f(\tau)]^2\, d\tau \ln 2.$$

In a normal limb-bud, not exposed to X-rays, there would be no unhealthy cells, and so $f(\tau) = 1$. Thus in the irradiated limb, the number of cells in the eventual structure $S(\tau, \tau + d\tau)$ is reduced below normal by a factor $[f(\tau)]^2$. We may call this the 'size reduction factor' $r(\tau)$ for structures whose rudiments emerge from the progress zone at age τ. To calculate $f(\tau)$, and hence the size reduction factor,

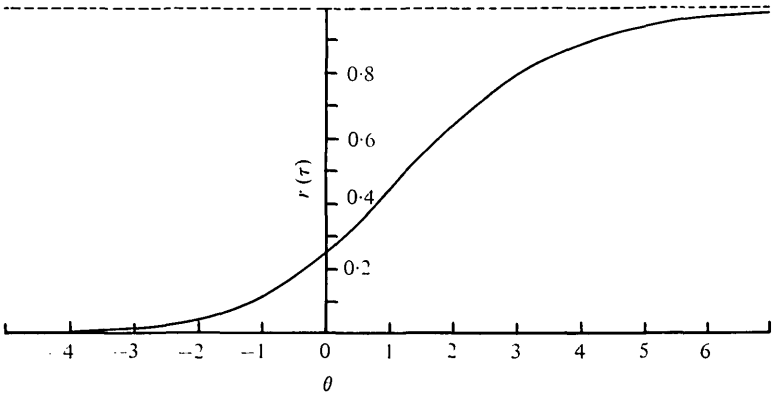

Fig. 14. Plot of r (τ) against θ.

consider a group of cells in the progress zone, just after X-irradiation, comprising n_h healthy, dividing cells and n_s sickly, non-dividing cells. Let τ_x be the age at X-irradiation. The fraction of healthy cells just after irradiation is thus

$$f(\tau_x) = \frac{n_h}{n_h + n_s}.$$

Hence

$$\frac{n_s}{n_h} = \frac{1}{f(\tau_x)} - 1.$$

After an age-interval $\Delta\tau = \tau - \tau_x$, the number of healthy cells will have grown by a factor $2^{\Delta\tau} = 2^{\tau-\tau_x}$ while the number of sickly cells will not have grown at all. Thus

$$f(\tau) = \frac{n_h 2^{\tau-\tau_x}}{n_h 2^{\tau-\tau_x} + n_s},$$

$$= \frac{2^{\tau-\tau_x}}{2^{\tau-\tau_x} + \frac{1}{f(\tau_x)} - 1},$$

$$= \left[1 + \left(\frac{1}{f(\tau_x)} - 1\right) 2^{-\tau+\tau_x}\right]^{-1}.$$

For short, let

$$\theta = \tau - \tau_x - \log_2\left(\frac{1}{f(\tau_x)} - 1\right).$$

Then

$$f(\tau) = \frac{1}{1+2^{-\theta}}$$

and the size reduction factor is

$$r(\tau) = [f(\tau)]^2 = \left(\frac{1}{1+2^{-\theta}}\right)^2.$$

Table 4. *The age τ and the Hamburger–Hamilton stage at which the rudiment of each part of the wing emerges from the progress zone*

Age τ	0		1		2		3		4		5		6		7
Stage	18	19	20	21	22	23	24	25	26	27	28				
Structure		Upper arm		Forearm		Wrist		Wrist		Meta-carpals		First phalanges		Second phalanges	

This table is probably accurate to within about ± one Hamburger–Hamilton stage. For a discussion of the errors, see Summerbell & Lewis (1975) and Lewis (1975).

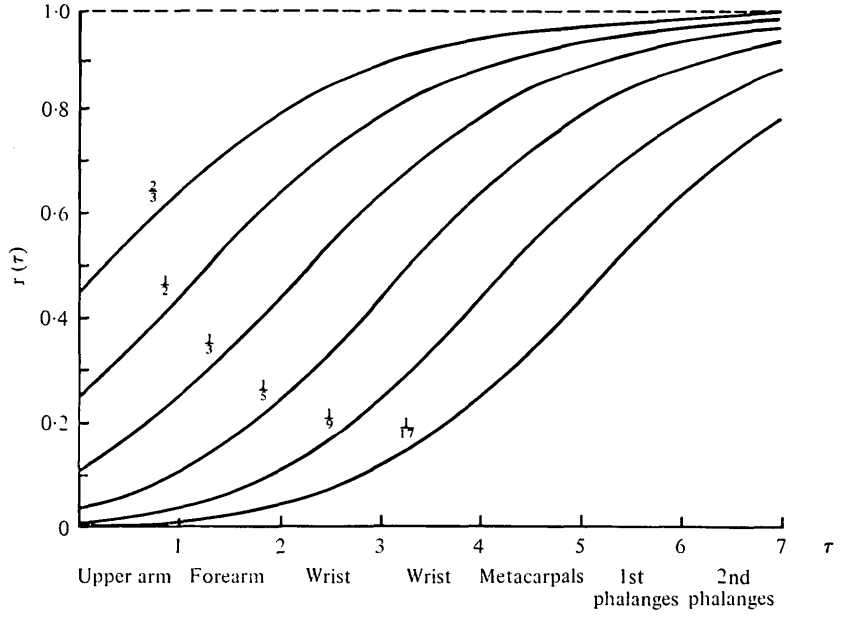

Fig. 15. Each curve shows the size reduction factor $r(\tau)$ for a particular value of $f(\tau_x)$, the proportion of unharmed cells just after X-irradiation at stage 18.

In Fig. 14, $r(\tau)$ is plotted against θ. This formula for the size reduction factor holds good for structures laid down after the X-irradiation, that is, for $\tau > \tau_x$, where τ_x is the age of the bud when it is irradiated.

The reduction factor is different for structures laid down before the irradiation, corresponding to $\tau < \tau_x$. At the time of irradiation, these structures are already represented by a rudiment of normal size. We assume that the X-rays affect the viability of the cells in this rudiment in just the same way that they affect the viability of cells that are still in the progress zone. Then the number of cells in the rudiment that remain healthy and so will go to form the final structure is reduced by the factor $f(\tau_x)$. This, then, is the size reduction factor for all the

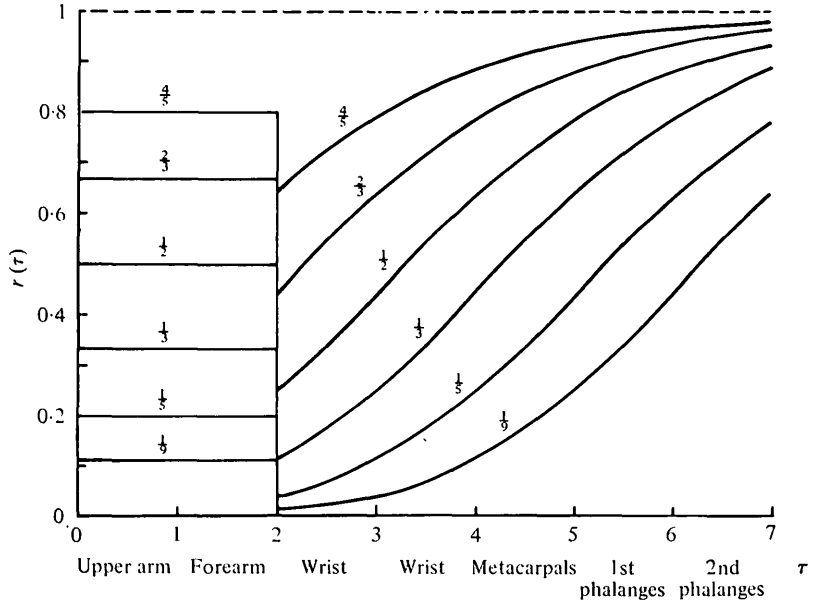

Fig. 16. Each curve shows the size reduction factor $r(\tau)$ for a particular value of $f(\tau_x)$, the proportion of unharmed cells just after X-irradiation at stage 21.

structures whose rudiments have emerged from the progress zone before X-irradiation. Putting these results together, the size reduction factor is given by

$$r(\tau) = \begin{cases} f(\tau_x) & \text{for} \quad \tau < \tau_x, \\ \left(\dfrac{1}{1+2^{-\theta}}\right)^2 & \text{for} \quad \tau > \tau_x, \end{cases}$$

where

$$\theta = \tau - \tau_x - \log_2\left(\frac{1}{f(\tau_x)} - 1\right).$$

To use the formula, we need to know the relation between the age τ and the morphological stage, and what the value of τ is for each structure in the limb. These data can be found in Summerbell & Lewis (1975) and Lewis (1975), and are set out in Table 4. From Table 3 we see that irradiation at stage 18 corresponds to $\tau_x = 0$ and irradiation at stage 21 corresponds to $\tau_x = 2$. Suppose, for example, that just one out of every nine cells is left unscathed by the X-rays, so that

$$f(\tau_x) = \tfrac{1}{9}.$$

Then

$$\log_2\left(\frac{1}{f(\tau_x)} - 1\right) = 3.$$

Hence for irradiation at stage 18

$$\theta = \tau - \tau_x - \log_2\left(\frac{1}{f(\tau_x)} - 1\right) = \tau - 3$$

and for irradiation at stage 21

$$\theta = \tau - \tau_x - \log_2\left(\frac{1}{f(\tau_x)} - 1\right) = \tau - 5.$$

Thus finally, using Table 4, it is easy to plot the size reduction factor for each part of the wing, given the value of $f(\tau_x)$ and the stage at X-irradiation.

Figures 15 and 16 show the results of the calculation for irradiation at stages 18 and 21 respectively, for each of a range of different values of $f(\tau_x)$.