Limb development in the polydactylyous talpid\textsuperscript{3} mutant of the fowl

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INTRODUCTION

The three groups of abnormal chick embryos known as talpids show a common pattern of remarkably widespread pleiotropic abnormalities, thought to represent the homozygous expression of one or other of three autosomal recessive genes, symbolized respectively as \textit{ta}\textsuperscript{1}, \textit{ta}\textsuperscript{2}, \textit{ta}\textsuperscript{3}. Ede & Kelly (1964\textit{a}, \textit{b}) described in detail the abnormalities of the \textit{talpid}\textsuperscript{3} embryos (\textit{ta}\textsuperscript{3}/\textit{ta}\textsuperscript{3}), which are essentially similar to those in Cole's \textit{talpid} (\textit{ta}\textsuperscript{1}/\textit{ta}\textsuperscript{1}) (Inman, 1946), while homozygotes for \textit{talpid}\textsuperscript{2} (Abbott, Taylor & Abplanalp, 1960) survive longer and, unlike the others, have relatively normal heads. All three possess at 11 days the following trunk abnormalities: (1) a shortening of the vertebral column accompanied by much fusion of adjacent vertebrae; (2) failure of cartilage replacement by bone; (3) substantial subcutaneous oedema and failure of the body wall to close ventrally round the viscera; (4) abnormal feather follicle formation; and (5) polydactyly in the shortened limbs. In the head of \textit{ta}\textsuperscript{1}/\textit{ta}\textsuperscript{1} and \textit{ta}\textsuperscript{2}/\textit{ta}\textsuperscript{2} embryos both the eyes and the maxillary processes are drawn together in the midline. Ede & Kelly (1964\textit{b}) suggest that there is a disturbance of the segregation of the mesenchyme, particularly in forming precartilaginous condensations, and that the basic effect may lie in the intercellular ground substance.

The most striking aspect of \textit{talpid}\textsuperscript{3} embryos is abnormal limb formation involving greatly increased numbers of digits. Ede & Kelly (1964\textit{b}) showed that at 11 days in both fore- and hind-limbs about 7 digits were formed, compared with 3 and 4 digits respectively in normal embryos. In the forelimb, carpals and metacarpals formed a single block while radius and ulna together formed a second block. The hind-limb showed similar fusions.

The investigations reported here represent an attempt to study the origin of the limb abnormalities. The development of the apical ectodermal ridge (AER)
Histochemical tests on the limbs were carried out in the hope of finding histochemical abnormalities preceding histological ones, and thus identifying more clearly the first site of developmental failure in *talpid*³. Tests were thus carried out on the acid mucopolysaccharides of the mesenchymal condensations ('membranous skeleton') and on alkaline phosphatase distribution, which shows a characteristic pattern of change during limb development (Milaire, 1961).

**METHODS**

(i) **Gross morphology and cell death**

Living *talpid*³ and normal embryos of 3½–6 days were vitally stained by Nile blue sulphate in Ringer's solution (Saunders *et al.* 1962), which colours areas of degeneration an intense dark blue. Detached limbs were photographed using yellow light, and enlargements made at a uniform magnification. The photographs, which show the general shape of the whole limb, with the AER which contains dying cells distally, and with areas of superficial mesenchymal necrosis picked out in black, were used to construct drawings. Areas of cell death were localized by histological and histochemical means. Nuclei of phagocytosed dead cells are intensely chromophilic (Chang, 1939; Ballard, 1965) and can be identified in iron-haematoxylin stained sections.

Acid phosphatase activity is associated with cell death and lysosomal activity (Novikoff, 1961), and can be identified by the Gomori lead method (Gomori, 1952) involving precipitation as lead phosphate of phosphate ions released at pH 5 at the site of enzyme activity. Unfortunately, paraffin wax embedding destroys 95% of enzyme activity (Barka & Anderson, 1963), and to avoid this loss the modification of Marit & Milaire (1961) was employed. In this technique, suitable for embryonic tissue, the tissue is fixed in cold (4 °C) formol-saline, washed in cold Locke's solution, and incubated for 2 h at 38 °C in the incubating medium. The tissue is then blocked and sectioned and the sections immersed in dilute ammonium sulphide solution. Black deposits indicate sites of enzyme activity. This method is open to the criticism that the amount of deposit depends on the rate of diffusion of substrate through the tissue as well as on enzyme concentration. The areas of cell death are, however, superficial, and the Marit–Milaire method gave consistently positive results for areas of cell death discovered by histological and vital staining methods.

(ii) **Mesenchymal condensations**

The distribution of the acid mucopolysaccharides (mps) of the condensations and of the neutral mucopolysaccharides of the basement-membrane, blood vessel walls and bone tissue, were studied by the Trevan technique (C. B. McLoughlin, personal communication). The Trevan technique involves first
the specific staining of acid mps with alcian blue at pH 2.5, the fixation of this stain in borax-saturated alcohol, and finally the PAS reaction which is specific for neutral mps and glycogen (Yamada, 1964). Acid mps are stained green, neutral mps and glycogen pink.

Alkaline phosphatase distribution was studied by the Gomori (1952) technique, in which slides are incubated with sodium glycerophosphate under alkaline conditions. Sites of enzyme activity liberate phosphate ions which are precipitated as calcium phosphate. The precipitate is made visible by conversion to cobalt sulphide. Some slight modifications were made to the Gomori technique: acetone, the fixative recommended in the Gomori technique, is physically very destructive to embryonic tissue, and limb buds were either infiltrated in celloidin (Gomori, 1952) after acetone fixation or fixed in cold 80% alcohol. Enzyme destruction during embedding was minimized by using wax of low melting point (45 °C). Control sections were used to distinguish false-positive staining from genuine enzyme reactions, using the methods suggested by Barka & Anderson (1963).

The sedation method of Hamburger & Hamilton (1951) has been adopted for description of the stage of development reached by normal embryos, and, as far as possible, for talpid³ embryos. The number of limbs examined by these various methods is given in Table 1.

### RESULTS

The following account compares firstly the gross morphology of normal and talpid³ limb development, and the apical ectodermal ridge (AER) changes. The absence of cell death in the talpid³ superficial mesenchyme, and finally the histological and histochemical development of the limb skeleton, is then described.
(i) **Limb development and the AER**

Text-figs. 1 and 2 are drawn from photographs of vitally stained fore- and hind-limbs, to show the length of normal and *talpid* AER from 3½ to 6 days. As the AER, particularly in the early stages, shades off into the surrounding ectoderm by degrees, it is not possible to obtain a very accurate measure of the AER length.

![Fig. 1 and 2](image)

**Fig. 1**

Text-fig. 1. Forelimb: left, normal; right, *talpid*. Text-fig. 2. Hind-limb: left, normal; right, *talpid*. Drawings from photographs of ventral aspect of vitally stained left limbs. Cell death is indicated by stippling. (Cell death in the AER and deeper mesenchyme not included.)

**Forelimb.** Stage 22 (about 3½ days) is the earliest at which it is possible to distinguish *talpid* forelimbs from normal. At stage 22 the base of the *talpid* forelimb is abnormally elongated anteriorly, while the outgrowth of the limb from the body wall is less than in the normal. In stages 22 and 23, the *talpid* AER appears to be about the same length as in normal limbs of a similar stage, notwithstanding the different limb shapes.
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During stages 24-29 (4-6 days) the characteristic ‘mushroom’ shape of \textit{talpid}^3 limbs develops, characterized by distal expansion, and a failure to shape the elbow angle. At stage 24 the \textit{talpid}^3 AER is noticeably longer than in the normal, and during stages 25-28 the AER becomes increasingly relatively longer than in the normal. At stage 28 the \textit{talpid}^3 AER is about 65\% longer than normal (this figure is based on two normal and two \textit{talpid}^3 limbs only). The \textit{talpid}^3 AER remains clearly visible until stage 29 (6-6\frac{1}{2} days) although by this stage in the normal the AER has regressed along most of its length.

**Hind-limb.** The \textit{talpid}^3 hind-limb is less abnormal than the forelimb and cannot be clearly distinguished from the normal by its morphology until stage 25. The appearance of the more extensive \textit{talpid}^3 AER in the hind-limb is also longer delayed than in the forelimb. During stages 22-24 (Plate 1, figs. A, D) the \textit{talpid}^3 AER is not noticeably more extensive than in the normal hind-limb, while at stage 25 it is barely more extensive. In both stages 26 and 27 the \textit{talpid}^3 AER is more extensive by about 20\% than in the normal (figures based on 4 normal, 6 \textit{talpid}^3 limbs only—see Plate 1, figs. B, E).

The \textit{talpid}^3 hind-limb is smaller than the fore until about stage 28, after which the hind-limb gradually becomes larger than the forelimb. The \textit{talpid}^3 AER is still clearly visible on stage 29, by which stage the normal AER has regressed. At stage 29 the \textit{talpid}^3 hind AER has become as extensive as the \textit{talpid}^3 fore AER of the same stage.

(ii) **Histology and histochemistry of the AER**

Ede & Kelly (1964b) reported that the \textit{talpid}^3 AER was histologically similar to the normal at 6 days, and in these studies haematoxylin staining revealed no morphological abnormality at 4 or 5 days. The possibility that the \textit{talpid}^3 AER was histochemically abnormal was investigated. Acid phosphatase distribution did not differ in the normal and \textit{talpid}^3 AER of fore- and hind-limbs of 4-6 days incubation, except that the normal AER underwent regression at 5\frac{1}{2}-6 days and showed diminished acid phosphatase activity. Acid phosphatase was localized in the dying cells found along the distal edge of the \textit{talpid}^3 and normal AER throughout the period examined. Acid phosphatase activity was also localized in the AER immediately adjacent to the basement membrane. This result is in disagreement with Milaire's finding (1961) that acid phosphatase was localized only in the AER adjacent to the basement membrane. Milaire's result is surprising as acid phosphatase is to be expected in the dying cells (Novikoff, 1961) found in the distal margin of the AER. Jurand (1965) also found acid phosphatase activity at this site in his electron-microscope study.

Alkaline phosphatase activity of the normal and \textit{talpid}^3 AER of fore- and hind-limbs of 4-6 days incubation was the same, being moderately active throughout, with the same intensity of staining as the undifferentiated limb mesoderm. This finding conflicts with other views on alkaline phosphatase activity of the chick AER. Zwilling (1961), quoting unpublished work by Loewenthal, claims
that the AER is devoid of alkaline phosphatase activity. By contrast, Milaire (1962) claims that ‘the activity of alkaline phosphatase is strong in the early AER’.

(iii) Absence of massive cell death in talpid³ superficial mesenchyme

Text-figs. 1 and 2, drawn from photographs of vitally stained limbs also show areas of massive necrosis in the superficial mesenchyme in normal fore- and hind-limbs, and the absence of such areas in talpid³ fore- and hind-limbs. Photographs of vitally stained hind-limbs (Plate 1, figs. A–F) show the development of the areas of necrosis in the normal, and their absence in talpid³ at comparable stages.

In the normal forelimb the pattern of cell death found is the same as that described by Saunders et al. (1962). Necrosis is first visible in the anterior mesenchyme separating the limb from the body wall at stage 21. This anterior necrotic zone (ANZ) spreads in a proximo-distal direction during later development, and according to Saunders et al. (1962) carves out the contours of the limb, especially the elbow. A second area of necrosis, the posterior necrotic zone (PNZ), appears in the posterior mesenchyme of the limb at stage 24 and also spreads in a proximo-distal direction. The distal ends of the necrotic zones mark the anterior and posterior limits of the AER.

In the normal hind-limb there is a similar pattern of cell death, illustrated in Text-fig. 2, which does not appear to have been described previously. The appearance of an ANZ is delayed in comparison with the forelimb, and does not appear until stage 23 (Plate 1, fig. A) though it is then more massive than that found in the forelimb. The ANZ underlies the anterior part of the AER during stages 23/24 to 25, and this is particularly clear at stages 23/24. At stage 24 the AER is a series of irregular waves over the ANZ, and can be seen to be breaking down. This observation confirms Gasseling & Saunders’s (1964) idea that the necrotic zones are not a source of AER maintenance factor, a conclusion based on their finding that grafting the PNZ under the AER resulted in the flattening of the latter. By stage 26 the AER over the ANZ has disappeared.

In talpid³ fore- and hind-limbs throughout the period 3½–6 days there is no sign of an ANZ or a PNZ in the superficial mesenchyme (Text-figs. 1, 2). The anterior and posterior mesenchyme of the limb is free from any massive necrosis.

(iv) Histology and histochemistry of superficial mesenchyme

Areas of necrosis were found corresponding to areas taking up the vital stain in normal limb development, and recognizable by the presence of the ‘giant cells’ described by Saunders et al. (1962), which contain intensely chromophilic granules (Plate 2, figs. G, H). According to Ballard (1965), in the interdigital tissue of the mouse-embryo limb these cells consist of transformed macrophages each of which has phagocytosed several dead cells whose shrunken dehydrated nuclei are represented by the chromophilic granules. It is likely that this interpre-
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The necrotic zones were positive for acid phosphatase, which was particularly rich in the dead cell group (Plate 2, fig. I). Many apparently normal mesenchyme cells contained acid phosphatase activity localized in a few small particles which according to Ballard are lysosomes. The dead cell groups contained much greater phosphatase activity localized in larger particles or vesicles presumably associated with ingested dead cells.

In sections of 3½–6 day talpid³ limbs stained with iron haematoxylin, although occasional dying cells were found, there were no areas of massive necrosis consisting of dead cell groups corresponding to those found in the normal limb (Plate 2, fig. J, K). Many of the apparently normal-looking talpid³ mesenchymal cells contained acid phosphatase localized in small particles, presumably lysosomes, but there were no dead cell groups with large acid phosphatase-rich vesicles (Plate 2, fig. L).

(v) Development of the limb skeleton as shown by acid mucopolysaccharide distribution

The cartilaginous skeleton of the limb originates as a mesenchymal condensation which forms the acid mucopolysaccharide (mps) characteristic of cartilage (probably chondroitin sulphate: Franco-Browder, de Rydt & Dorfman, 1963). Thus staining sections of a series of limbs for acid mps gives a clear picture of the development of the limb skeleton. Text-fig. 3 is drawn from photographs of sections of limbs stained for acid mps, and shows the developing skeleton of normal and talpid³ forelimbs.

Searls (1965) showed radioactive sulphate uptake, probably as chondroitin sulphate, by prospective chondrogenic areas as early as stage 22–23, but these areas are not visible with the alcian blue technique until stage 25. In the normal at this stage for the first time a central core of acid mps has appeared, forked at its distal end into radial and ulnar condensations. The condensation is still in one piece, but by stage 26 the separation of radius and ulna is more clearly marked, and less well-defined condensations representing the metacarpals have appeared distally (Plate 3, fig. M). By stage 29–30 the radius and ulna have sharply defined edges, and intercellular material is clearly visible between the cartilage cells which are arranged transversely to the long axis, but are not yet hypertrophic (Plate 3, fig. O). Indistinct condensations representing the wrist bones are visible for the first time, and the 3rd and 4th metacarpals are clearly defined. In the talpid³ a central core of acid mps does not appear until stage 26, but there is no sign of a separation into radius and ulna (Plate 3, fig. P). At stage 27–28 the staining intensity is less than normal and the positive regions are divided rather imprecisely into two major regions, the proximal block representing the fused radius–ulna, and the distal condensation representing the fused metacarpals (Plate 3, fig. Q). The edge of these condensations is imprecise.
and quite different from the more sharply defined edges of the normal cartilage condensations at this stage.

At stage 29–30 (Plate 3, fig. R) there are three major blocks rich in acid mps (not counting the humerus). The leading edge of the proximal radius-ulna condensation shows heavy staining, presumably indicating an ‘attempt’ at the formation of the radius. However, this ‘radius’ condensation is joined on its trailing edge to the remainder of the condensation (ulna) which shows intense staining proximally. The edges of the condensation, with the exception of the leading ‘radius’ edge, are not clearly defined, and the chondrocytes, although showing signs of orientation, are not clearly orientated at right-angles to the long axis of the condensation as they are in the normal. The joint region between

Text-fig. 3. Localization of acid mucopolysaccharides in normal (left) and talpid³ (right) forelimb development. Drawings from photographs of PAS/alcian-blue stained sections. Intensity of stippling is proportional to intensity of staining. C, Carpals; C1, centrale 1; H, humerus; MC, metacarpal; R, radius; R–U, fused radius-ulna; U, ulna.
humerus, radius and ulna is slightly more poorly differentiated in talpid\textsuperscript{3} than in the normal, in that acid mps material is more continuous between the three elements. Talpid\textsuperscript{3} thus resembles talpid\textsuperscript{1}, in which Inman (1946) claims there is 'a less distinct joint between humerus, radius and ulna', which are indistinctly separated at 8 days. Distal to the radius–ulna is a clear but thinly stained narrow band of acid mps material, presumably the fused carpals. This band has a single thickening anteriorly, probably centrale 1, which is the first of the wrist elements to become clearly defined (Montagna, 1945) and which is also the only clearly defined wrist element found by Inman (1946) in talpid\textsuperscript{1}. Distal to the carpal block is the third major block, broad and fairly heavily stained, representing the metacarpals. The band shows no sign of any separate elements, it is poorly defined round its edges, and the cartilage cells are not arranged transversely to the long axis of the limb. Distal to the metacarpal band is a very poorly defined band of acid mps which may represent the phalanges.

(vi) \textit{Cell death in the deeper mesenchyme}

An ‘opaque patch’ of degenerating cells appears in normal hind-limb development at stage 24. Fell & Canti (1934) found by extirpation experiments that it appears to correspond to prospective femur tissue. The ‘opaque patch’ reaches its maximum size at stage 26, and at stage 28–29 when it occupies the area between femur, tibia and fibula, it begins to disappear. There is a similar opaque patch in the forelimb (Saunders \textit{et al.} 1962). At stage 26 it appears to be present between the arms of the Y representing the radius–ulna–humerus condensation, and by stage 28 the patch, which has begun to disappear, separates the humerus from radius and ulna in the prospective elbow region.

In both fore and hind talpid\textsuperscript{3} limbs the opaque patch can be identified first at stage 25 and is still present at stage 28. It is difficult to identify, as the necrotic cells are much more scattered than in the normal in which areas of necrosis are strictly localized, particularly in the later stages.

The function of the opaque patch remains a mystery (Fell, 1964), though several workers (Saunders \textit{et al.} 1962; Zwilling, 1964) suggest it may be important in the differentiation in the skeleton of the joint. Jackson (1964) has suggested that cell death is often involved in the reshaping of connective tissue, and Fell & Dingle (1963) have demonstrated that lysosomal preparations release chondroitin sulphate from chick embryo cartilage. In talpid\textsuperscript{3} the scattering of the opaque may contribute to the poor joint differentiation at 6 days between humerus, radius and ulna, and possibly to the earlier failure of the last two to separate.

(vii) \textit{Alkaline phosphatase distribution}

The changing pattern of alkaline phosphatase distribution in normal and talpid\textsuperscript{3} limbs is summarized in Text-fig. 4, constructed from photographs of which examples are shown in Plate 4, figs. S–X.
In normal forelimbs at stage 24, the enzyme is homogeneously distributed in the mesenchyme, but at stage 25 there is a greater concentration (Plate 4, fig. S) in the mesenchymal condensation representing radius, humerus and ulna. As the intercellular material of the mesenchymal condensations increases, these contain an increasingly smaller concentration of the enzyme than in the periphery (Plate 4, fig. T). At stage 28, the perichondrium shows high alkaline phosphatase concentration for the first time, and by stage 30 this activity has greatly increased.

In talpid³ forelimbs, the enzyme is at first uniformly distributed in the mesenchyme (stages 24, 25). Later in stages 26, 27 (Plates 4, fig. V) and 28 (Plate 4,
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fig. W) greater activity is found in the mesenchymal condensations which retain high activity much longer than those of normal embryos. By stage 30 (Plate 4, fig. X), however, activity in the condensations has declined, though to a lesser extent than in normal forelimbs at this stage. The decline in activity is particularly marked in the radius part of the radius–ulna fusion, but is less marked in the carpal and metacarpal fusions. There are three bands of mesenchyme between the skeletal blocks which show high activity, namely (1) in the area between radius, ulna and humerus and corresponding to a similar area in the normal forelimb, (2) in the area distal to the radius–ulna fusion, and (3) distal to the metacarpal fusion. At stage 30 there is a striking absence in the *talpid*³ perichondrium of the increased enzyme activity found in the normal perichondrium.

DISCUSSION

(i) The *talpid*³ apical ectodermal ridge and the absence of cell death in the superficial mesenchyme

Glücksmann (1951) has drawn attention to the frequent occurrence of cell death in embryogenesis, and Saunders *et al.* (1962) have described the massive zones of cell death which occur in the superficial mesenchyme during chick limb morphogenesis. A striking aspect of *talpid*³ limb development is the complete absence in both fore- and hind-limb development of these massive zones of cell death and phagocytosis in the superficial mesenchyme. Histological evidence of the absence of cell death is paralleled by the absence in *talpid*³ of the very high acid phosphatase activity which is characteristic of areas of cell necrosis in normal embryos, though, as in normal embryos, living *talpid*³ mesenchymal cells possess low acid phosphatase activity, presumably localized in lysosomes. The absence of cell death in these areas is all the more remarkable because dying cells are found in the ectoderm of the AER and in the ‘opaque patch’ of the deeper mesenchyme, as in the normal.

The mechanism of cell death has been the subject of much recent research. Saunders *et al.* (1962) showed by extirpation and grafting that a ‘death clock’ is set as early as stage 17 in the presumptive posterior necrotic zone (PNZ) of the forelimb, where necrosis does not manifest itself until stage 24, but they were unable to identify the initial cause of death. Recently much attention has been given to the role of the lysosomes and related cytoplasmic particles containing acid phosphatase and other hydrolytic enzymes (Novikoff, 1961). It is often maintained (e.g. by Zwilling, 1964) that cell death is caused by intracellular release of acid hydrolases from lysosomes of cells destined to die. However, according to Ballard (1965), in the interdigital zones of cell death of the mouse embryo, dead cells contain acid phosphatase localized as in living mesenchymal cells, and in fact do not show any increase in acid phosphatase activity, which is a later characteristic of the macrophages which ingest dead cells after they have died.
Control of cell death, therefore, remains an open question, as much in the normal as in talpid3. However, its absence in, and only in, very specific areas in the mutant indicates that its occurrence is under genetic control. A number of genes are known to influence cell death in areas other than the limbs, and in general they seem to increase normally occurring processes of necrosis, for example in Rumplessness in the fowl (Zwilling, 1942); the extent to which normally occurring necrosis is increased in the tail-bud tissues at 2–4 days determines the degree of rumplessness at later stages. In the mouse several mutant genes increasing cell death in the embryonic tail are known (reviewed by Grüneberg, 1963). Talpid3 is unique in that cell death in a particular region is decreased rather than extended.

The consequences of the absence of cell death are easier to show than its causes. They appear to be three: (1) failure of limb contour shaping, (2) effect on limb symmetry, and (3) abnormal extension of the AER resulting in polydactyly. These consequences are now discussed more fully.

(1) Saunders et al. (1962) suggest that cell death in the superficial mesenchyme plays a role in shaping the contours of chick limb development, particularly in ‘carving out’ the elbow angle. In talpid3 forelimbs the absence of cell death presumably prevents the carving out of the elbow angle.

(2) Gasseling & Saunders (1964) suggest the forelimb PNZ may have some responsibility for the pattern of limb symmetry. When they grafted the PNZ preaxially in the wing-bud rim they obtained immediately posterior to the graft ‘a supernumerary wingtip of left-hand asymmetry, mirror-twinning the normal right hand’. The absence of the PNZ may be responsible for one of the unique features of talpid3 limb development in that none of the digits can be recognized as a specific toe or finger. This contrasts with most polydactylous conditions in both the fowl and the mouse in which there is either twinning of the limb but with each limb plate relatively normal (e.g. eudiplopodia (Goetinck, 1964) and Duplicate (Landauer, 1956b) in the fowl), or the addition of one or more digits preaxially to an otherwise normal limb-plate (e.g. Polydactyly in the fowl (Baumann & Landauer, 1944) and preaxial polydactyly in the mouse (Chang, 1949).

(3) The PNZ and anterior necrotic zone (ANZ) may, according to Gasseling & Saunders (1964), play another role in limb development. Experimental study of normal limb development has led Zwilling (1961) to identify two important factors. The first is that the apical ectodermal ridge (AER) is responsible for stimulating the outgrowth of the underlying mesoderm (Saunders, 1948). The second is that the AER is dependent on the existence of an apical ectodermal maintenance factor (AEMF) in the underlying limb mesoderm. Gasseling & Saunders found experimentally that ‘apical ectoderm flattens when underlaid by a graft of PNZ mesoderm’. This suggests the necrotic zones have a role to play in limiting the AER length through limiting the area of mesoderm from which AEMF is available.
In talpid the AER is never terminated, either posteriorly or anteriorly, by mesenchymal necrotic zones. The excessive AER extension in talpid in stages 25–28 coincides with the period during which the AER in the normal is terminated by the PNZ and ANZ. In addition, excessive talpid AER extension is recognizable at an earlier stage in the forelimb than in the hind-limb, which is not surprising if AER extension is limited by the ANZ as this appears in the forelimb considerably earlier than in the hind-limb. The excessive extension in talpid appears to be due to an abnormal increase in the area of talpid mesoderm concerned in AEMF production or distribution as a result of the failure of cell death to occur in the mesoderm.

(ii) Skeletal abnormalities

The first visible abnormality in the talpid limb skeleton is in the shape of the initial mesenchymal condensation. The distal part of the condensation shows no sign of the separation of radius and ulna found in the normal. The later combined radius–ulna can be traced to this early failure to form separate condensations.

This failure of the talpid mesenchyme to form the normal pattern of skeletal condensations persists throughout later development. The mesenchyme also behaves abnormally in that alkaline phosphatase activity persists unusually long in the condensations, and in that cartilage, when it is formed, is abnormal in that the chondroblasts are not arranged transversely to the long axis of the rudiment, but are disorganized. The mesenchymal condensation seems to have no histochemically detectable abnormality in the acid mps ground substance which appears to play a part in integrating mesenchymal cell activity (Moscona, 1960).

The first abnormality in alkaline phosphatase distribution in talpid limb development is abnormally long retention of high enzyme activity by the mesenchymal condensations. The functional significance of alkaline phosphatase activity in normal limb development is still not understood (Zwilling, 1961), though Moog (1944) found that most chick embryo tissues when undergoing differentiation underwent an increase in alkaline phosphatase activity, and Milaire (1962) detects a changing pattern of distribution common to all vertebrate species he studied. The first detectable abnormality in talpid limb skeleton is in the pattern of condensation, while the long retention of phosphatase activity by the condensation appears later, simultaneously with a morphological abnormality: failure of orientation of the chondroblasts in the condensation. The fact that this first sign of histogenetic abnormality follows the anomaly in the pattern of condensation suggests that anomalous alkaline phosphatase distribution should not be regarded as indicating an initial effect of abnormal gene activity.

Later, at stage 28, the talpid perichondrium fails to develop an inner layer of osteoblasts rich in alkaline phosphatase. This point will be taken up in a
further paper, concerned with ossification with particular reference to the shoulder girdle in \textit{talpid}³.

This analysis reveals two apparently unrelated abnormalities in the \textit{talpid}³ limb:

(1) The first identifiable defect is a difference in shape of the forelimb at stage 22, apparently as a result of failure of the ANZ to appear. Later, this absence of superficial mesenchymal cell death in the PNZ and ANZ accounts for the mushroom shape of the \textit{talpid}³ limb. \textit{Talpid}³ polydactyly probably results from excessive AER development resulting in an excess of mesenchyme available distally for digit formation.

(2) The initial failure in mesenchymal cell death cannot be held responsible for the widespread failure of separate cartilaginous condensations to appear. Many mutations possess an increased AER without, for example, fusion of carpals and metacarpals into bands (e.g. the polydactylous monster in the guinea-pig; Scott, 1938). \textit{Talpid}³ cartilage is \textit{in itself} abnormal: this is confirmed histochemically in the limbs, histologically and histochemically in the shoulder girdle (unpublished) and by the fusions between vertebrae (Ede & Kelly, 1964b). The observations reported here support Ede & Kelly's claim that the \textit{talpid}³ gene has a general effect on mesenchymal condensation, and give this view histochemical support. But the relation between the absence of necrotic zones in the limb superficial mesenchyme and the \textit{talpid}³ cartilage anomaly remains obscure.

(iii) \textit{Other cases of the development of polydactyly}

Zwilling & Ames (1958) have put forward the hypothesis, based on Zwilling's theory of AER/AEMF interaction in normal limb development, that polydactyly in general is the consequence of alterations in the distribution of the AEMF found in the mesoderm. This hypothesis is based on experiments on the \textit{Duplicate} mutant in the fowl, which causes preaxial polydactyly. Zwilling & Hansborough (1956) obtained limb-buds composed of reciprocal crosses between normal and polydactylosous mesoderm and ectoderm. \textit{Duplicate} mesoderm and normal ectoderm formed a polydactylous limb, but the complementary combination resulted in a normal limb. In the developing combination limb which later became polydactylosous, they observed the excessive preaxial extension of the AER. Zwilling & Hansborough postulate that the \textit{Duplicate} limb is formed through preaxial excess of the AEMF in the mesoderm.

Similarly Goetinck & Abbott (1964) investigated the polydactylosous mutant \textit{talpid}² and, finding by reciprocal exchanges of normal and mutant tissues in grafts that \textit{talpid}² mesoderm and not ectoderm is responsible for the polydactyly, they concluded there is excess formation of the AEMF. \textit{Talpid}² shows striking resemblances to \textit{talpid}³ (limb formation, feather retardation) together with certain differences (comparatively normal hand development), and it is reasonable to suppose that reciprocal crosses in \textit{talpid}³ would similarly indicate the source of the abnormality in the mesoderm.
### Table 2. Characteristics of known polydactylous conditions

<table>
<thead>
<tr>
<th>Mutation/strain</th>
<th>AER extension</th>
<th>Extra digits preaxial</th>
<th>No. of additional digits</th>
<th>Time of 1st abnormality (days) (1)</th>
<th>Reference</th>
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<td><strong>Fowl</strong></td>
<td></td>
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<tr>
<td>Polydactyly</td>
<td></td>
<td></td>
<td>Fore 1, Hind 2</td>
<td>11</td>
<td>Carter, 1954</td>
</tr>
<tr>
<td>Duplicate</td>
<td></td>
<td></td>
<td>Fore 0, Hind 1</td>
<td>11</td>
<td>Carter, 1954</td>
</tr>
<tr>
<td>Diplopodia-1 (3)</td>
<td>+</td>
<td>+</td>
<td>Fore 1, Hind 1</td>
<td>11</td>
<td>Carter, 1954</td>
</tr>
<tr>
<td>Diplopodia-2 (3)</td>
<td>?</td>
<td>+</td>
<td>Fore 1, Hind 1</td>
<td>11</td>
<td>Carter, 1954</td>
</tr>
<tr>
<td>Eudiplopodia (3)</td>
<td>(6)</td>
<td>No</td>
<td>Fore 4, Hind 3</td>
<td>3½-4, St 22-3</td>
<td>Goetinck, 1964</td>
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<tr>
<td>Splitfoot (3)</td>
<td>Assumed</td>
<td>+</td>
<td>Fore 0, Hind 1</td>
<td>1 or 2</td>
<td>Abbott, 1960</td>
</tr>
<tr>
<td>Talpid-1 (3)</td>
<td>?</td>
<td>No</td>
<td>Fore 2 or 3, Hind 4</td>
<td>4½</td>
<td>Goetinck &amp; Abbott, 1964</td>
</tr>
<tr>
<td>Talpid-2 (3)</td>
<td>+</td>
<td>No</td>
<td>Fore 2 or 3, Hind 4</td>
<td>4½</td>
<td>Goetinck &amp; Abbott, 1964</td>
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<tr>
<td>Talpid-3 (3)</td>
<td>+</td>
<td>No</td>
<td>Fore 4 or 5, Hind 3</td>
<td>3½-4, St 22-3</td>
<td>Goetinck &amp; Abbott, 1964</td>
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<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Luxate (3)</td>
<td>+2 (7)</td>
<td>+</td>
<td>Fore 0, Hind 0</td>
<td>11</td>
<td>Carter, 1954</td>
</tr>
<tr>
<td>Luxoid (Green’s) (3)</td>
<td>+ 2</td>
<td>+</td>
<td>Fore ½-2, Hind 1-4</td>
<td>11½</td>
<td>Forsthoefel, 1959</td>
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<tr>
<td>Luxoid (Strong’s) (3)</td>
<td>+ 2</td>
<td>+</td>
<td>Fore 1-3, Hind 1½-3 (4)</td>
<td>11½</td>
<td>Forsthoefel, 1963</td>
</tr>
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<td>Fortuyn’s strain</td>
<td></td>
<td></td>
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<tr>
<td>Preaxial polydactyly</td>
<td>+2 (8)</td>
<td>+</td>
<td>Fore 0, Hind 0</td>
<td>12</td>
<td>Chang, 1939</td>
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<tr>
<td>Postaxial polydactyly (9)</td>
<td>+</td>
<td>No</td>
<td>Fore 0, Hind 0</td>
<td>12</td>
<td>Chang, 1939</td>
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<tr>
<td><strong>Guinea-pig</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Polydactylyous monster (heterozygota)</td>
<td>?</td>
<td>No (10)</td>
<td>Fore 1, Hind 2</td>
<td>17½ or 18½ (11)</td>
<td>Wright, 1934, 1935</td>
</tr>
<tr>
<td>(homozygota)</td>
<td>+</td>
<td>No</td>
<td>Fore 6, Hind 7</td>
<td>17½ or 18½ (11)</td>
<td>Scott, 1937, 1938</td>
</tr>
</tbody>
</table>

**Key.** (1) In limb development: 4 days (st. 23) chick = 11-day mouse = 19-day guinea-pig. (2) Extension of AER preaxially. (3) Homozygote. (4) ‘½’ indicates replacement of hallux or pollex by triphalangous digit. (5) ‘Polydactylous. (6) Second dorsal AER and limb-plate. (7) Carter claims AER reduced preaxially. (8) Own observation from Chang’s photographs. (9) Strain. (10) Forelimb normal post-axially. (11) 18½ days equivalent to 3½ days (st. 20–21) in chick and 10½ days in mouse.
The success of the Zwilling & Ames (1958) excess AEMF hypothesis in explaining polydactyly in general should not obscure its inability to explain the various polydactylyous patterns of the limb skeleton. Why should not an excess AEMF lead to broader rather than more digits? In addition to the excess AEMF hypothesis, cases of polydactyly require a subsidiary hypothesis about the distribution of condensation centres. Morphological evidence indicates major differences between Duplicate (unaltered normal wingplate) and talpid\(^a\) (completely reorganized limb-plate) even though the excess AEMF hypothesis may apply to both, and different developmental pathways may be involved. In order to discuss this issue more fully, a comparison is made in Table 2 of the best-known cases of polydactyly, as to the number and position of extra digits, the time of their first appearance, and what is known of AER development.

Two polydactylous conditions appear to be unique. In eudiplopodia there is an additional AER, dorsal to the primary one, which produces the supernumerary digits. In postaxial polydactyly in the mouse the single additional digit on each forelimb is produced postaxially.

The remainder are all included in one or other of two groups. The larger group, of which Duplicate is representative, is characterized by the occurrence of a small number of supernumerary digits (1 or 2 per limb), added preaxially as a result of preaxial extension of the AER earlier in development. The postaxial part of the limb is normal, and in general (Duplicate and luxate are exceptions) the abnormality does not appear until the formation of a distinct footplate. Included in this group are Duplicate, diplopodia\(^1\), luxate, Strong's luxoid, Green's luxoid and Fortuyn's strain. Polydactyly, diplopodia\(^2\) and splitfoot require further investigation, but they should probably also be included.

The Zwilling & Ames (1958) hypothesis that polydactyly depends on an excess (in these cases preaxial) of the AEMF, and hence on a preaxial extension of the AER, may well apply to this group. Extra digit formation may result either from excess preaxial mesenchyme, or from surviving preaxial mesenchyme that normally undergoes necrosis, or from mesenchyme diverted from its normal role in radius or tibia formation. This latter would account for the fairly common occurrence in this group of mutants of hemimelia of the radius or tibia. In view of the observations reported here on talpid\(^3\), it would be interesting to know whether the forward spread of the AEMF, in the fowl mutants of this group, is accompanied by any reduction in the ANZ. Unfortunately in the mouse, although interdigital degenerations are known (Forsthoefel, 1959; Milaire, 1962; Ballard, 1965), it is not known whether cell death plays a role in limb morphogenesis similar to that in the chick.

The smaller group comprises Scott's homozygous polydactylyous monster (Px/Px) and the three talpid mutants. In contrast to the 'preaxial' group there is no recognizable unaltered postaxial foot or wing in these cases, the plate being so altered that none of the fingers and toes are recognizable as normal digits. The number of additional digits (+5 in the polydactylyous monster, +3 to +6...
in the *talpid* group) is greater and in most cases the abnormality is detectable earlier: at stage 23 in *talpid*², stage 22 in *talpid*³, and in the guinea-pig monster at the equivalent of stage 20–21 in the chick.

In spite of the similarities within this group, the abnormalities of the skeleton in the polydactylous monster are quite different from those in the *talpid* mutants. Bone formation in the polydactylous monster is according to Scott (1937) 'a little slow but normal'. The bones of the limbs show no general tendency to fusion beyond a limited proximal fusion of two of the seven or eight metacarpals, and of two of the five metatarsals (Scott, 1938). By contrast in all the *talpid* mutants, the cartilaginous condensations of the skeleton show moderate (*talpid*²) and extreme (*talpid*¹, *talpid*³) forms of fusion, and there is no ossification in *talpid*³ while that in *talpid*¹ is extremely retarded (Abbott et al. 1960). The abnormalities in the tissue of the cartilage skeleton of *talpid* embryos distinguish them both from the ‘preaxial’ group of mutants and from the guinea-pig polydactylous monster. Presumably different developmental pathways, all involving AER enlargement, are involved in the preaxial polydactyly group, in the *talpids* and in the polydactylous monster. In the case of the three *talpid* mutants this developmental pathway leads to a very extreme form of polydactyly, characterized by great enlargement of the AER, greatly increased digit number, and widespread skeletal fusion.

**SUMMARY**

1. *Talpid*³ limb development is characterized by excessive elongation of the apical ectodermal ridge (AER). In the forelimb the *talpid*³ AER is of normal length during stages 22 and 23, but at stage 28 it exceeds the normal by about 65%. In the hindlimb the *talpid*³ AER is of normal length during stages 22–24, but at stage 27 it exceeds the normal by about 20%.

2. Massive cell death is absent from the superficial mesenchyme of both fore and hind *talpid*³ limbs. Histological and histochemical studies show that areas of dead cell groups rich in acid phosphatase activity are absent in *talpid*³ from the positions in the superficial limb mesenchyme in which they occur in the normal.

3. This absence of cell death in the limb superficial mesenchyme is thought to account for (a) the abnormal sculpturing of *talpid*³ limbs, and (b) the excessive elongation of the *talpid*³ AER, through apical ectodermal maintenance factor (AEMF) being available from areas of mesenchyme normally undergoing necrosis and lacking AEMF.


5. The relationship between the abnormal pattern of limb mesenchymal
condensation and the absence of massive cell death in the superficial mesenchyme is not understood.

6. *Talpid*\(^3\) limb development is discussed in relation to other cases where genes affect the pattern of cell death, and in relation to other examples of polydactyly.

**RÉSUMÉ**

_Développement du membre chez le mutant polydactyle talpid\(^3\) du poulet_

1. Le développement du membre chez _talpid\(^3\)_ est caractérisé par un allongement excessif de la cape apicale ectodermique. Dans le membre antérieur, la cape apicale ectodermique de _talpid\(^3\)_ est de longueur normale aux stades 22 et 23, mais au stade 28 elle dépasse la normale de 65\% environ. Dans le membre postérieur, la cape apicale ectodermique de _talpid\(^3\)_ est de longueur normale aux stades 22–24, mais au stade 27 elle dépasse la normale de 20\% environ.

2. Une mortalité cellulaire massive est absente dans le mésenchyme superficiel, aussi bien celui des membres antérieurs que celui des membres postérieurs de _talpid\(^3\)_.

3. Cette absence de mortalité cellulaire dans le mésenchyme superficiel du membre rendrait compte: (a) du modelage anormal des membres de _talpid\(^3\)_ et (b) de l'allongement excessif de la cape apicale ectodermique de _talpid\(^3\)_ sous l'action du facteur de maintien de l'ectoderme apical disponible à partir de zones du mésenchyme qui normalement subissent la nécrose et manquent de ce même facteur.

4. Le squelette anormal du membre antérieur de _talpid\(^3\)_ tire son origine d'un type anormal de la condensation mésenchymateuse formant le squelette membraux au stade 26. Le mésenchyme du membre de _talpid\(^3\)_ présente un type anormal de distribution de la phosphatase alcaline qui débute au stade 26.

5. La relation entre le type anormal de la condensation mésenchymateuse du membre et l'absence de mortalité cellulaire massive dans le mésenchyme superficiel n'est pas expliquée.

6. Le développement du membre de _talpid\(^3\)_ est discuté en liaison avec d'autres cas où des gènes affectent le type de la mortalité cellulaire, et en liaison avec d'autres exemples de polydactylie.
Limb development in fowl

REFERENCES


(Manuscript received 24 October 1966)
EXPLANATION OF PLATES

*a.n.z.*, Anterior necrotic zone; *a.e.r.*, apical ectodermal ridge;
*d.c.*, dead cell group; *p.n.z.*, posterior necrotic zone.

PLATE 1

Figs. A–C. Ventral view of *normal* left hind-limb vitally stained to show necrosis in the superficial mesenchyme and AER. Fig. A, stage 23; Fig. B, stage 25; Fig. C, stage 27.

Figs. D–F. Ventral view of *talpid³* left hind-limb vitally stained to show absence of necrosis in the superficial mesenchyme, and necrosis in the AER. Fig. D, stage 23; Fig. E, stage 25; Fig. F, stage 27.

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facing p. 404
Fig. G. Left normal stage 27 hind-limb section stained with iron haematoxylin.

Fig. H. High-power enlargement of the ANZ indicated by arrow in fig. G.

Fig. I. High-power view of section of ANZ stained for acid phosphatase by the method of Marit & Milaire (incubated for 2 h). Note presence of small sharply localized areas of high acid phosphatase activity, presumably lysosomes, in viable mesenchymal cells. Groups of phagocytosed dead cells are indicated by high acid phosphatase activity.

Fig. J. Left talpid³ stage 27 hind-limb section stained with iron haematoxylin to show ANZ absence.

Fig. K. High-power enlargement of area indicated in fig. J, showing absence of necrotic zone.

Fig. L. High-power view of section of the anterior part of talpid³ hind-limb stained for acid phosphatase, and corresponding with the area shown in fig. K. Note acid phosphatase activity, with the possible exception of one dead cell group, is found only in the lysozymes of viable mesenchymal cells.
Figs. M–O. Sections of normal forelimb stained with alcian blue/PAS. Fig. M, stage 26; Fig. N, stage 27–28; Fig. O, stage 29.
Figs. P–R. Sections of talpid³ forelimbs stained with alcian blue/PAS. Fig. P, stage 26; Fig. Q, stage 27–28; Fig. R, stage 29–30.
Figs. S–U. Sections of normal forelimbs stained for alkaline phosphatase. Fig. S, stage 25; Fig. T, stage 27; Fig. U, stage 29.
Figs. V–X. Sections of talpid forelimbs stained for alkaline phosphatase. Fig. V, stage 27; Fig. W, stage 28; Fig. X, stage 29–30.

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