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Cell death and the development of limb form and skeletal pattern in normal and *wingless* (*ws*) chick embryos

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SUMMARY

The *wingless* condition resulting from the action of the sex-linked *wingless* (*ws*) gene arises from the precocious appearance of cell death in the anterior necrotic zone (ANZ) of the forelimb-bud at stage 19 (3 days) and its progressive extension beyond its normal area during stages 20–23. A similar though less pronounced effect occurs in the hindlimb-bud. Although some *wingless* hindlimb-buds are normal, others are affected by the precocious appearance of cell death in the ANZ.

The *ws wingless* mutant resembles the different *wingless* mutant investigated by Zwilling (1956) in that the apical ectodermal ridge (AER) is absent in most *ws* wing-buds. AER absence could be due to *ws* mesenchymal cell death interfering with the production of apical ectodermal maintenance factor (AEMF), which Zwilling claims is necessary to maintain the AER which plays an essential role in inducing limb outgrowth.

Wingless mutant phenotypes range from birds with rudimentary wings and normal legs through a modal type with forelimbs absent and hindlimbs normal to wingless and legless forms showing a high degree of expressivity. Individual *wingless* embryos vary in the degree to which the precocious ANZ appearing at 3 days is extended into the limb-bud and the wide range of *wingless* phenotypic expression is attributed to this variation.

Electron microscopic and histochemical analysis of the cell death process in *wingless* wing-buds revealed the presence of both isolated dead cells and macrophages, which contained intense acid phosphatase activity. These findings are interpreted as showing that isolated dead cells are ingested by neighbouring mesenchymal cells which thus become transformed into macrophages, first ingesting and then digesting further dead cells.

A study was made of the origin of the anomalous hindlimb condition, including absence or reduction of the tibia and digits, characteristic of severely affected *wingless* embryos. Autoradiographic analysis of the pattern of $^{35}SO_4$ uptake revealed that at stage 24/5 ($4\frac{1}{2}$ days) *wingless* hindlimb-buds which were smaller than normal had a normal prospective fibula region, but that the prospective tibia region was small or absent. Thus the effect of a precocious hindlimb ANZ at stages 19–22 is to reduce or delete the pre-axial prospective tibia at stage 24/5.

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INTRODUCTION

A case of genetic winglessness in the fowl was first reported by Waters & Bywaters (1943), who described the effects of an autosomal recessive lethal gene, *wingless*, later given the symbol *wg* by Hutt (1949). This gene not only affected the development of the wings, eliminating them completely or reducing them to small stumps, and to a lesser extent the legs, but caused the non-appearance of the lungs and air sacs and of the metanephric kidneys and their ureters.

Zwilling (1949) reported that in this mutant the early wing-bud was formed at 3 days but that it developed no further and he correlated this with the absence of a typical apical ectodermal ridge, which Saunders (1948) had shown to be essential for continued distal growth. Later, Zwilling (1956) investigated the relationship between ectoderm and mesoderm in producing winglessness experimentally using reciprocal interchanges of mutant and normal ectodermal caps and mesenchymal cores. But the original mutant having become extinct, a second wingless mutant was used which had arisen in a flock at Storrs. This closely resembled the first in its effect on the limbs and kidneys but differed in that the lungs and air sacs were unaffected. The experiments established that ectoderm and mesoderm were mutually dependent on each other in limb development and Zwilling concluded that some mesodermal factor which was absent or reduced in the mutant was required for the maintenance of the apical ectodermal ridge. Thus, this wingless mutant has made an important contribution to the evidence for the Saunders/Zwilling hypothesis of a reciprocal interaction between apical ectodermal ridge and limb mesenchyme in chick limb morphogenesis and, more specifically, to the existence of an apical ectoderm maintenance factor (AEMF) supposed to be produced by the normal limb-bud mesenchyme.

The *wingless* mutant described here is therefore the third. It results from the action of a sex-linked gene discovered by Pease (1962) and given the symbol *ws* by Lancaster (1968) who made linkage studies and described its wide range of phenotypic expression. He found a modal type comprising 35·6 % of wingless birds in which the hindlimbs were normal and the forelimbs absent, every other major feature being normal. Lower degrees of expressivity produced rudimentary wings of various sizes, often showing assymmetry, with a higher degree of abnormality on the right side than on the left. Higher degrees of expressivity caused abnormalities of the hindlimbs also, including reduction of the number of digits and a peculiar condition of the tibia, 'which appeared to have broken and fused together again in an abnormal position before hatching'.

In this mutant the development of the internal organs, including the kidneys, is normal and the gene is not lethal. The development of the limbs has been briefly described by Ede (1968), who reported that the apical ectodermal ridge was absent from the wing bud of most embryos and that the mesenchymal cells

were necrotic at 4 days, and also that the tibial buckling in the hind-limb appeared to be caused by weak cartilage matrix formation. Since the occurrence of cell death has come to be recognized as an important but little understood factor in normal limb development (Saunders, Gasseling & Saunders, 1962; Saunders, 1966; Dawd & Hinchliffe, 1971) the more detailed investigation reported here has been made with particular attention to the problem of mesenchymal cell death and its relation to the apical ectodermal ridge and to the pattern of hind-limb chondrogenesis.

MATERIAL AND METHODS

Wingless embryos were obtained from Light Sussex descendants of the *wingless* stock originally discovered by Pease (1962). Carrier cocks (*ws*/+) were crossed with either normal (*Y*/+) or *wingless* (*Y*/*ws*) hens, giving a 1:3 and 1:1 ratio of *wingless* to normal embryos respectively. Some wingless cocks (*ws*/*ws*) were crossed with *wingless* hens (*Y*/*ws*) which produced embryos which were all *wingless*.

Embryos were classified according to the Hamburger-Hamilton (1951) series. For histological studies embryos were fixed in Bouin's solution, embedded in paraffin wax, sectioned at 8 μm, and stained with haematoxylin and eosin, or alcian blue and Mayer's haemalum. Alizarin red clearance preparations of whole embryos were made to show bone development in the hind limbs.

(1) *Vital staining for cell death*

Areas of cell death in normal and *wingless* embryos were mapped *in ovo* by the application for $\frac{1}{2}$–1 h of a 1:40000 solution of Nile blue sulphate or neutral red in Ringer's solution to the vitelline circulation.

The changing pattern of areas of cell death was observed at approximately 4 h intervals over a 24 h period in embryos stained vitally using several successive applications of sterile vital dye solution.

For photography, limbs were dissected out and mounted in Ringer's solution in a cavity slide and photographed immediately. A method, partly following Pertusa (1966) and Fallon & Saunders (1968) was used to preserve the vital dye in fixed and cleared whole mounts.

The method was as follows:

(1) Fix overnight at 4 °C in formol-calcium (4 % formaldehyde, 1 % calcium chloride), neutralized to pH 7 before use.

(2) Wash for $\frac{1}{2}$ h at 4 °C in 4 % formaldehyde neutralized to pH 7.

(3) Dehydrate directly in 100 % isopropyl alcohol at 4 °C for 1 h.

(4) Clear in toluene, and mount in Canada balsam made up in toluene.

(2) *Electron microscopy, thin (1 μm) sections and acid phosphatase staining*

Limb-buds, or parts of limb-bud, were fixed in 4 % glutaraldehyde at pH 7·4, rinsed for 24 h at 4 °C in cacodylate buffer at pH 7·4, post-fixed in 1 % osmium tetroxide, dehydrated via ethanol and propylene oxide and orientated and embedded in TAAB resin (mix C). Thin (1 μm) sections were cut and stained in toluidine blue for light microscopy, while ultra-thin sections were stained with uranyl acetate and lead citrate and photographed using an AEI 6B electron microscope.

For acid phosphatase staining, procedure was as described above, except that after fixation in 4 % glutaraldehyde and rinsing in cacodylate, the small (0·5 mm square) tissue blocks were incubated in Gomori medium for acid phosphatase (Barka & Anderson, 1963) at pH 5·2 for 1 h at 37 °C, washed briefly in four changes distilled water, treated with dilute (0·5 %) ammonium sulphide solution, washed in distilled water and post-fixed as above in 1% osmium tetroxide. Final procedures were as described above, and the resin blocks were sectioned for both light microscopy (the 1 μm sections were mounted without further staining in Clearmount) and electron microscopy.

Deposits of black lead sulphide indicate sites of acid phosphatase activity. No diffusion artifacts were observed in the sections.

(3) *Autoradiography using radiosulphate*

The pattern of chondroitin sulphate synthesis in developing normal and *wingless* hindlimbs was analysed using a labelled precursor: radiosulphate (Searls, 1965; Amprino, 1955). 0·02 ml of a sterile aqueous solution of ^{35}S-labelled sulphate (Amersham Radiochemical Centre) with a total activity of 40 μCi was applied through a window to the area vasculosa, and the sealed egg returned to the incubator for 2 h. The embryos were then fixed in Bouin's fluid, and the limbs dissected off, blocked in paraffin wax and sectioned at 8 μm. The sections were dewaxed, coated with stripping film (Kodak Autoradiographic Stripping Plates AR 10) and exposed for 12 days at 4 °C, after which they were developed in Kodak D 19 developer and mounted in 'Clearmount'.

A total of 15 normal and 24 *wingless* hindlimbs at stages 24/5, 25–8 and 30 was examined in this way.

RESULTS

(1) *Overall morphology*

The *wingless* condition can be first recognized at stage 19 (3 days) through the appearance in the wing-bud of a region of cell death detectable by vital dyes. At stage 20, *wingless* embryos develop smaller wing-buds than normal, and outgrowth soon ceases (Fig. 1B) so that by stages 23 and 24 in most embryos there is no wing-bud (Fig. 2). In some *wingless* embryos the expression

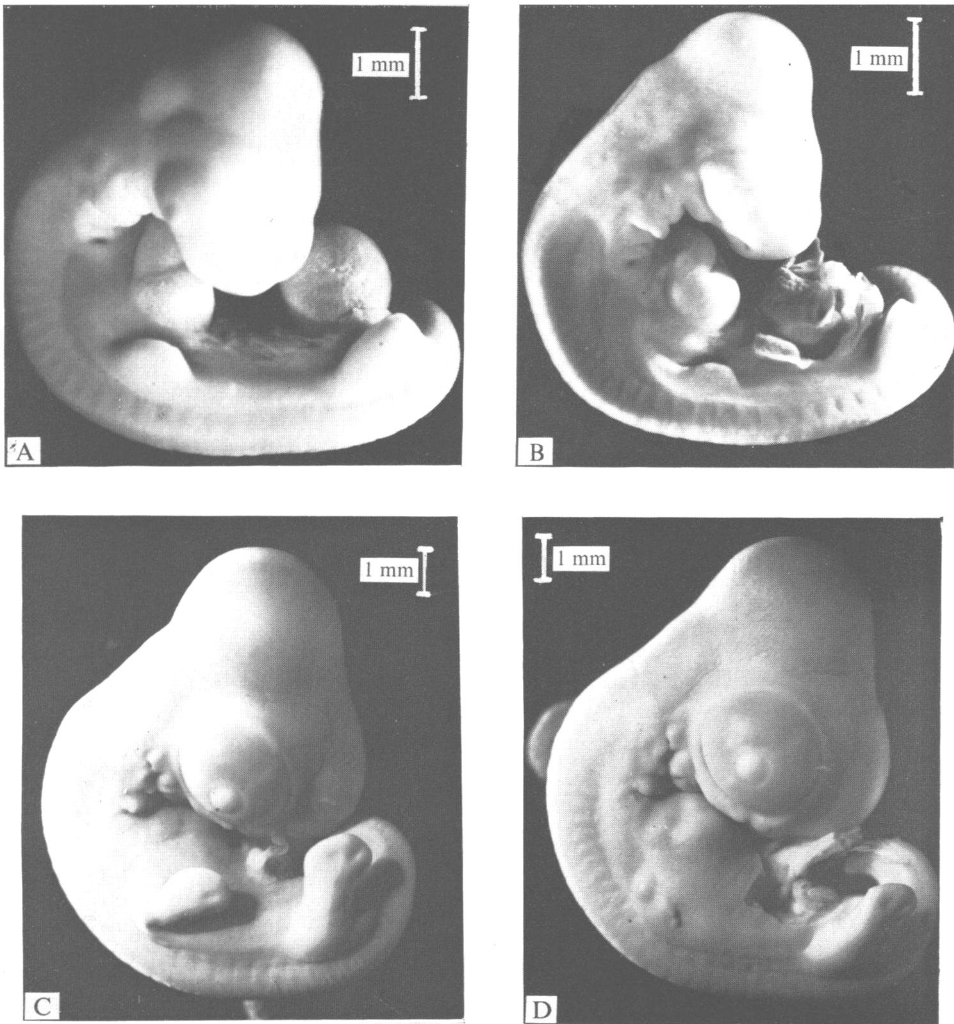


Fig. 1. Fixed normal embryos, stage 22(A) and stage 26(C), and *wingless* embryos, stage 22(B) and stage 26(D).

of the gene is mild, and posterior parts of the wing-bud are formed (Fig. 4I). In a minority of older embryos (7 days) proximal and posterior elements (e.g. humerus and ulna, but not radius) are recognizable.

The development of the leg-bud is less affected and stages 19–21 are difficult to distinguish from normal in almost all cases. In some *wingless* embryos, normal leg development takes place. In other *wingless* embryos the size of the leg-bud is reduced, beginning at stage 22, due to the absence of anterior and distal parts (Fig. 5F, G). In later stages the digit number is reduced from 4 to 3–1. A series of *ws* leg-buds illustrating such anterior and distal deletions is

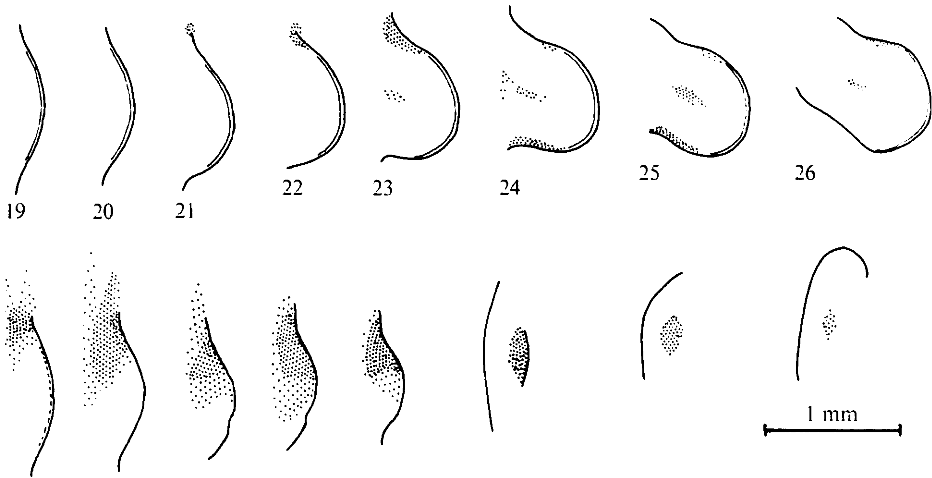


Fig. 2. The pattern of cell death in normal (top) and *wingless* (bottom) wing-buds. Stippling indicates areas of cell death.

illustrated in Fig. 3. In extreme cases *wingless* embryos lack any hind-limb development.

(2) *The pattern of cell death*

Using the *in vivo* vital staining method, *wingless* embryos can first be detected at stage 19 through the concentration of Nile blue sulphate in macrophages in the anterior part of the wing-bud and in the mesenchyme of the body wall anterior to this. In later stages (20–23), the area of cell death spreads into the remainder of the wing bud (Fig. 4F–H) until by stage 25 most *wingless* embryos show no sign of any wing outgrowth. The pattern of cell death in *ws* wing-buds from stage 19–26 is shown in Fig. 2. There is considerable variation in the extent of cell death, which may be restricted to the anterior quarter of the limb-bud (Fig. 4G), or may extend as far as the centre (Fig. 4F). Where the area of cell death is more restricted, the growth of the posterior part of the forelimb may take place normally, and the PNZ and 'opaque patch' regions can be identified (Fig. 4I, compare with Fig. 4D). In some embryos there is asymmetry in the extent of cell death with the right wing bud being more affected than the left.

In normal embryos the anterior necrotic zone (ANZ) makes its appearance at stage 21 at the junction of body wall and wing-bud and moves progressively into more distal positions until stage 26 (Figs. 4C, 2). Thus it appears that in the *wingless* embryos the ANZ make an earlier appearance than in the normal and that it is more extensive and spreads into the central limb-bud mesenchyme.

In the hindlimb development of *wingless* embryos there is a similar, though less-pronounced effect (Figs. 5E, 3). A small patch of dead cells appears at stage 19 anterior to the hindlimb-bud and extending into the base but not the

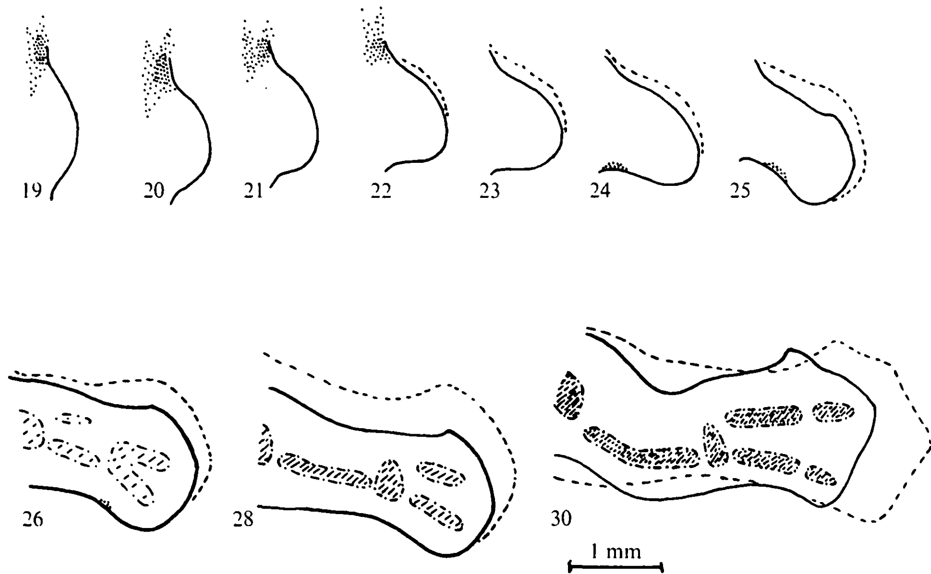


Fig. 3. The pattern of cell death in *wingless* hindlimbs (selected to show severe effects of the *ws* gene). Stippling indicates areas of cell death. Precartilage condensations and cartilage rudiments are indicated in stages 26–30. The dotted line indicates the outline of the normal limb.

apex of the limb-bud. The area of cell death is variable in extent: in some cases it is scarcely visible. Although the hindlimb-bud after stage 23 is often smaller than normal, there is no sign at this later stage of an ANZ (Fig. 5F, G). A PNZ appears, as in the normal, during stages 24–26 (Fig. 5G). The variation in size of the hindlimb-bud thus appears to be caused by the extent by which cell death in the limb base at 3 days has reduced the number of limb forming cells.

(3) *The* ws *limb ectoderm*

The relationship between mesenchymal cell death and the apical ectodermal ridge (AER) is of considerable interest. In normal embryos the AER makes its first appearance at stage 19, but in the majority of *wingless* forelimb-buds there is no thickened ridge (Figs. 4F, H, 6A) between stages 19 and 23. Some *wingless* wing-buds show an accumulation of dead cells in the distal region of the ectoderm, though not strictly localized in the ridge region (Fig. 4G). It is possible that these dead cells represent a regressing ridge. In those *wingless* forelimb-buds in which cell death is limited to the anterior part of the bud, sections reveal that where the ectoderm is underlaid by necrotic mesenchyme cells there is no AER, but that in the posterior part where the mesenchymal cells are viable, an AER is present. The AER may persist during later stages (e.g. at stage 24: Fig. 4I) in cases of mild expression where posterior parts of the forelimb develop.

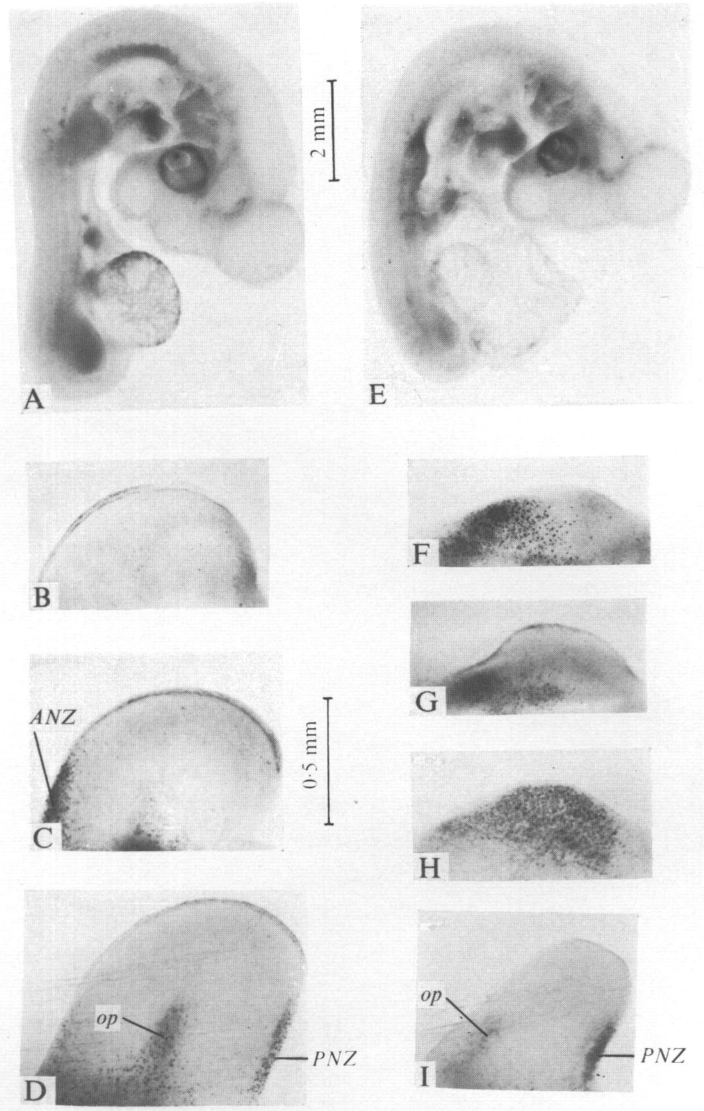


Fig. 4. Cell death in *wingless* and normal forelimbs. Photographs of vitally stained limb-buds. (A–D) Normal. (A) Stage 22 normal embryo, (B) stage 21, (C) stage 23, (D) stage 24. (E–I) *Wingless*. (E) Stage 22 *wingless* embryo, (F) stage 21, (G) stage 21 (limited increase ANZ: sections of this *ws* wing-bud reveal no ridge, but dead cells in the distal ectoderm). (H) Stage 23, (I) stage 24 (note opaque patch and presence of posterior part of limb, including PNZ and a small length of AER: a mild expression of *ws* gene). (ANZ and PNZ and *op* (opaque patch) indicated.)

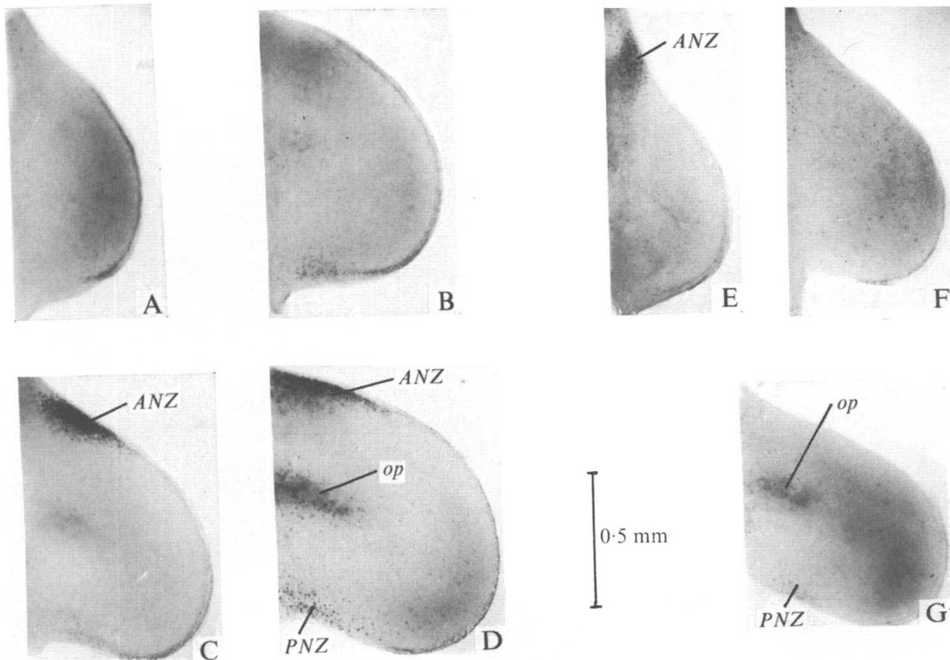


Fig. 5. Cell death in *wingless* and normal hindlimbs. Photographs of vitally stained limb-buds. (A–D) Normal. (A) Stage 20, (B) stage 22, (C) stage 23, (D) stage 24. (E–G) *wingless*. (E) Stage 20 (note precocious ANZ), (F) stage 23, (G) stage 24. (ANZ, PNZ, *op* (opaque patch) indicated.)

Wingless hindlimbs may have a normal AER. However, in *ws* hindlimbs characterized by anterior and distal deletions (presumably resulting from substantial cell death in the anterior leg-bud mesenchyme) the ridge is shortened and lacks its full anterior extension (Fig. 4F, G). One curious feature is that in all *wingless* legs after $4\frac{1}{2}$ days, whether normal or abnormal, the anterior AER terminates in a thickening (illustrated in Fig. 1D and in section in Fig. 8C) which gives the limb a characteristically sharp angle anteriorly.

(4) *The process of cell death*

Thin 1 μm sections and electron micrographs reveal that many of the forelimb-bud mesenchyme cells are dead, and isolated dead cells showing chromatopycnosis (Fig. 7A) or hyperchromatosis (Fig. 7B) can be identified. Such cells show simultaneous deterioration of the nucleus and the cytoplasm, which is vacuolated and in some cases in process of being shed (Fig. 7A). Phagocytes can be classified into early stages, identifiable as apparently normal viable mesenchymal cells whose cytoplasm contains a single dead cell (Fig. 7A), and into later stages or mature macrophages containing many dead cells within well-defined digestive vacuoles (Figs. 6B, 7A, B). Dense and vacuolated pieces of shed cytoplasm may be found (Fig. 7B). The ingested material con-

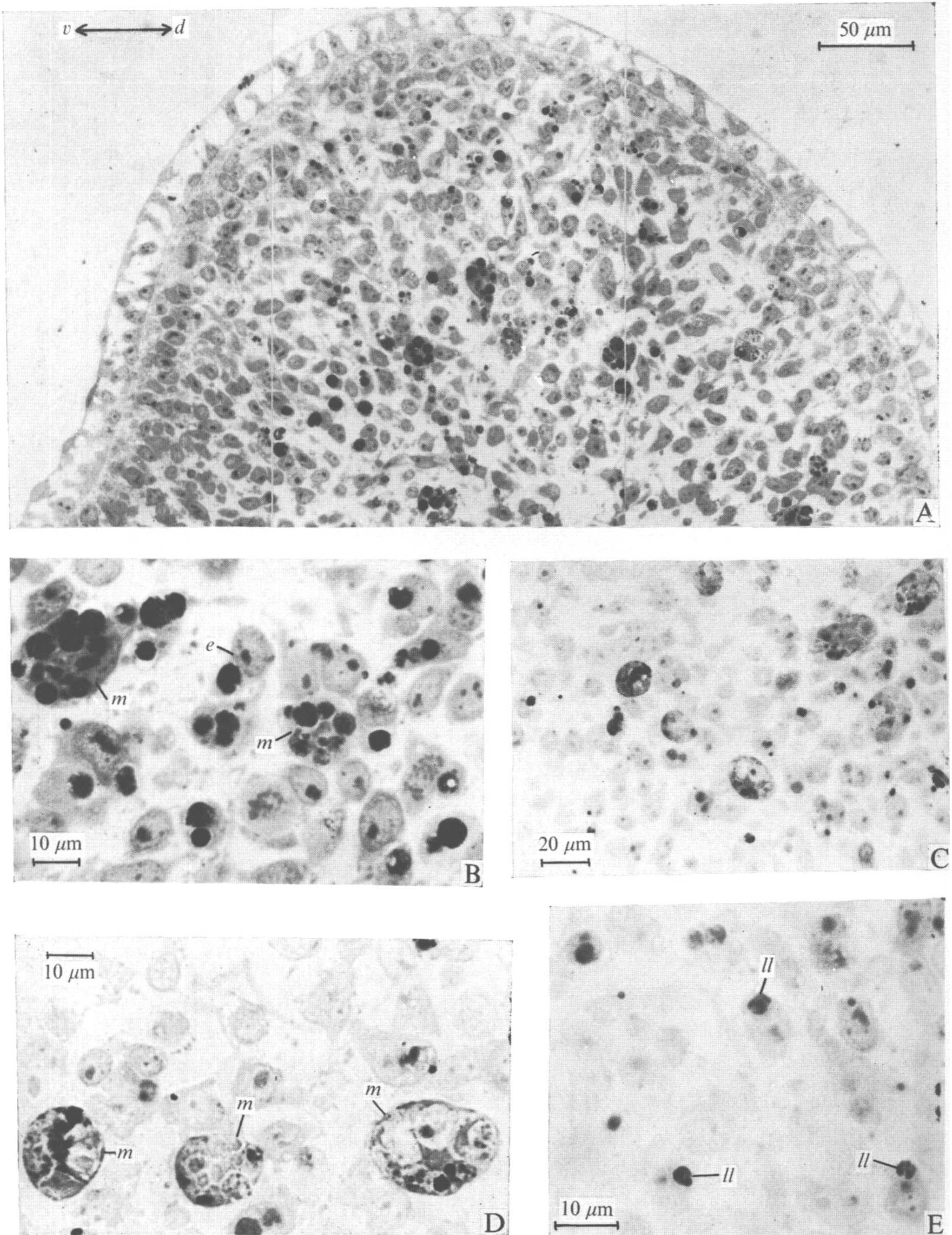


FIGURE 6

Fig. 6. Histology and histochemistry of stage 21 ($3\frac{1}{2}$ days) *wingless* forelimb. (A) Transverse section (1 μm, stained with toluidine blue). Note AER absence (*d–v*: dorso-ventral axis). (B) Detail of (A). Note mature macrophages (*m*) and early phagocyte (*e*) with one ingested dead cell. (C) Part of section of acid phosphatase stained wing-bud. (D) Detail of (C) with three macrophages (*m*). (E) Detail of part of same section as (C) showing the 'large lysosomes' (*ll*).

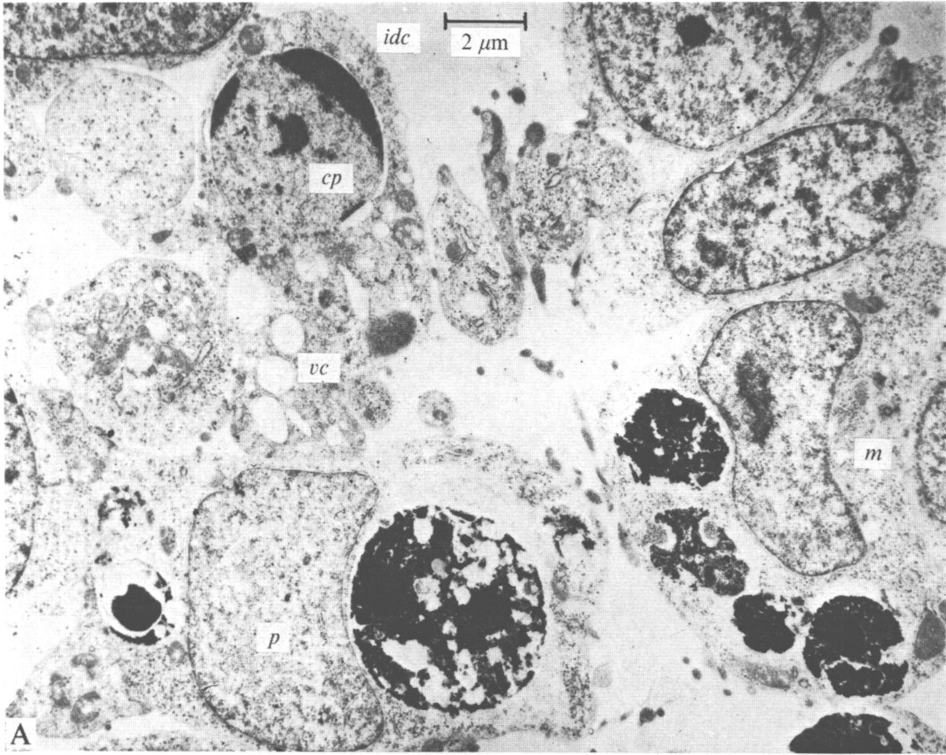
tained by macrophages takes on various forms: recently ingested material is dense and often contains banded granules (Fig. 7B) (resembling those found originally by Bellairs (1961) in dead cells of the chick blastoderm) while partially digested material is granular and pitted, and the final stage is represented by myelin figures. Similar stages of cell death and digestion by macrophages may also be found in the ANZ and PNZ of normal chick limbs. These results are interpreted as evidence that the following sequence of events is taking place: isolated mesenchymal cells deteriorate, die and are then ingested by neighbouring viable mesenchymal cells which thus become transformed into macrophages, first ingesting and then digesting further dead cells. Such a sequence has been proposed in the cases of cell death in the developing rat limb (Ballard & Holt, 1968) and in the 'opaque patch' region of the central mesenchyme of the developing chick limb (Dawd & Hinchliffe, 1971).

Acid phosphatase activity is higher in the *wingless* limb-bud (Fig. 6C) than in the normal. The enzyme is found in apparently viable mesenchymal cells discretely localized in lysosomes (siez 0·3–0·7 μm), but the most striking areas of intense activity are the macrophages (Fig. 6D) which contain several discrete areas of intense activity within the macrophage cytoplasm, and more diffuse activity associated with the dead cells in the digestive vacuoles. These findings are consistent with the theory that the intense acid phosphatase activity characteristic of a region of regression indicates digestion of dead cells within macrophages by acid hydrolases, rather than that cell death results from intracellular release of lysosomal enzymes. However, there are some mesenchyme cells which contain a large phagosome (approximately 2 μm) rich in acid phosphatase but also containing dense material (Fig. 6), which may be an ingested dead cell or autolysed cytoplasmic material, whose interpretation remains at present obscure, but which may be recovering from sublethal deterioration.

Mitosing cells in *wingless* wing mesenchyme are rare. Preliminary counts suggest that at 3 and $3\frac{1}{2}$ days the mitotic index is $\times$3–6 lower than the normal. It is intended to investigate this issue further, using tritiated thymidine as an experimental tool.

(5) *Autoradiographic* ($^{35}SO_4$) *analysis of skeletal development in the* ws *hindlimb*

A chondrogenic pattern emerges in the normal hindlimb first at stage 24/5 in the form of a central Y-shaped region of increased sulphate uptake into chondroitin sulphate. The base of the Y represents the femur, the arms the tibia and fibula which are present as precartilaginous condensations at this stage. In *wingless* hindlimbs the pattern may be completely normal, or it may show a range (illustrated in Fig. 8B–D) of abnormality from a slight reduction to complete absence of the tibial areas. *Wingless* hindlimbs showing abnormalities do not have a clearly defined reduction in the area of chondroitin sulphate synthesis corresponding with the 'opaque patch' region.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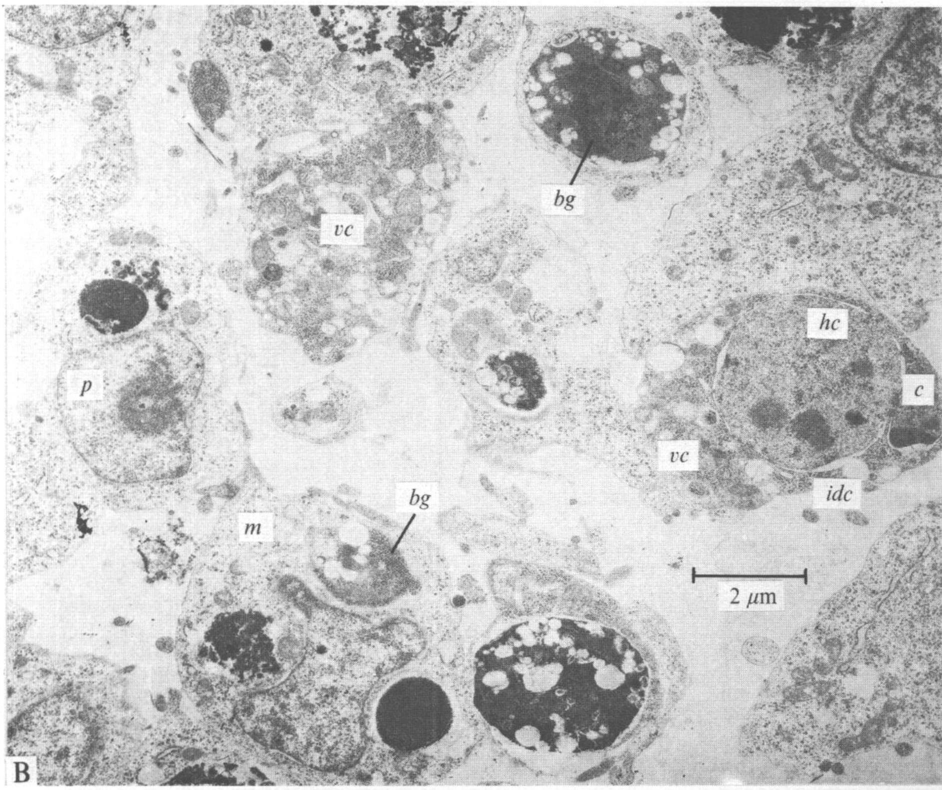
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At $5\frac{1}{2}$ days (stage 27) of development the normal hindlimb shows separate regions of sulphate uptake, representing the larger tibia and smaller fibula, with the ankle and metatarsal areas now identifiable. *Wingless* hindlimbs show a range of abnormality from reduction in tibia size to the complete absence of tibia and the reduction in the number of ankle and metatarsal elements illustrated in Fig. 8F. Skeletal development in this wingless hindlimb resembles that of the posterior half of a normal limb of this stage.

At 7 days (stage 30) of development, *wingless* hindlimbs show a similar range of abnormality, from a tibia reduced in cross-section area to that of the fibula (Fig. 8H) to complete omission of the tibia (Fig. 8I). However, in the cases examined, the intensity of staining in the reduced *wingless* tibia was the same as that in the normal tibia, and autoradiography did not reveal a case similar to that described by Ede (1968) in which the 7-day *wingless* tibia showed reduced quantities of acid mucopolysaccharide in the intercellular material, as indicated by alcian blue staining.

In summary, autoradiography reveals that the *wingless* embryos showed a range of skeletal abnormalities starting at stage 24/5 involving the reduction or deletion of the anterior skeletal elements.

The degree of reduction in skeletal elements is greatest in limb-buds showing the greatest reduction in size.

(6) *The wingless hindlimb skeleton*

One-day old *wingless* chicks show a range of expressivity in the varying degree of abnormality shown by the leg skeleton, which ranges from normal to a 1- to 2-digit structure. The missing parts of the leg skeleton are the anterior elements, distal to the femur. The tibia may be smaller in size, it may buckle (Fig. 9B), or it may be completely absent (Fig. 9C). Buckling of the tibia is probably associated with poor development of the matrix which stained poorly with alcian blue (specific for the acid mucopolysaccharide matrix component) in a proportion (3 out of 10) of the 7-day *wingless* legs examined. Complete absence of the tibia is associated with extreme reduction of digit number. All *wingless* legs have a well-developed fibula (Fig. 9B, C).

FIGURE 7

Electron micrographs of the mesenchyme of a stage 21 ($3\frac{1}{2}$ days) *wingless* forelimb, showing various stages of cell death and macrophage digestion. Note isolated dead cells (*idc*) with nuclei showing chromatopycnosis (*cp*) or hyperchromatosis (*hc*) (with a separate cap (*c*) of chromatic material) and with vacuolated cytoplasm (*vc*); banded granules (*bg*) in phagocytosed cytoplasm; phagocytes (*p*) containing small numbers of dead cells and mature macrophages (*m*) containing larger numbers of dead cells.

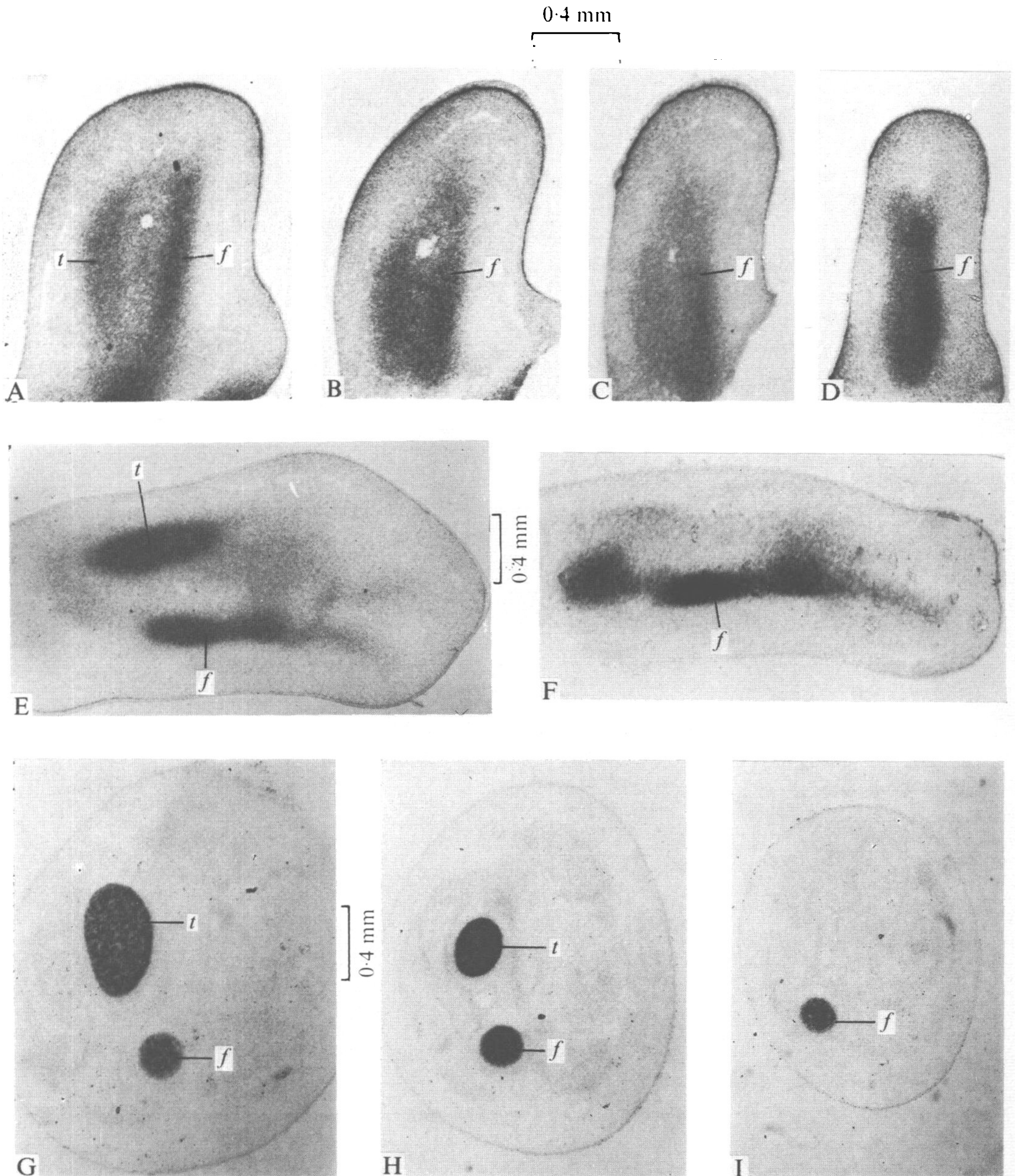

Fig. 8. Autoradiographs of ^{35}S uptake into chondroitin sulphate in normal and *wingless* hindlimbs. (A–D) Stage 24/5. (A) Normal, (B–D) increasing degrees of abnormality involving reduction of the tibia in *wingless* hindlimbs. (E–F) Stage 27. (E) Normal, (F) *wingless* (note absence of tibia and the reduction in number digit). (G–I) Stage 30 (transverse sections). (G) Normal, (H, I) *wingless* (reduction and absence of tibia). *f*, Fibula; *t*, tibia.

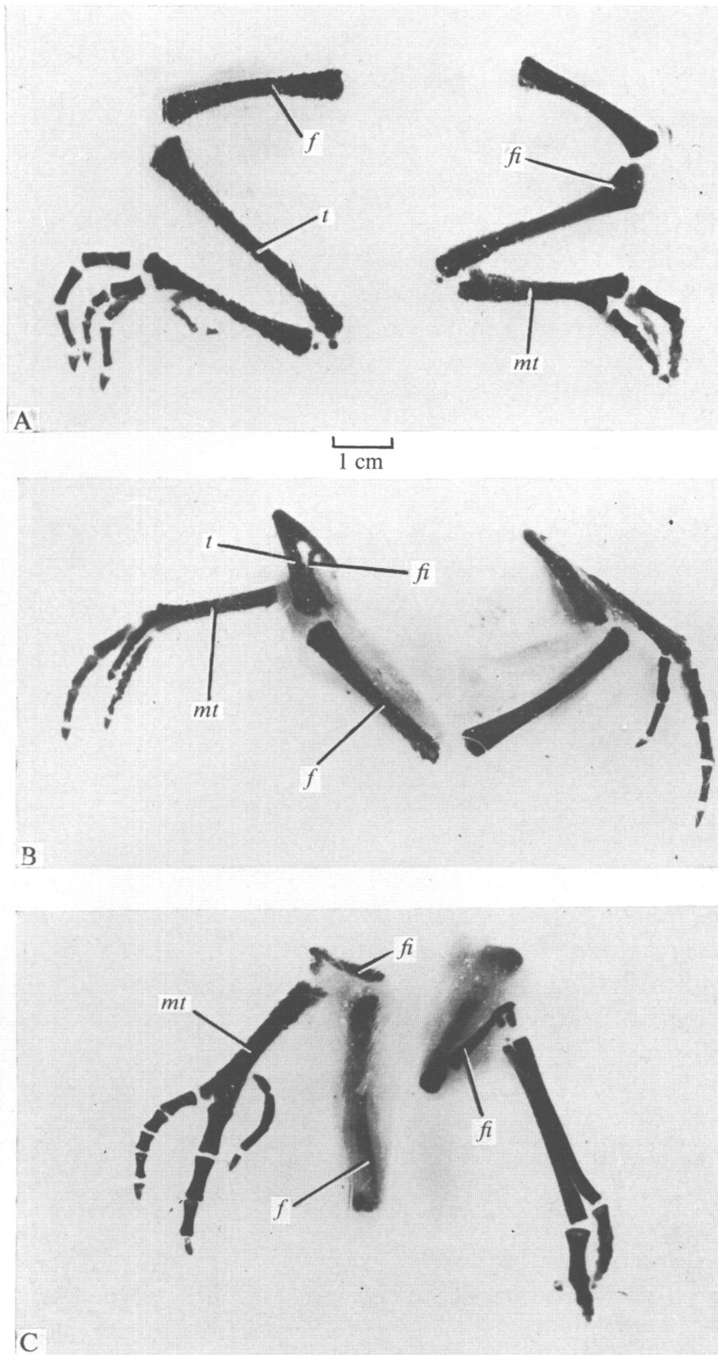

FIGURE 9

Hindlimbs from 1-day chick stained with Alizarin Red to show the skeleton. (A) Normal. (B) *Wingless* showing bent tibia and well-developed fibula. (C) *Wingless* showing well-developed fibula and absence of tibia. Right leg has only two digits, *f*, Femur; *fi*, fibula; *mt*, metatarsal; *t*, tibia.

DISCUSSION

(1) *Extension of cell death in the* wingless (ws) *mutant*

Our results show that the wingless condition arises from the precocious appearance of cell death in the ANZ and its extension beyond its normal area in the 3- to 4-day limb-bud. This effect is more pronounced in the forelimb than in the hindlimb. Individual *wingless* embryos vary in the degree to which the ANZ is extended and it is reasonable to suppose that this variation is responsible for the wide range of phenotypic expression noted by Lancaster (1968).

Textbooks of embryology (e.g. Ebert & Sussex, 1970) often imply that cell death in development is always associated with re-modelling and re-shaping processes, but in fact in the limb, which is frequently quoted as an example, the role of cell death is obscure. In the case of interdigital cell death in the digital plate there certainly seems no reason to doubt that its function, as suggested by Saunders & Fallon (1966) is to sculpt out the digits; its role in the case of the 'opaque patch' in the deep mesenchyme is less evident, though Dawd & Hinchliffe (1971) believe that in the wing it plays a part in separating the radial and ulnar condensations; but no convincing role has been suggested for the anterior and posterior necrotic zones found in the chick fore- and hindlimb-buds. Saunders *et al.* (1962) early suggested that the ANZ and PNZ played a part in shaping limb outgrowth but the absence of corresponding necrotic zones in other birds and in mammals made this unlikely and when Saunders (1966) blocked cell death in the PNZ experimentally by inserting a piece of dorsal mesoderm over it, a limb bud of normal shape was produced.

It might be that the ANZ and PNZ represent regions of a particular sort of stress which is registered as cell death in the fowl but not in other species. Hinchliffe & Ede (1967) suggest that these zones represent 'end-stops' to the apical ectodermal ridge and that in their absence, in the *talpid*^{3} mutant, the ridge is abnormally long. In the *wingless* mutant, the extension of the ANZ inhibits or eliminates ridge formation. It is noteworthy that *talpid*^{3} mesenchyme cells from all regions of the limb-bud are more resistant to injury and death in other stress situations (Ede & Flint, 1972). It may be that these zones represent the centre of a stress situation where a threshold of cell 'resistance' determines whether or not there is actual necrosis: according to this hypothesis the threshold of *talpid*^{3} cells would be sufficiently high to resist necrosis at all points, normal cells would have a threshold which would produce cell death at the centre, but the resistance threshold of *wingless* cells would be so low that they would undergo necrosis over a far wider area. In some other species of birds and mammals (e.g. the mouse and Japanese quail), as already mentioned, cell death does not occur in these regions, and several other mutant genes are known which act by extending or restricting areas of cell death in other organ systems (reviewed Saxén & Rapola, 1969).

There is clearly a difference between anterior and posterior regions of the

limbs with respect to the manifestation of the *wingless* gene, and this may be an indication of the greater susceptibility of the anterior limb mesenchyme to cell death. Two sets of observations support this view: firstly the fact that in the normal limb the anterior necrotic zone appears before the posterior zone, and secondly Cairns' experimental observation (unpublished, reported in Ede, 1971) that stripping off the ectodermal ridge is followed by an antero-posterior wave of cell death in the subridge mesenchyme.

(2) *Mesenchymal cell death and the AER*

One of the most interesting aspects of the *wingless* mutant is its possible contribution to understanding ectodermal/mesenchymal interactions in limb development (reviewed Saunders & Gasseling, 1968). Zwilling (1956) investigated this aspect in the second *wingless* mutant by separating ectodermal and mesodermal components in 3-day embryos and recombining them in various ways. When a normal ectodermal cap was combined with a *wingless* mesodermal core the apical ectodermal ridge regressed after 2 or 3 days and distal outgrowth ceased; when *wingless* ectoderm was combined with normal mesoderm there was no further development at all; controls of normal ectoderm recombined with normal mesoderm developed more or less normally. Zwilling interpreted these results to indicate a reciprocal dependence between the two components, the mesoderm responding to the stimulus of the AER by growing distally and at the same time providing some factor which is necessary for the continued maintenance of the apical ectoderm; in the mutant this factor appeared to be deficient. The weak point of Zwilling's experiments was in the relatively late stage at which the operation was done, so that a direct effect of the gene upon the ectoderm cannot be excluded.

The *ws wingless* mutant resembles Zwilling's in the absence of an AER in most *ws* wing-buds. This absence could be attributed to mesenchymal cell death interfering with the production of apical ectoderm maintenance factor (AEMF) and consequent failure to maintain a ridge. It may be objected that since according to Zwilling AEMF is predominantly localized in the postaxial mesoderm, extension of the ANZ in *ws* would not interfere with AER maintenance during initial stages of limb development. In answer to this it should be pointed out (i) that although the increased *ws* ANZ at stage 19 is initially preaxial, by stage 21 it has spread postaxially and would then interfere in postaxial AEMF production and (ii) that postaxial *ws* cells may cease AEMF production along with other vital activities some time before the cells are recognizably dead since many prospective PNZ cells are known to cease DNA synthesis at stage 22, well before the death of the cells at stage 24 (Saunders & Fallon, 1966).

This hypothesis of cell death interference in *ws* AEMF production is essentially identical with Zwilling's hypothesis, though he published no histological observations giving information about cell death in the mesenchyme. Gasseling

& Saunders (1964) have shown that AER cannot survive if it is underlaid by grafted PNZ mesenchyme, and in the *talpid*^{3} mutant, as mentioned above, absence of ANZ and PNZ is associated with a greatly extended ridge. Our finding that in the *ws* mutant failure of AER initiation and anterior mesenchymal cell death are observed simultaneously at stage 19 does not allow us to decide between this hypothesis and two others: that the mesenchymal cell death might prevent initiation of the AER, which Kieny (1960) has shown to be dependent on induction by limb mesenchyme, or that the primary effect of the gene is on the ectoderm, which leads to degeneration of the mesoderm. It is planned to determine the initial effect of the *ws* gene by making reciprocal combinations of normal and *ws* mesoderm and ectoderm, but using the method of Kieny (1960) which allows recombination of stage 15 limb mesoderm with flank ectoderm, i.e. ectoderm which has never been in contact with determined limb mesoderm, when, if the gene has no primary effect on the ectoderm, *wingless* ectoderm combined with normal mesoderm should produce a normal limb.

Winglessness resembles the limblessness of certain reptiles such as snakes and the slow worm which possess only rudimentary limbs, and it is of interest to compare the development of these two forms of limblessness. Raynaud (1972) & Raynaud (1963) have shown in the slow worm *Anguis fragilis* that a reduced number of somites contribute to the limb-bud mesenchyme and that though an ectodermal ridge is formed it soon degenerates and this process is followed by reduced protein and RNA synthesis and finally cell death in the underlying limb mesenchyme. Mesenchymal cell death is thus a secondary event in limb bud regression in the slow worm.

(3) *Hindlimb development*

Although the legs of *wingless* (*ws*) embryos may develop normally, it is clear that in many cases an extension of the ANZ at stage 19 (3 days) removes a substantial number of anterior leg-bud cells, and this accounts for the skeleton deficiencies (e.g. absence or reduction of tibia and reduction in digit number) in leg development of many *wingless* chicks. The greater or lesser degree of abnormality can be ascribed to the degree by which the size of the *wingless* limb-bud has been reduced by a greater or lesser extension of the ANZ. However, extension of cell death is restricted to the ANZ and does not occur in the 'opaque patch' region, and one of the features of some of the reduced *wingless* 4-day leg-buds is the reduced amount of cell death in the 'opaque patch' or its total absence. Hence, the absence or reduction of the tibia results from the reduction in number of leg-bud cells, and not from an extension of the 'opaque patch'.

On the other hand, development of the tibial rudiment is inhibited even where there is still plenty of mesenchyme for the formation of a reduced tibia if not one of normal size. In a preliminary study of the *ws* mutant based on a small number of embryos, Ede (1968) reported that the tibial matrix stained only

weakly with alcian blue relative to the fibula. This was not confirmed in the present investigation, but it appears to be necessary that some such weakness should occur to account for the tibial buckling which is common in later embryos.

The reduction of tibial development and the comparatively strong development of the fibula is interesting in view of the observations of Hampé (1960) on competition between the two rudiments. He found that where the material available for the development of both was reduced, it was monopolized by the tibial rudiment, whereas if excess was available an extended fibula was produced: it was as if the tibia took precedence, using whatever was available until a normal rudiment was produced, after which anything left over went into formation of the fibula. It is interesting therefore that in the *ws* mutant the tibial rudiment fails to achieve its normal dominance over the fibula. This may be related to the fact that in Hampé's experiments the reduction or addition of material was of the zeugopod region in general and not specifically of one or the other rudiments. In the *ws* mutant the abnormal necrosis affects only the preaxial region, and there must be some early effect on the tibial rudiment which causes the normal dominance relation to be reversed.

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Note added in proof

Recently Professor J. W. Saunders has made observations (personal communication) on cell death in limb development in the American *wingless* mutant originally investigated by Zwilling (1956). He finds the lateral plate mesoderm in the region of the prospective wing is extensively necrotic beginning at stage 16 or 17, and the AER does not form. Leg bud development is relatively normal to stage 19 or 20, when mesenchymal cell death and AER regression may then take place, but in an unpredictable pattern. The complete foot is never formed, and a range of expression is found from complete absence of the hindlimb to a reduction in the number of toes. While the American *wingless* mutant resembles sex-linked winglessness (*ws*) in the involvement in limb morphogenesis of cell death which has more pronounced effects on wing rather than on leg development, there is no evidence for precocious appearance and abnormal extension of the ANZ, as in *ws*.