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Cell death in the 'opaque patch' in the central mesenchyme of the developing chick limb: a cytological, cytochemical and electron microscopic analysis

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SUMMARY

Cell death in the 'opaque patch' of central mesenchyme of the developing chick forelimb was investigated by a variety of light and electron-microscope cytological and cytochemical techniques. Cell death appears first at stage 23/4 (4 days) and reaches its maximum extent at stages 24 and 25 (4½ and 5 days), at which it separates the ulnar and radial mesenchymal condensations. It then decreases in size to a small area separating the proximal parts of radius and ulna and disappears at stage 28.

Cytological studies show the presence of a few isolated dead cells, of mesenchymal cells containing 1–3 ingested dead cells and of macrophages containing up to 18 dead cells in various stages of digestion. 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.

Histochemical studies show that isolated dead cells and recently ingested dead cells contain no more acid phosphatase activity, either discrete or diffuse, than either neighbouring living mesenchymal cells, or mesenchymal cells which have ingested 1–3 dead cells.

Increased acid phosphatase activity is found within the macrophages, where activity is localized within the digestive vacuoles ('secondary lysosomes') containing the dead cells, and within the Golgi apparatus and Golgi vesicles ('primary lysosomes') of macrophage cytoplasm. Loss of staining capacity by the dead cell is correlated with high acid phosphatase activity: this is interpreted as indicating the digestion of dead cells within the macrophage by acid hydrolases.

There is circumstantial evidence that viable mesenchyme cells in the 'opaque patch' area autophagocytose part of their own cytoplasm in secondary lysosomes (1·2–2 μm).

The role of the 'opaque patch' in relation to the pattern of limb chondrogenesis is discussed. It is suggested that cell death may play a role in separation of radius and ulna, and that autophagocytosis may indicate a change in the pathway of differentiation of the mesenchyme cells lying between radius and ulna.

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INTRODUCTION

Cell death, whose role in morphogenesis has long been recognized (Glücksmann, 1951), is a prominent feature of the developing chick limb, although in general its role remains obscure. Dead cells are found in the ectoderm in the apical ectodermal ridge (AER) (Chang & Hwei-Yün, 1964; Jurand, 1965) in two regions of the superficial mesenchyme, the anterior and posterior necrotic zones (ANZ and PNZ), (Saunders, Gasseling & Saunders, 1962) and in the interdigital areas. Initially it was thought that the ANZ and PNZ might contribute to the shaping of the limb (Saunders *et al.* 1962) but it is now known that experimental blocking of death in the PNZ does not prevent the development of a normally shaped limb (Saunders, 1966). Saunders & Gasseling (1968) have shown that the PNZ area plays a role in determining the antero-posterior limb axis, but it is not clear whether the dead or dying cells make any contribution to this morphogenetic role. Interdigital areas of mesenchyme undergo regression and are thought to contribute to the shaping of the digits in the chick (Menkes & Deleanu, 1964; Saunders & Fallon, 1966) and in other amniote embryos (e.g. in the mouse (Chang, 1939; Milaire, 1967) and in man (Menkes, Deleanu & Ilies, 1965)).

In addition there is a region of cell death (called the 'opaque patch' on account of its opacity to transmitted light in the living embryo) in the central mesenchyme of the chick limb at 5 days of development, and which is the subject of the present investigation. Previously the 'opaque patch' has been described for the forelimb by Saunders *et al.* (1962); and for the hindlimb by Fell (Fell & Canti, 1934; Fell, 1964), who claimed that the opaque patch played no role in the knee joint formation and was able to assign to it no morphogenetic role. A homologous area of cell death is present in the rat and mouse. Milaire (1967) describes an area of cell death in the central mesenchyme of the mouse limb at 11 days, and at 12 days he states that an important area of degeneration lies between the presumptive tibia and fibula. Schwarz (1966) describes dead cells in the central axis of rat limb buds at 12 days of development.

Recently, the possible role of lysosomes in causing cell death has aroused particular interest (de Duve, 1959; Saunders & Fallon, 1966) and there has been controversy as to whether cells die through the intracellular release from lysosomes into the cell cytoplasm of acid hydrolases synthesized by the prospectively necrotic area or whether the increased acid hydrolase activity which is associated with cell death is concerned with the digestion of dead cells in macrophages. Ballard has carried out detailed investigations, using the electron microscope and thin Epon sections for cytochemical techniques, of the problem of lysosomal participation in cell death and phagocytosis in the interdigital areas of cell death in the rat embryo limb (Ballard, 1965; Ballard & Holt, 1968). However, in the case of the chick limb, little work has been done with recent techniques to investigate the process of cell death.

The work described in this paper was undertaken, using cytochemical and electron microscope techniques not available to Fell and Canti, to try to solve the problem of (1) the relationship between the 'opaque patch' and the developing mesenchymal and cartilage skeleton; (2) the possible morphogenetic significance of the opaque pattern particularly in relation to the pattern of limb chondrogenesis; (3) the part played by lysosomes, lysosomal enzymes and phagocytosis in the process of cell death in the 'opaque patch'.

MATERIALS AND METHODS

Normal chick embryos of $3\frac{1}{2}$–$6\frac{1}{2}$ days were classified as Hamburger & Hamilton (1951) stages, and the right forelimbs fixed, after which a variety of staining methods was used. Full details of solutions and preparation methods are given in Dawd (1969).

(1) *PAS (periodic acid–Schiff) and alcian blue*

The Trevan technique was used to stain 8 μm wax sections of Carnoy-fixed limb-buds. Acid mucopolysaccharides (mps) are specifically stained by 1% alcian blue at pH 2·5, the stain is fixed in borax-saturated 80% alcohol, and neutral mps are stained by the PAS method. Acid mps are stained green, neutral mps and glycogen pink.

(2) *Thin (1–2 μm) Epon sections, stained by Feulgen and toluidine blue*

The thin sections obtained with the Epon method give much greater resolution under the light microscope than wax sections. Limb-buds were fixed in 4% glutaraldehyde buffered in sodium cacodylate at pH 7·4 for 24 h and rinsed in cacodylate-buffered 2 M sucrose (pH 7·4) for up to 24 h. A cube of about 1 mm sides containing the opaque patch was dissected out and post-fixed in osmium tetroxide buffered with phosphate (pH 7·4) (Millonig, 1962) for 1 h. The specimen was then dehydrated via alcohol and propylene oxide and embedded in Epon (Luft, 1961).

Sections 1–2 μm thick were cut, serially as far as possible, using a glass knife, and dried on to glass slides. The sections were stained using the Feulgen method and hydrolysing for 20 min in N-HCl at 60 °C, and counter-stained in 0·2% toluidine blue in 0·05 M pH 7 phosphate buffer. Sections were air-dried, cleared in xylene and mounted in balsam.

(3) *Acid phosphatase staining*

(*a*) *Formol-calcium fixed thick frozen sections*

The Gomori (1941) lead method (described in Gomori, 1952) as modified by Barka & Anderson (1963) was used. Black lead sulphide indicated sites of enzyme activity. Limb-buds were fixed in cold (0–4 °C) formol-calcium (10%

formalin containing 1 % calcium chloride adjusted to pH 7) for 24 h, and frozen 20 μm sections cut and incubated (Trismaleate buffer pH 5·2, sodium β-glycerophosphate and lead nitrate – Barka & Anderson, 1963) for 1 h at 37 °C. Sections are washed, treated with 1 % ammonium sulphide, mounted in glycerine jelly and immediately photographed.

(*b*) *Glutaraldehyde-fixed thin Epon sections*

A clearer picture of acid-phosphatase distribution within the cell can be obtained by combining Epon embedding with the Gomori method and counter-staining with toluidine blue (Ballard, 1965). Limbs were fixed in 4 % glutaraldehyde, rinsed in sucrose as above (in section 2) and thick sections (40 μm) cut on the freezing microtome. The sections were incubated in Gomori incubating medium as above (Section 3*a*) for 1 h at 37 °C. The sections were washed, treated with 1 % ammonium sulphide, and the opaque patch which now became visible was dissected out and post-fixed in osmium tetroxide buffered with phosphate (pH 7·4) for 1 h. The opaque patch area was embedded in Epon as described in Section 2, and 1–2 μm sections cut and dried on to glass slides. The sections were counter-stained in 0·2 % toluidine blue (pH 7, phosphate buffer), washed and air-dried, cleared in xylene, mounted in balsam.

(*c*) *Control experiments on the specificity of the lead deposits*

Control experiments, either omitting substrate or using known inhibitors (sodium fluoride, citrate and D(+) tartrate), were carried out as in Ballard & Holt (1968). The complete absence of staining provides evidence that the lead deposits obtained by the modified Gomori acid-phosphatase technique used were due to the enzyme activity, and not to non-specific lead binding by the tissue.

Enzyme latency. Unfixed frozen sections, incubated in modified Gomori acid-phosphatase incubating medium, and treated in the same way as the fixed frozen sections, show very little or no acid-phosphatase activity in the dead cells of the opaque patch, and no activity in normal mesenchymal cells. This demonstration of enzyme latency, interpreted as representing the period necessary for the fixative to increase lysosomal membrane permeability and allow substrate to enter, supports the idea that the bodies staining for acid phosphatase are lysosomal (de Duve, 1963).

(4) *Electron microscopy*

Cubes of about 1 mm sides including the opaque patch were dissected out from right forelimb-buds, fixed for 2 h at 0 °C in 4 % glutaraldehyde, rinsed overnight in cacodylate-sucrose at pH 7·4, post-fixed for 1 h at 0 °C in Palade's osmium tetroxide at pH 7·5, rinsed in cacodylate-sucrose for 15 min and stained in 2 % uranyl acetate-sucrose for 2 h at 0 °C. The block was dehydrated via

alcohol and infiltrated with Araldite/Epon (1:1) mixture which was then hardened at 75 °C for 2 days.

For acid phosphatase, the Gomori lead method as modified for the electron microscope by Holt & Hicks (1962) was adopted, using glutaraldehyde as the fixative. The lead deposits are electron dense and show up clearly under the electron microscope. There are two drawbacks: incubation of the sections in the (acid pH 5) incubating medium damages cell ultrastructure, and the lead deposit obscures details in the area of deposition.

The first method used was to fix tissue for 24 h in glutaraldehyde, but this severely damaged the cells and in later experiments fixation time was cut as indicated in the following method, which is modified after Holt & Hicks (1962):

(1) Fix in 4 % glutaraldehyde buffered with cacodylate (pH 7·4) for 3 h at 0 °C.

(2) Rinse for 24 h at 0 °C in cacodylate-sucrose buffer (pH 7·4).

(3) Cut 40 μm sections on a freezing microtome and incubate in Gomori medium for acid phosphatase (pH 5·2 – Section 3*a*) for 1 h at 37 °C. The ammonium sulphide stage was omitted.

(4) Transfer sections to cacodylate-sucrose buffer. The area of the opaque patch ($\frac{1}{2}$–1 mm^3) is dissected out after identification within the section through the presence of small white dots.

(5) Post-fix in 1 % Palade's osmium tetroxide in cacodylate buffer (pH 7·4) for 1 h.

(6) Rinse in cacodylate-sucrose buffer for 15 min.

(7) Two per cent uranyl acetate-sucrose for 2–3 h.

(8) Dehydrate and embed in Araldite/Epon.

(9) Cut 90–150 nm sections with a glass knife.

RESULTS

(1) *The position and duration of the opaque patch*

Fig. 1 summarizes the location of the opaque patch in relation to the system of mesenchymal condensations. The first appearance of the opaque patch is at stage 23, when no mesenchymal condensation can be detected. There is an increase in intensity of acid-phosphatase staining of the central mesenchymal core, but dead cell groups and phagocytes cannot be found. There is also a well-marked area of cell death and phagocytosis in the prospective shoulder girdle region.

During stage 24 the opaque patch reaches its maximum extent and appears as a progressively more distal area of strongly acid phosphatase-positive dead cell groups, separated from that in the girdle region (Fig. 3A–C). At stage 25 the area of dead cell groups lies between the radius and ulna which form the two arms of the Y-shaped mesenchymal condensation (Fig. 3D).

The area occupied by dead cells now becomes smaller, separating the proximal

parts of radius and ulna at stage 26 (Fig. 3E), and shrinking in stages 27 and 28 to a small number of dead cell groups located in the triangular area bounded by the bases of radius and ulna and by the head of the humerus. The opaque patch has disappeared by stage 29.

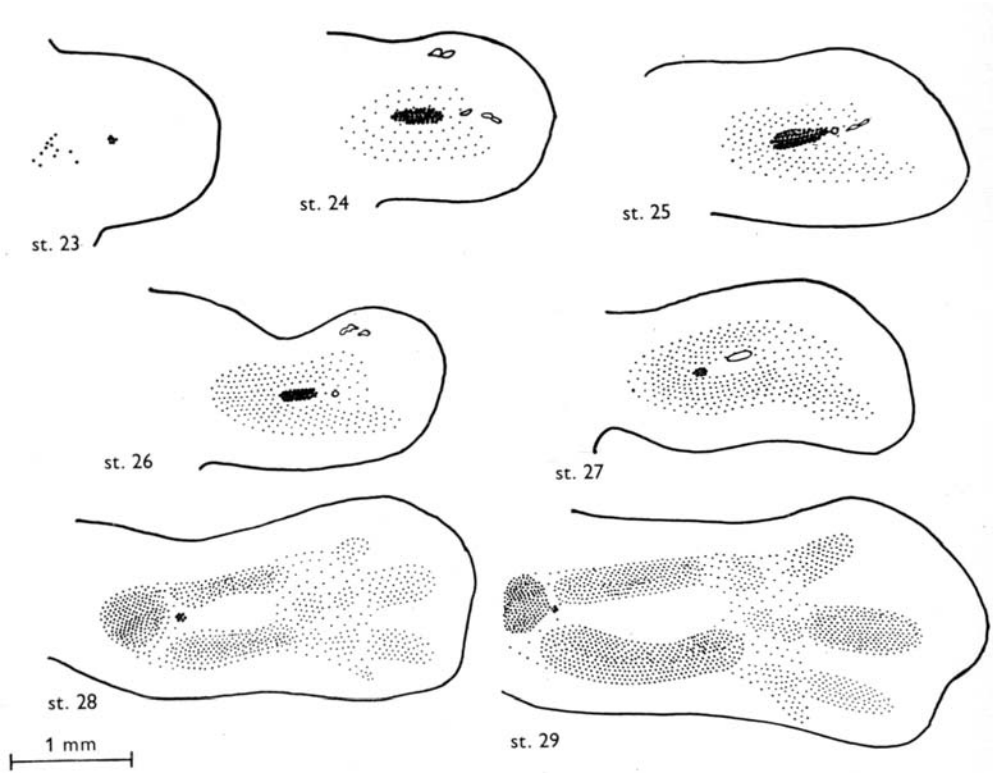


Fig. 1. Distribution of cell death (heavy stippling) in the opaque patch region of chick forelimbs in relation to the mesenchymal condensations (light stippling).

These results are in disagreement with the widely accepted accounts of cell death in the opaque patch in the forelimb by Saunders *et al.* 1962 and the hind-limb by Fell & Canti (1934). Saunders' drawings show (i) the opaque patch present at stages 24, 26 and 27 but absent from stage 25, and (ii) cell death extending into the ulna-humerus joint at stage 26, a condition which was not found in the limbs described here (Saunders *et al.* (1962), fig. 5, and Saunders, 1966, fig. 2).

Fell & Canti (1934) described the position of the hind-limb opaque patch, in relation to the supposed prospective fate of the surrounding mesenchyme cells. According to this interpretation the opaque patch is present in prospective femur tissue at stages 23 and 24, begins to diminish by stage 25, and at stage 26 is T-shaped, with the top of the T across the joint region between femur, tibia and fibula (Fig. 2). Fell and Canti's drawings place the hind-limb opaque patch in a

substantially more proximal position than was found in the forelimb in this analysis, in which the opaque patch was found between radius and ulna, but not in prospective humerus material.

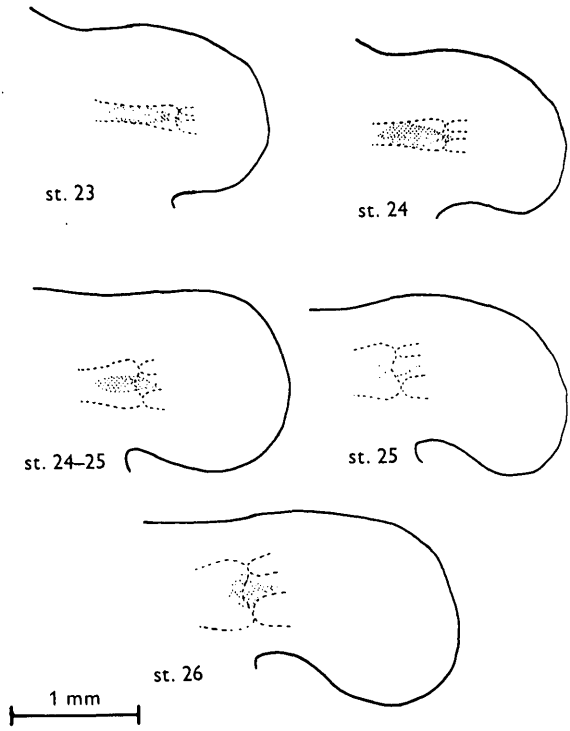


Fig. 2. Distribution of cell death (stippling) in the opaque patch of chick hind-limbs in relation to prospective femur, tibia and fibula as indicated by the development in culture of isolated limb fragments. Cell death occurs at stages 23–25 in the prospective femur region. Redrawn from Fell & Canti (1934).

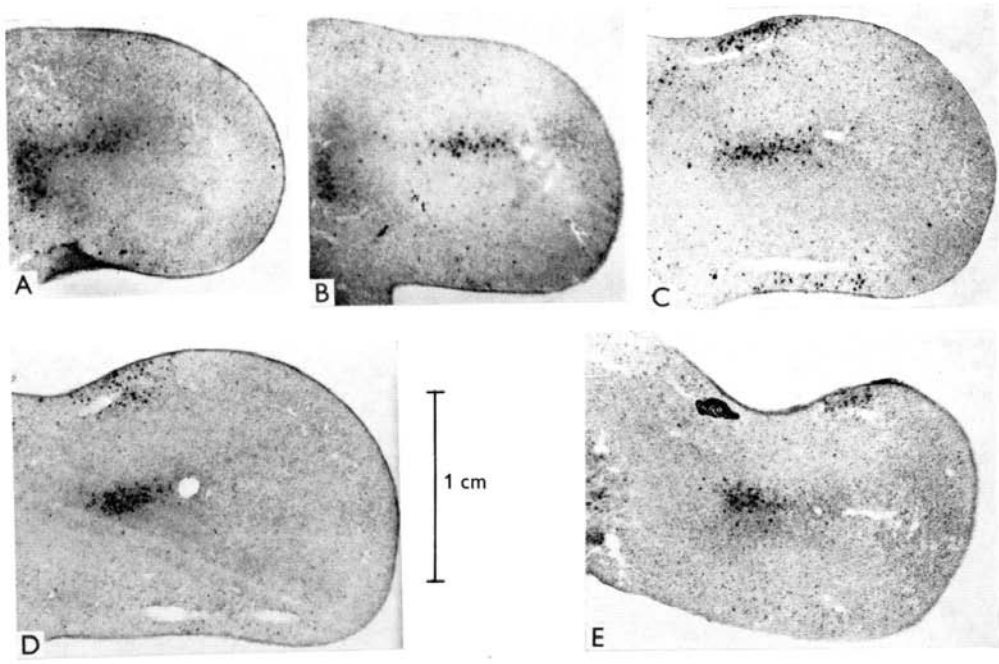
(2) *The cytology and cytochemistry of cell death in the opaque patch*

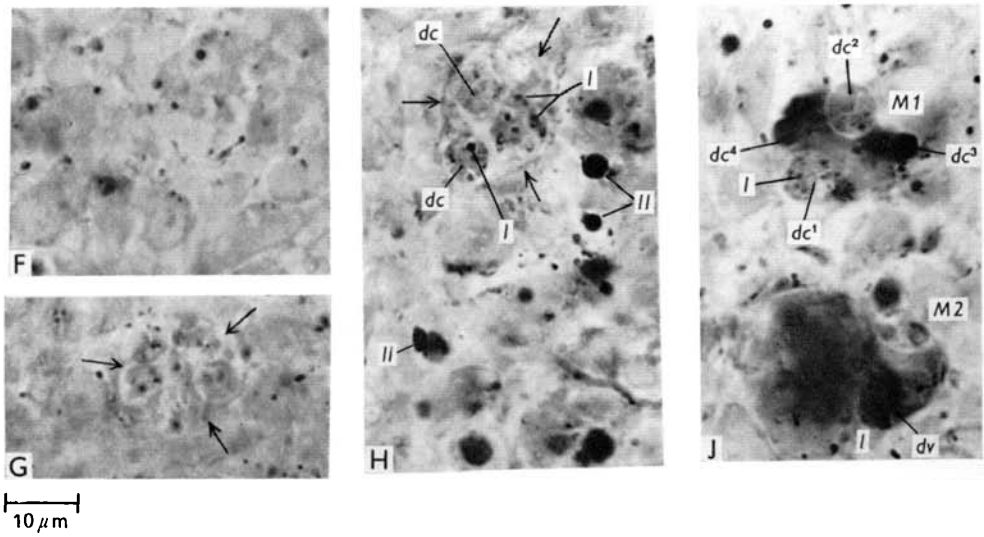
(*a*) *Viable mesenchymal cells*

Healthy mesenchyme cells are found both within and immediately adjacent to the opaque patch. The nuclei contain one or two prominent nucleoli and the rather sparse cytoplasm which forms processes making contact with those of neighbouring cells contain large numbers of free ribosomes, a small amount of rough endoplasmic reticulum, fairly few mitochondria and Golgi apparatus (Fig. 5F). Both light and electron microscope studies show that acid-phosphatase activity is confined to a few discrete areas (size 0·3–0·7 μm) thought to be lysosomes (Figs. 3F, 4A, 7A). The electron microscope also reveals acid-phosphatase activity in the Golgi apparatus and in closely associated Golgi vesicles or 'primary lysosomes' (Fig. 7C).

(*b*) *Isolated morbid and dead cells*

Morbid cells are found under the electron microscope, but they are rare (Fig. 5G). The marginal chromatin of the nucleus is denser than that of neighbouring cells. The cytoplasm is rounded up, and cytoplasmic vacuoles are present, including a dense body containing membranous material which may be





evidence of autophagy of morbid cell cytoplasm. Similar dense bodies thought to indicate autophagy were found in regressing chick mesonephric epithelium by Salzgeber & Weber (1966). Some mitochondria and both rough and smooth endoplasmic reticulum are swollen and apparently deteriorating.

Small numbers of individual dead cells lie free between viable mesenchymal cells. They can be divided into two categories. (1) Those showing chromatopycnosis (Fig. 5A), in which the rounded nucleus has a thick chromatic margin and in which most chromatic material is mainly located in two or three areas of the nucleus. Sometimes small amounts of rounded cytoplasm are recognizable. (2) Hyperchromatic dead cells (Fig. 5H) have deteriorated further: the shrunken nuclear part becomes very densely stained, and in general the cytoplasm is lost.

The electron microscope reveals details of cytoplasmic deterioration in dead cells. Where large amounts of cytoplasm are still present, small vacuoles are found and the mitochondria and endoplasmic reticulum are swollen. In some cases the aggregation of many cytoplasmic granules into parallel bands is a characteristic similar to the banded granules found by Bellairs (1961) in dead cells of the chick blastoderm. Banded granules are found in cytoplasm which has undergone considerable deterioration and in which cytoplasmic bodies are difficult to identify (Fig. 5H).

In some examples part of the cytoplasm of degenerating cells seems to be in process of being cut off from the rest of the cells into the intercellular spaces (Fig. 5H). Isolated pieces of cytoplasm can be seen which sooner or later are probably ingested by neighbouring mesenchymal cells. Isolated dead cells show no more total acid-phosphatase activity than neighbouring viable mesenchymal cells (Fig. 4E). Apparently intact lysosomes can be seen of the same size as those in viable mesenchymal cells. There is no evidence of dying cells synthesizing

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Fig. 3. Frozen sections, 15 μm, of chick forelimbs, stained for acid-phosphatase activity using the modified Gomori method. The macrophages in the 'opaque patch', the shoulder girdle region (in A and B) and the ANZ (in C–E) stain black.
(A-E) Low power. A, Early stage 24; B, middle stage 24; C, late stage 24; D, stage 25; E, stage 26.
(F–J) Oil immersion. F, Normal mesenchyme cells adjacent to opaque patch, with small (0·32–0·66 μm) stained cytoplasmic sites. G, Class I phagocyte showing no increased activity above the level of surrounding cells. H, Early class 2 phagocyte with small discrete areas of staining (*l*) associated with some of the dead cells (*dc*). Note large discretely stained areas (*ll*) away from phagocytes. J, Class 2 and 3 macrophages. Class 2 macrophage (*M1*) with acid phosphatase localized in discrete areas (*l*) in association with two large dead cells (dc^1, dc^2 which is fragmented), and with high activity apparently diffused through two other smaller dead cells (dc^3, dc^4). Class 3 macrophages (*M2*) show high activity throughout. In some parts more or less uniformly stained areas are identified as digestive vacuoles (*dv*), while in others small discrete areas of intense activity (*l*) are found in association with recently ingested dead cells.

additional acid phosphatase or of this enzyme leaking into the cytoplasm as was suggested by de Duve's 'suicide bag' hypothesis.

(*c*) *Ingestion of dead cells by mesenchymal cells*

Ingestion is inferred from two stages which are thought to be in succession. In the first, a dead cell is surrounded by arms of cytoplasm from one or two apparently viable mesenchymal cells (Figs. 5B, 7B), and in the second, a dead cell with no sign of digestion appears within the cytoplasm of a viable cell, otherwise indistinguishable from its neighbours (e.g. irregular oval nucleus – Fig. 5C). The morbid cell undergoing ingestion in Fig. 7B provides convincing evidence against de Duve's 'suicide bag' hypothesis that cell death results from intracellular release of lysosomal enzymes; the dead cell cytoplasm contains an intact lysosome-like body (size 0·4 μm) strongly positive for acid phosphatase.

(*d*) *Early phagocyte* (*stage 1*)

The phagocytes can be conveniently grouped into three successive stages, following the classification of Ballard & Holt (1968). At this early stage, mesenchymal cells are found containing ingested dead cells, but are otherwise similar in amount and type of cytoplasm and in the appearance of the nucleus to non-ingesting mesenchymal cells (Figs. 5C, 6A). The dead cell shows little sign of digestion and the nucleus and cytoplasm can frequently be distinguished. Acid-phosphatase distribution is the same as in normal mesenchyme cells, i.e. it is localized in the Golgi apparatus and in lysosome-like bodies near the Golgi apparatus but not in the ingested dead cell (Figs. 3G, 4B). The level of acid-phosphatase activity remains at the same low level as that of the surrounding viable mesenchymal cells.

(*e*) *Stage 2 phagocyte*

The number of ingested dead cells increases to 4–8 dead cells in one phagocyte (Figs. 4B, 5D). Some of these dead cells are recently ingested, while others show signs of digestion, such as presence of myelin figures, decrease in size, loss of staining capacity, and in some cases appearance of pits. Within some phagocytes there are some small dead cells strongly and uniformly stained. Digestion when it begins seems to be uniform through the whole dead cell. In some cases dead cells lie in distinct digestive vacuoles. The phagocytes mainly take on an oval shape, with a single common membrane and, swollen with ingested material, they are now much larger than the mesenchymal cells. The phagocyte nucleus can usually be identified, taking a convoluted shape between the dead cells. In certain cases phagocytes appear to have ingested other phagocytes and their contents.

Acid-phosphatase activity has increased above the background level of living mesenchyme cells. Stage 2 phagocytes contain several dead cells, most of which appear to be recently ingested since they are still fairly large (about 6 μm)

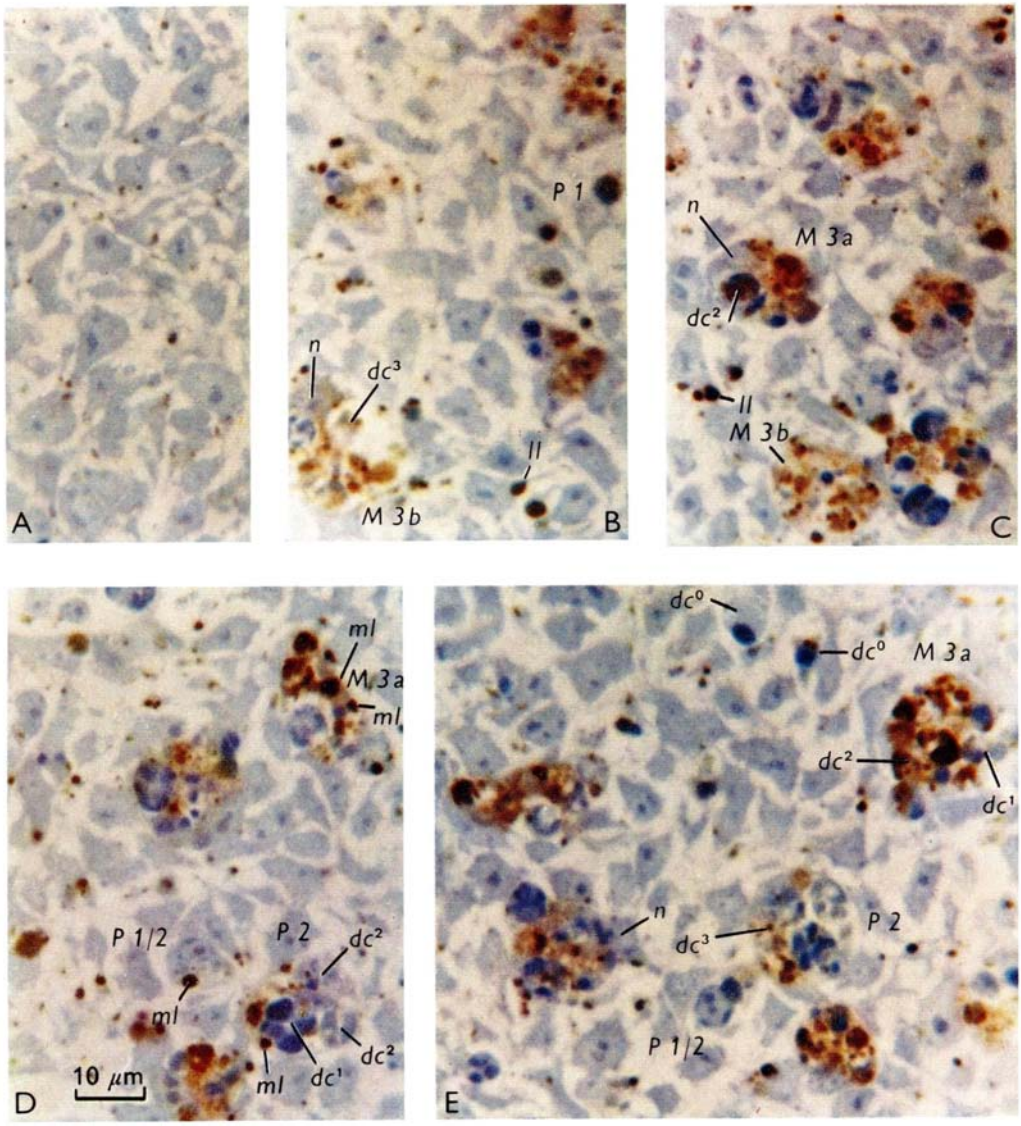
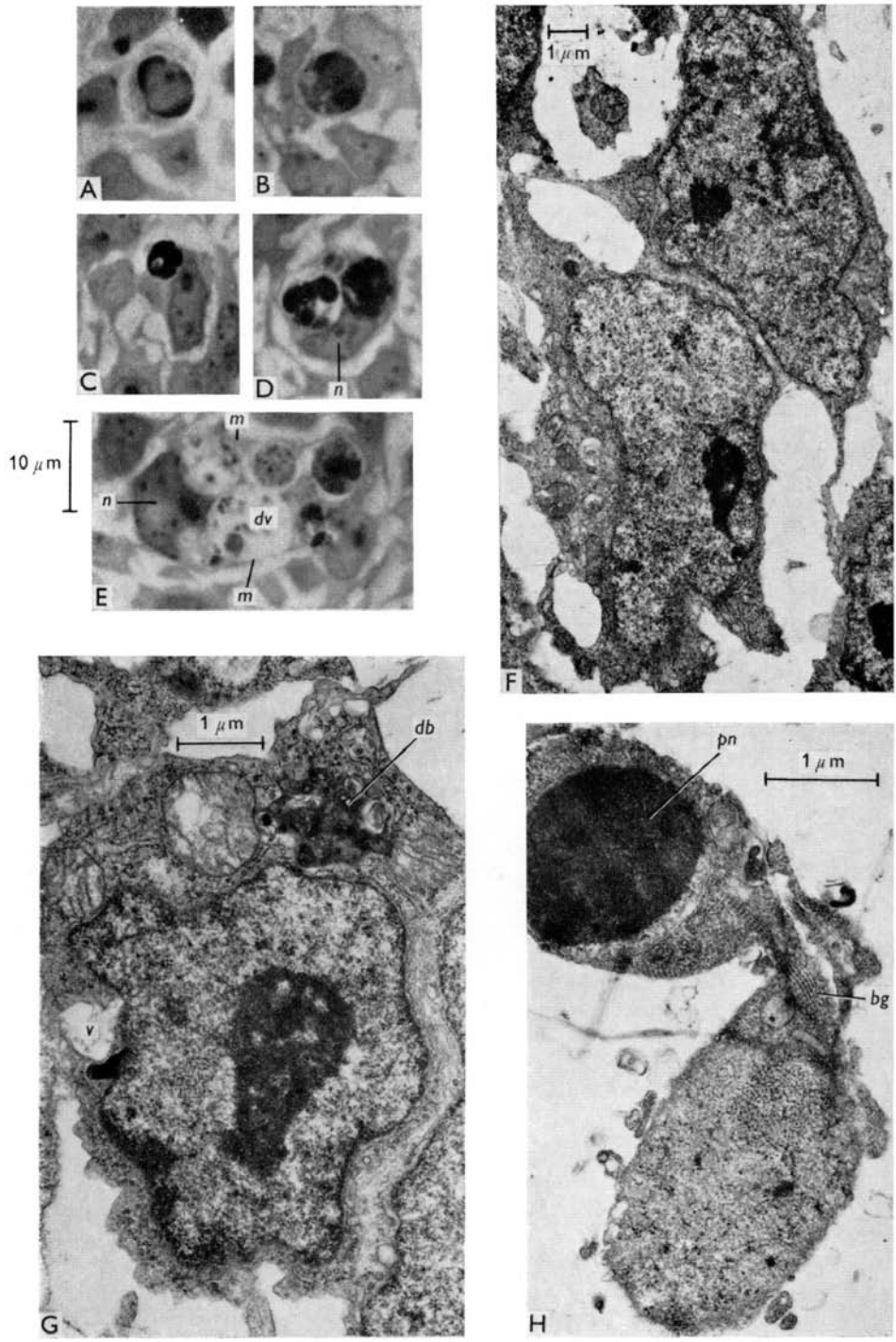


Fig. 4. Glutaraldehyde-fixed frozen 40 μm sections incubated in Gomori medium for acid phosphatase, post-fixed in osmium, Epon embedded, sectioned at 1–2 μm and stained in 1% toluidine blue. (A) Mesenchyme cells containing 0·32–0·66 μm lysosomes from near opaque patch. (B–E) Stage 25 opaque patch (D and E are sections at different levels through the same cells). *dc*, Dead cell: 0, isolated; 1, recently ingested; 2, partly digested; 3, fully digested; *ll*, large lysosomes (autophagic vacuoles?) in viable mesenchyme cells; *M*, macrophage; *3a*, early stage 3; *3b*, late stage 3; *ml*, lysosome in macrophage cytoplasm; *n*, macrophage nucleus; *P*, phagocyte; *1*, stage 1; *1/2*, stage 1/2; *2*, stage 2.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with fragmented nuclei and showing little sign of digestion and with little acid-phosphatase activity (Fig. 3H). Phagocyte cytoplasm adjacent to some of the dead cells contains large lysosomes (Fig. 4D). Other dead cells are at an advanced stage of digestion in digestive vacuoles of about 2·2 μm possessing strong acid-phosphatase activity (Figs. 3J, 4D, E).

(f) Mature macrophages (stage 3)

In the later stages large phagocytes (up to 25 μm in diameter – Figs. 5E, 7B) contain up to 15 dead cells whose granular appearance and loss of staining capacity indicates an advanced stage of digestion. Dead cells at late stages of digestion may still contain dense material or may be composed principally of myelin figures. They are often found in clearly defined digestive vacuoles. Such macrophages often contain one or two dead cells, showing little sign of digestion and apparently recently ingested. An outer membrane to the phagocyte can usually be distinguished, but there is often only a thin rim of phagocyte cytoplasm round the contained dead cells.

Some of the phagocytes appear to have free chromosomes within the cytoplasm indicating that they can undergo mitosis (Fig. 6B). This supports the interpretation that the dead cell groups are found within a single living cell and provides evidence against the idea, which has sometimes been put forward, e.g. by Saunders *et al.* (1962), that the dead cell groups are formed simply from the aggregation of several dead cells.

FIGURE 5

(A–E) Epon-embedded sections, 1–2 μm, of opaque patch of chick forelimb buds, stained with Feulgen and toluidine blue.

(A) Isolated dead cell showing chromatopycnosis.

(B) Isolated dead cell in process of being phagocytosed by neighbouring mesenchyme cells.

(C) A prospective phagocyte – a mesenchyme cell containing one dead cell.

(D) A small macrophage containing two dead cells and a digestive vacuole with cell fragments.

(E) Macrophage with well-defined digestive vacuoles containing well-digested contents and bounded by well-defined membrane (*m*). A, from stage 26; B, C, E, from stage 24; D, from stage 25/6. *dv*, digestive vacuole; *n*, macrophage nucleus.

(F–H) Stage 24 forelimb tissue fixed for 2 h in cacodylate buffered glutaraldehyde post-fixed in osmium tetroxide and embedded in Araldite/Epon mixture.

(F) Viable mesenchymal cell with normal mitochondria and sparse endoplasmic reticulum.

(G) Rounded up morbid cell with dense marginal chromatin in the nucleus, vacuoles (*v*) and a dense body (*db*) in the cytoplasm.

(H) Isolated dead cell apparently in process of shedding cytoplasm containing banded granules (*bg*), with a rounded pycnotic nucleus (*pn*) with concentrated chromatic material ('hyperchromatosis').

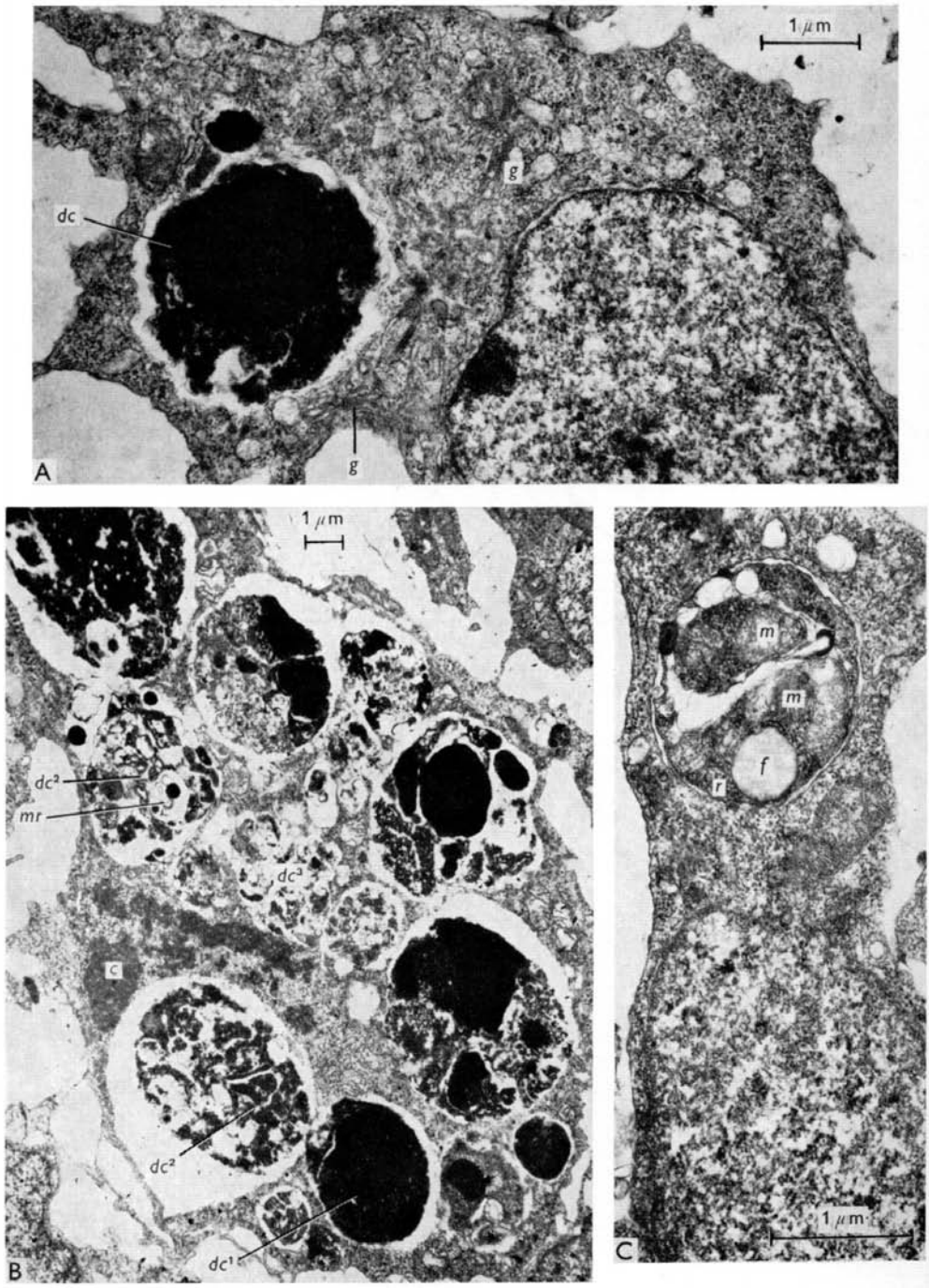

Fig. 6. (A) and (B) treated as Fig. 5 F–H. (C) Fixed in glutaraldehyde, freezing sections cut and incubated in Gomori medium for acid phosphatase, post-fixed in osmium tetroxide and embedded in Araldite/Epon. All from stage 25 forelimb. (A) Mesenchymal cell containing one dead cell (in which nucleus and cytoplasm can be distinguished – *dc*); *g*, extensive Golgi apparatus. (B) Mature macrophage containing 11–14 dead cells in well-marked digestive vacuoles. *dc*, Dead cells; dc^1, recently ingested; dc^2, partly ingested; dc^3, fully digested. *mr*, Myelin remnants of digestion; *c*, free chromosomes. (C) Normal mesenchyme cell with vacuole (autophagic?) containing swollen mitochondria (*m*), ribosomes (*r*) and fat droplets (*f*).

Stage 3 macrophages possess high acid-phosphatase activity (Fig. 3J). Some dead cells at an early stage of digestion show only low acid-phosphatase activity, but are associated with a few small discrete areas of intense activity, thought to be lysosomes. Other dead cells are associated with high acid-phosphatase activity in digestive vacuoles of about 4·5 μm. In these dead cells loss of chromatic material indicates digestion is under way (Fig. 4C, E). In the final stages of digestion only a few small remnants of digested dead cells are found with a high diffuse acid-phosphatase activity within clearly defined digestive vacuoles (Fig. 4B, C).

The electron microscope shows that late phagocytes possess intense acid-phosphatase activity within some of the digestive vacuoles (Fig. 7D). The activity tends to be discrete, associated with particular bodies and not distributed evenly through the contents of the digestive vacuole. These discrete areas of activity correspond with the particulate distribution of activity within vacuoles of stage 3 phagocytes observed under the light microscope (e.g. Fig. 4C, E). Acid-phosphatase activity does not seem to be present within the digestive vacuoles either in the form of lysosomes or primary lysosomes (Golgi vesicles). In the phagocyte cytoplasm outside the digestive vacuole, phosphatase activity is found in the lumen of the extensive Golgi system and in the primary lysosomes which are often located near the digestive vacuole (Fig. 7D). These findings are in accordance with the theory that lysosomal enzymes are transported from the Golgi apparatus in the form of primary lysosomes which fuse with pinocytic vacuoles containing material foreign to the cells, thus forming secondary lysosomes or digestive vacuoles (Cohn & Fedorko, 1969). In addition, large lysosomes are found in the macrophage cytoplasm (Fig. 7D): these may represent secondary lysosomes which according to Cohn and Fedorko may also fuse with pinocytic vesicles. These large lysosomes may correspond with intensely stained bodies found in macrophage cytoplasm under the light microscope (Fig. 4C, D). In very late phagocyte stages, in which digestive vacuole contents consist mainly of myelin figures, activity is dispersed widely throughout the vacuole contents, though some areas may be totally devoid of activity. Myelin figures are an indication of the last stages of digestion of dead cells by macrophages (Ballard, 1965). These vacuoles probably correspond with those which appear to show diffuse activity (Fig. 4C, E) in late stage 3 phagocytes under the light microscope.

(*g*) *Change of cell type with time*

At stage 23/4 isolated dead cells are fairly common, while those mesenchymal cells which contain dead cells have ingested only small numbers (1–4) of dead cells, mostly at an early stage of digestion. At stages 24–26 there are few isolated dead cells, and all stages of phagocytes are found, including both those with dead cells at middle and at later stages of digestion. Phagocytes in which dead cells are almost completely digested predominate at stage 26.

(h) Evidence for autophagic vacuoles in viable mesenchymal cells

Under the light microscope, some apparently viable mesenchymal cells contain lysosomes which are both more numerous and larger (i.e. 1·2–2·0 μm) than those from mesenchymal cells adjacent to the necrotic zone (Fig. 4B, C). Owing to the lead deposits the nature of the contents of such lysosomes is not clear. Similar 'large lysosomes' are found in the opaque patch but not associated with phagocytes, using the Gomori thick-section technique (Fig. 3H). Bodies corresponding to these 'large lysosomes' were sought under the electron microscope. It was thought that these bodies might be either autophagic vacuoles, or lysosome-like dense bodies.

In a small number of apparently normal mesenchymal cells the electron microscope revealed vacuoles which might represent autophagic vacuoles containing swollen mitochondria, ribosomes and probably endoplasmic reticulum and fat droplets but no nuclear material. These vacuoles (Fig. 6C) are interpreted as being autophagic on the grounds that the cytoplasm which is shed from dead cells and which might be phagocytosed by normal mesenchyme cells has deteriorated further and is more granular and frequently contains 'banded granules' (compare with Fig. 5H). The size of the 'autophagic vacuole' in Fig. 6C is 1·6 μm, which compared with a size range of 1·2–2·0 μm obtained for the large lysosomes seen under the light microscope following acid-phosphatase staining. Further work, such as the examination of alternate sections by light and electron microscope, is necessary before it can be shown that the large lysosomes found under the light microscope represent the autophagic vacuoles revealed by the electron microscope.

DISCUSSION

(1) *Anatomy of the opaque patch*

The work reported here shows that the opaque patch reaches its maximum development separating prospective radius and ulna at stages 24–26, after which it disappears. This account of the distribution of cell death differs from that of Fell & Canti (1934), who claim a more proximal position for the opaque patch and interpret the results of their experimental study of the developmental potential of cultured isolated fragments of limb mesoderm as showing that in early stages (stages 23–24/5) the opaque patch corresponds with prospective femur tissue. In more detail, their results show that if the cut is made proximal to the opaque patch at stage 24 or before, then in the developing isolated mesodermal fragment the opaque patch corresponds to prospective femur tissue, but that if the cut is made later, at stage 26, the opaque patch corresponds to the area between femur, tibia and fibula. The interpretation of these results was based on the assumption, then current (e.g. Murray & Huxley, 1925), that limb development was of a mosaic type. More modern work – for example, that of

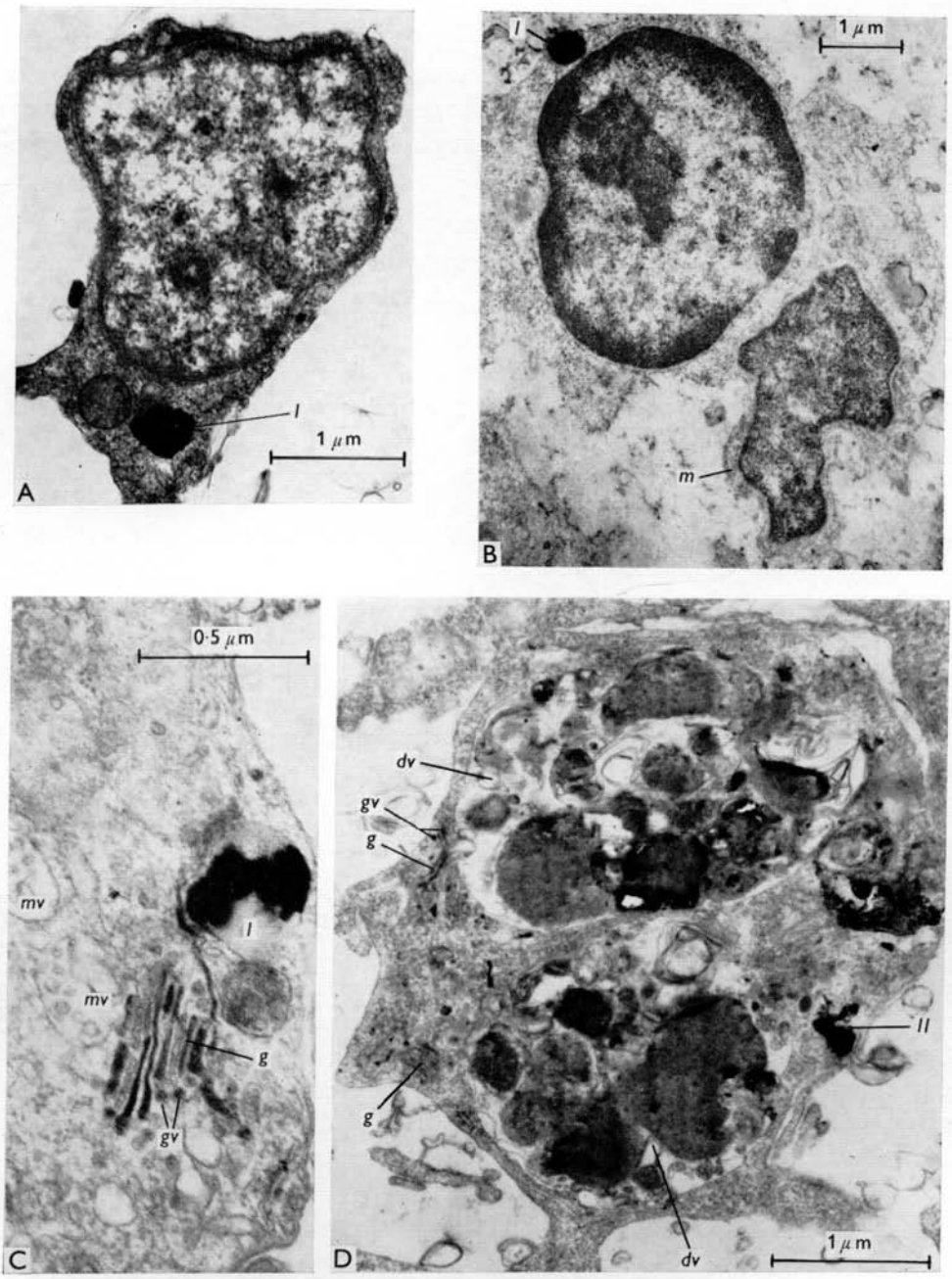

Fig. 7. Stage 25 forelimb tissue fixed for 4 h (B for 24 h) in glutaraldehyde, frozen sections cut and incubated in Gomori medium for acid phosphatase, post-fixed in osmium tetroxide, stained in uranyl acetate and embedded in Araldite/Epon. (A) Mesenchyme cell with lysosome (*l*). (B) Isolated dead cell with 0·4 μm intact lysosome (*l*) apparently in process of ingestion by the adjacent mesenchyme cell (*m*). (C) Detail of mesenchyme cell showing close association of Golgi apparatus (*g*), Golgi vesicles (primary lysosomes, *gv*), multivesicular bodies (*mv*) and lysosome (*l*). (D) Late class 3 macrophage from stage 25 opaque patch. Note two well-defined digestive vacuoles (secondary lysosomes, *dv*) showing high but heterogeneously distributed acid phosphatase activity. Macrophage cytoplasm contains large lysosomes (*ll*), Golgi apparatus (*g*) and vesicles (*gv*), all positive for acid phosphatase.

Wolff (1958), Amprino (1965) and Zwilling (1968) – emphasizes that, particularly in the early stages, regulation can occur in limb development. In the light of this, it is possible that regulation may have occurred in Fell and Canti's isolated mesodermal fragments, particularly in those taken at an early stage, and their conclusion that the opaque patch corresponds with prospective femur tissue can be no longer justified.

(2) *Changes in dying cells*

A characteristic pattern of ultrastructural changes takes place in the isolated dead and dying limb mesenchyme cells. The finding that these changes take place in dying cells *before* ingestion by a phagocyte indicates that phagocytes are not the cause of death. This is in agreement with the interpretation by Ballard (1965) and Manasek (1969) of their studies on cell death in the rat embryo limb and in the embryo chick heart, but by contrast Saunders (1966) was unable to find morbid cells prior to ingestion in the chick limb PNZ.

The question of lysosomal participation in cell death is of interest. Evidence from material stained for acid-phosphatase activity indicates that dying cells contain no more lysosomes than neighbouring viable cells, that the lysosomes they contain are of normal size, and that there is no sign of the lysosomes releasing acid phosphatase (and presumably other enzymes) into the cytoplasm. Thus there is no evidence in this case of lysosomal participation in causing cell death, a conclusion which contradicts the 'suicide bag' theory formerly suggested by de Duve (1959) that dying cells synthesize additional lysosomal enzymes which destroy the cell through release of hydrolytic enzymes into the cytoplasm.

(3) *Phagocytosis and the transformation of mesenchyme cells into macrophages*

Convincing evidence is provided by these results that the ingestion of an isolated dead cell by a neighbouring mesenchymal cell sets off a pathway of differentiation leading to the formation of a macrophage engaged in the digestion of many dead cells. Direct evidence of the presence of macrophages in the degenerating zone is provided by the thin Epon sections and the electron-microscope studies. In previous light-microscope studies of regression of areas of the chick limb mesenchyme (e.g. Saunders *et al.* 1962; Hinchliffe & Ede, 1967; Fell & Canti, 1934) the presence of macrophages was either not known or was only inferred.

There is also conclusive evidence that the digestion of the dead cells is brought about within the phagocyte. The isolated dead cells show no sign of digestion and possess acid-phosphatase activity at about the same level as the ingesting mesenchyme cell. Stage 1 phagocytic mesenchyme cells have low acid-phosphatase activity and this correlates with the relatively undigested state of a few dead cells which they contain. Stage 3 phagocytes are interpreted as being the final stage in the sequence of macrophage differentiation as the dead cells they

contain show well marked signs of digestion correlated with greatly increased hydrolase activity.

The increased hydrolase activity within the stage 3 phagocytes is thought to originate within the macrophage cytoplasm, where Golgi, primary lysosomes and lysosomes all show acid-phosphatase activity, rather than within the ingested cell. The finding that within early stage 3 phagocytes some dead cells shows signs of digestion and intense hydrolase activity while others appear to have been ingested recently and show low activity clearly indicates that the increase in acid-phosphatase activity takes place within the phagocyte after the death and ingestion of the mesenchyme cells.

A similar interpretation of the digestion of dead cells by macrophages which have differentiated from mesenchyme cells has been put forward by a number of students of embryonic cell death, e.g. by Ballard (1965) and Chang (1939) working on the rat and mouse limbs respectively, by Weber (1969) on amphibian tail regression in metamorphosis and by Scheib (1964) on Müllerian duct regression. Kelley (1970) claims that degenerating interdigital cells in human embryo limbs undergo autophagic breakdown and ingestion by mature macrophages, but his illustrations are consistent with the theory of ingestion of dead cells by neighbouring mesenchyme cells which become transformed into macrophages. Maximow (1932) states generally that where mesenchymal cells remain relatively undifferentiated in embryonic tissues, fixed mesenchymal cells may differentiate into macrophages. Saunders (1966) and Saunders & Fallon (1966) state that dead cells of the PNZ undergo digestion in macrophages, but they provide no evidence to show whether macrophages differentiate *in situ* from mesenchymal cells or whether macrophages migrate into the tissue. In all of the above cases which have been investigated the increased acid-hydrolase activity characteristic of involuting tissue is found to be localized within the macrophages rather than within the dead cells before ingestion. Ericcson (1969) states a general rule that involuting mesenchyme tissue is characterized by the transformation of some mesenchyme cells into macrophages which digest the dead cells, while in contrast involuting epithelial cells undergo autophagic breakdown of their cytoplasm.

(4) *Role of opaque patch*

The earliest appearance of skeletal pattern in the limb is at stage 24, when the cells along the central axis start to form mesenchymal condensations and simultaneously to synthesize acid mps at a greater rate than the surrounding non-chondrogenic areas. The interest of the opaque patch in relation to this is in (i) the role that cell death may play in the distal separation of the two arms (radius and ulna) of the 'Y', and (ii) the indication provided by autolysis of a changed pathway of differentiation of the cells lying between radius and ulna. Cell death is likely to result in alteration in adhesive properties which would split a central condensation distally into two. Experiments with dissociated

mixtures of cartilage-forming and heart-forming cells (or liver-forming cells) indicate that the cartilage-forming cells have a greater affinity for each other than do heart or liver cells for their own kind (Steinberg, 1964). On the other hand, as Angelici & Pourtois (1968) claim in relation to cells involved in secondary palate fusion, dying cells are likely to lose their tendency to stick together. This is indicated also by their 'rounding up'. This contrast between the extremely 'self-adhesive' chondrogenic cells of radius and ulna and the poor adhesive properties of the cells of the opaque patch is likely to result in the division distally of the axial condensation into two separate centres of chondrogenesis. Such primary condensation centres may, as Holtfreter (1968) suggests, rapidly acquire inducing properties, assimilating peripheral cells to form the cartilage rudiment.

The mesenchymal condensation appears simultaneously with an identical pattern of increased synthesis of the acid mps chondroitin sulphate, which is a component of the intracellular material of cartilage. Searls (1965) and Thorogood (1971) mapped the pattern of radioactive sulphate incorporation into chondroitin sulphate. Uptake takes place throughout the whole central area at stages 22 and 23, but by stage 24 a Y-shaped pattern of increased uptake emerges in which the area between radius and ulna incorporates less radioactive sulphate than the radius and ulna themselves. This suppression of acid-mps synthesis in the inter radius–ulna region is synchronous with the period of maximum cell death in the inter radius–ulna region, which in itself will account for the suppression. The death of some mesenchyme cells and the autophagic processes in others may represent extreme and mild expressions of the same phenomenon of cellular deterioration. Viable cells with signs of autophagocytosis of parts of their own cytoplasm may be involved in dedifferentiation, i.e. in a change in their pathway of differentiation, through digestion of cytoplasm programmed for production of cartilage intercellular material. Ericcson (1969) states that autophagic activity is common in cell remodelling during differentiation. Schwarz (1966) interprets in a similar way the processes of degeneration in the central blastema of the rat embryo limb-bud, where he finds primary lysosomes and cytolysomes, though he fails to acknowledge the macrophages clearly seen in his photographs. Schwarz claims that after 'autodigestion of the old template and ribosomes a new phase of cytodifferentiation to precartilage with new template and ribosomes begins'. It seems more likely that these processes, occurring in the centre of the blastema in a similar position to the chick opaque patch, are concerned in the differentiation of the non-cartilaginous cells separating radius and ulna.

The phenomenon by which a specific organ product is first synthesized throughout a wide embryonic region, and then as regionalization continues, either disappears or becomes undetectable except in the rudiment of which it is characteristic, is known in other organ systems (e.g. chick heart development, Ebert, 1953). In the limb, too, there is evidence that chondroitin sulphate

synthesis takes place in myogenic areas throughout stages 22–24 (Searls, 1965; Thorogood, 1971), showing that initially acid mps are synthesized by a much greater area of limb mesenchyme than that involved in formation of the cartilage rudiments.

Additional evidence linking cell death in the opaque patch with pattern formation in the precartilaginous skeleton is provided by the *talpid*^{3} mutant of the chick embryo. In *talpid*^{3} homozygotes there is fusion of radius and ulna in a single cartilaginous block and poor differentiation of the humerus–radius–ulna joint (Ede & Kelly, 1964; Hinchliffe & Ede, 1967). Cell death is completely or partially suppressed in the 'opaque patch' area of the limb mesenchyme of *talpid*^{3} embryos (J. R. H., personal observation). This is circumstantial evidence supporting the view that the opaque patch is involved in the determination of the 'Y' pattern of limb chondrogenesis. However, caution must be observed in interpreting the evidence from *talpid*^{3}: the simultaneous occurrence of the two events does not necessarily imply a causal relationship. Further, Ede & Agerbak (1968) interpret *in vitro* work as indicating that *talpid*^{3} limb mesenchyme cells are abnormally adhesive, thus inhibiting the cell movement involved in condensation and resulting in fusion of adjacent cartilaginous elements. However, many time-related factors are responsible for a particular morphogenetic pattern and absence of cell death and increased mesenchyme cell adhesiveness may both contribute to the fusion of radius and ulna in *talpid*^{3} embryos.

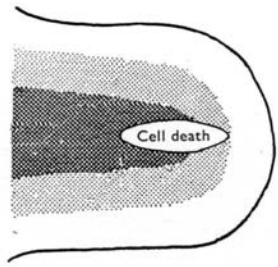

Fig. 8. A model limb formed by interaction of the four 'conditions' (see text). Stippling indicates areas of condensation and of high acid mps synthesis.

(5) *A model for the formation of the early limb skeleton*

From the information reported here, a model modified from Waddington (1962, pp. 123–5) can be constructed which formally explains (Fig. 8) through the interaction of four 'conditions' the formation of the Y-shaped pattern of humerus, radius and ulna. The conditions are as follows:

(1) The cells along the central axis of the limb cylinder tend to condense, and to increase their synthesis of acid mps. This 'rule' is followed by limb-mesenchyme aggregates (Ede, 1971) and by cultured pieces of limb mesenchyme whether from prospective chondrogenic or myogenic areas (Zwilling, 1966).

(2) When the concentration of the condensing cells increases beyond a certain limit they die or undergo partial autolysis.

(3) There is a proximo-distal decrease in the threshold for such deterioration.

(4) Areas of primary condensation act as inducing centres recruiting peripheral cells and thus forming the cartilage rudiment.

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