SUMMARY

Physiological cell death and degeneration in the interdigital mesenchyme of the hind foot of the rat foetus have been studied using classical staining methods and staining methods for enzyme localization. Individual mesenchymal cells die and shrink as the result of some unknown mechanism. Their acid phosphatase and esterase activities are not significantly different from those of viable loose mesenchymal cells. The dead cells are engulfed by viable neighbouring cells which resemble other loose mesenchymal cells in their morphology and in their acid phosphatase and esterase activities. These phagocytes then differentiate and become typical macrophages. Expressions of this process are the altered appearance of their nuclei and the increase in cytoplasm and in acid phosphatase and esterase activities. Many dead cells may be engulfed by a single macrophage and are then digested by its acid hydrolases. No evidence was found suggesting that cell death might be initiated by the intracellular release of lysosomal enzymes.

INTRODUCTION

Cell death and degeneration are extremely common phenomena during embryonic and foetal development in vertebrates (Glucksmann, 1951; Saunders, 1966) and in insects (Lockshin & Williams, 1965). They occur in many parts of the body including developing limbs of the chick (Saunders, Gasseling & Saunders, 1962; Menkes, Deleanu & Ilies, 1965), the mouse (Chang, 1939; Forsthoefel, 1959; Milaire, 1963; Menkes et al. 1965), the duck, rat and man (Menkes et al. 1965), and are now described in detail in the hind foot of the rat foetus.

Because the cells die in a regular pattern in normal animals without the need for experimental treatment, they provide a good opportunity for studying the factors controlling and mediating cell death and the changes involved. Little is yet known about these important developmental events, the disturbance of which can lead to abnormal development (Zwilling, 1964; Hinchliffe & Ede, 1967) and may have relevance to the origin of foetal tumours.

Advances in biochemical cytology have led to the suggestion that the intracellular release of lysosomal enzymes might initiate cell death and autodigestion in adult (de Duve, 1959, 1963; de Duve & Beaufay, 1959) and embryonic tissues (de Duve, 1959, 1961; Brachet, Decroly-Briers & Hoyez, 1958; Scheib-Pfleger & Wattiaux, 1962;
Scheib, 1963; Zwilling, 1964). De Duve & Wattiaux (1966) state that autophagy and autolysis are implicated in regression phenomena during development, possibly reaching their 'ultimate suicidal conclusion', but also recognize that other mechanisms of cell removal may be involved.

Evidence partly supporting the autodigestion hypothesis has come from electron-microscope studies showing the presence of large lysosomes in regressing tissues such as mouse apical ectodermal ridge (Jurand, 1965), chick Mullerian duct (Scheib, 1965a, b) and chick mesonephros (Salzgeber & Weber, 1966). However there is no evidence that the lysosomes kill the cells; neither has any direct evidence been provided for the release of enzymes from these bodies as envisaged in the original hypothesis of de Duve (1959). A different pattern of cell digestion has been observed by many authors who have suggested that macrophages are the main agents of digestion during cell and tissue regression in developing animals (Metchnikoff, 1883; Gräper, 1914; Chang, 1939; Glucksmann, 1951; Weber, 1964; Saunders, 1966; Saunders & Fallon, 1967).

The present study was undertaken to try to answer the following questions. (1) Do cells destined to die have a higher content of acid hydrolases than viable neighbouring cells? (2) Is there any evidence to support or contradict the idea that cell death might be initiated by the intracellular release of lysosomal enzymes? (3) Are dead cells in the course of degeneration associated with greater amounts of acid hydrolases than are found in neighbouring viable cells? (4) What is the origin of such large amounts of acid hydrolases—do the enzymes originate within the dead cells or within other cells? (5) Do the dead cells complete their own digestion or are they engulfed and then digested by macrophages? (6) What is the origin of such macrophages?

MATERIALS AND METHODS

Animals

Albino rats of the Wistar strain fed ad lib. on Rowett Research Institute Diet no. 86 were bred at random from the Courtauld Institute closed colony. Male rats were placed with virgin or first-litter females on specified nights and removed by 9 a.m. the following morning, which was counted as the first day of pregnancy and day 1 of development. No attempt was made to record the time of mating and to date the foetuses in hours, for litter-mates vary in their stages of development as pointed out by Nicholas (1949) and confirmed in the present study.

Pregnant females were killed on the required day by a blow on the head followed by dislocation of the neck.

For vital staining, freshly dissected whole foetuses were transferred rapidly to a 0.1% solution of Nile blue sulphate in Ringer at 37 °C for 1 h and rinsed in plain Ringer (after Saunders, personal communication).

Fixation, embedding, sectioning and staining

Foetuses were again rapidly dissected from their membranes and dropped entire into cold fixative, except when Altmann's fluid and glutaraldehyde fixatives were used. On these occasions, only the hind limbs were fixed. For details of the fixatives and sub-
Cell death in foetal rat foot

sequent procedures, see Table 1. The paraffin-embedded, Carnoy-fixed limbs were serially sectioned at 5–10 μ. Limbs fixed by the other methods and infiltrated with cacodylate-buffered hydroxyethylcellulose/sucrose (HEC), (Holt, Ballard, Katchburian & Barrow, in preparation) were cut at 10 μ in a cryostat and stained while free floating or attached to gelatinized coverslips. Epon-embedded tissues were serially sectioned at 1–2 μ with a glass knife, the sections floated out on drops of water on slides, allowed to dry and stained by the Feulgen method, employing a previously determined optimal hydrolysis time of 20 min in 1 N HCl at 60 °C. Sections were counterstained with 0.2% toluidine blue in 5 mM phosphate buffer, pH 6.9.

Table 1. Fixation, embedding and staining methods

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Conditions</th>
<th>Rinse</th>
<th>Embedding/sectioning</th>
<th>Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnoy</td>
<td>24 h room temp.</td>
<td>—</td>
<td>Paraffin</td>
<td>Feulgen; methyl green/pyronin (Kurnick, as in Pearse, 1960)</td>
</tr>
<tr>
<td>Altmann</td>
<td>24 h room temp.</td>
<td>HEC*</td>
<td>Cryostat</td>
<td>Heidenhain iron haematoxylin</td>
</tr>
<tr>
<td>Formal calcium</td>
<td>18 h ice-cold</td>
<td>HEC</td>
<td>Cryostat</td>
<td>Oil red O (after Chiffelle &amp; Putt, 1951); Baker's acid haematein (Baker, 1946); periodic acid/Schiff; esterase; acid phosphatase</td>
</tr>
<tr>
<td>3% glutaraldehyde</td>
<td>4 h ice-cold</td>
<td>HEC</td>
<td>Cryostat</td>
<td>Esterase; acid phosphatase</td>
</tr>
<tr>
<td>in 0.067M cacodylate buffer+9 mM CaCl₂ (after Sabatini et al. 1963)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same,</td>
<td>4 h ice-cold</td>
<td>0.25 M sucrose in cacodylate buffer</td>
<td>Epon (Luft, 1961)</td>
<td>Feulgen+ toluidine blue</td>
</tr>
<tr>
<td>followed by osmium tetroxide</td>
<td>1 h ice-cold</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(Millonig, 1962)</td>
<td></td>
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</table>

* Hydroxyethylcellulose medium (see above).

Enzyme survival

The survival of enzyme activity (Table 2) in foetal tissues fixed for 20 h in formalin solutions and infiltrated 24 h in HEC was assessed using liver from 20-day rat foetuses as test material. This was chosen to obtain sufficient reasonably homogeneous tissue for enzyme assays. Fixed and unfixed livers were homogenized in ice-cold distilled water in a Potter–Elvehjem homogenizer with a tapered Teflon pestle. Homogenates were frozen and thawed once and the acid phosphatase activity measured by a modification of the method of Gutman & Gutman (Varley, 1962) using β-nitrophenyl phosphate as substrate. Esterase activity was measured using a pH-stat to record acetic acid liberated by the enzymic hydrolysis of 10⁻³ M indoxyl acetate at pH 7.4.
Enzyme stains

Acid phosphatase activity was demonstrated by the method of Gomori (1952) and by using a medium containing $2.2 \times 10^{-4}$ M naphthol AS-BI phosphate and $2 \times 10^{-3}$ M hexazotized pararosaniline in $0.1$ M acetate buffer, pH 5.3 (after Barka & Anderson, 1962). Esterase activity was demonstrated by the indoxyl method (Holt, 1958), using

<table>
<thead>
<tr>
<th>Tissue treatment before homogenization</th>
<th>Acid phosphatase</th>
<th>Esterase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg nitrophenol liberated per g tissue per 60 min</td>
<td>% initial activity present (average)</td>
</tr>
<tr>
<td>None</td>
<td>9.0/9.8</td>
<td>100</td>
</tr>
<tr>
<td>Kept at 4°C for 44 h</td>
<td>10.2/10.8</td>
<td>108</td>
</tr>
<tr>
<td>Formol calcium 20 h, then HEC 24 h</td>
<td>2.0/2.0</td>
<td>21</td>
</tr>
<tr>
<td>Formol calcium in cacodylate buffer 20 h, then HEC 24 h</td>
<td>2.0/1.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2. Acid phosphatase and esterase assays of 20-day foetal rat-liver homogenates

Table 3. Influence of inhibitors on acid phosphatase staining

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Molarity Naphthol AS-BI medium</th>
<th>Naphthol AS-BI medium</th>
<th>Ferric chloride</th>
<th>Cupric acetate</th>
<th>Sodium d(+)-tartrate</th>
<th>Sodium citrate</th>
<th>Sodium arsenate</th>
<th>Sodium molybdate</th>
<th>Sodium parachloromercuribenzoate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.002</td>
<td>Precipitate</td>
<td>+ + + + +</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>Precipitate</td>
<td></td>
<td></td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>Precipitate</td>
<td></td>
<td></td>
<td>+ + + +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>Precipitate</td>
<td></td>
<td></td>
<td>+ +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>Precipitate</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>Precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>Precipitate</td>
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<td></td>
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</tbody>
</table>

Key: +, slight inhibition; + +, moderate inhibition; + + +, high inhibition; + + + +, very high inhibition; + + + + +, complete inhibition.

half the usual concentration of ferro-ferricyanide redox buffer and also by incubating sections in a medium containing $6.6 \times 10^{-5}$ M naphthol AS acetate and $10^{-3}$ M hexazotized pararosaniline in $0.067$ M phosphate buffer, pH 6.8 (after Holt & Hicks, 1962). In all cases freshly prepared staining media were used and incubation times did not exceed 1 h. Some sections stained by the Gomori acid phosphatase and indoxyl esterase methods were counterstained with acid haemalum and picric acid (Holt, 1958).
Acid phosphatase inhibitors were used to study any differences between the enzymes demonstrated by the two staining methods and between the enzymes in normal and degenerating tissue. The inhibitors (Table 3) were added to the substrate media immediately before adding the tissue sections. The effect of some of the inhibitors could not properly be assessed since they caused precipitation of lead salts, and it is known that a lowered lead concentration affects the staining properties of the medium (Holt, 1959). In other experiments, sections of unfixed tissue were incubated in acid phosphatase media in an attempt to compare the degree of enzyme latency (Bitensky, 1963) in normal and degenerating tissue.

After using the Gomori procedure, counts were made of the number of stained sites of acid phosphatase activity in about 100 viable loose mesenchymal cells close to the degenerating interdigital zones. The number of stained sites of similar size (0.5–1 μ) was counted in dead cells in the degenerating zones.

**RESULTS**

Cells which have condensed, darkly staining pyknotic nuclei and rounded up cytoplasm, but which are not erythrocytes, are regarded as dead on the basis of Glucksmann’s (1951) detailed descriptions and analysis of cell death. The presence and identity of the pyknotic nuclei was confirmed by their positive Feulgen reaction.

**Distribution of dead cells**

These observations apply to peripheral regions of the hind foot.

On day 15 of development the hind foot is at the stage shown in Fig. 1a. There is a well-developed apical ectodermal ridge which contains dead cells throughout its length, but none are found in the underlying mesenchyme. The marginal blood sinus is well developed.

On day 16 (Fig. 1b) the apical ectodermal ridge is reduced in height and contains dead cells throughout its length. Dead cells are also present in the interdigital mesenchyme, mainly between the marginal sinus and the ectoderm. Some dead cells occur in the mesenchyme over the tips of the digits, immediately below the regressing apical ectodermal ridge.

On day 17 (Fig. 1c) the apical ectodermal ridge is further reduced in height and can barely be distinguished from normal ectoderm. It contains few dead cells. Vital staining clearly shows that cell death in the interdigital mesenchyme is at a maximum and that it occurs in a wedge-shaped zone approximately 300–400 μ wide and deep. Dead cells are absent from the tips of the digits, which have by now occluded the marginal sinus. For some studies the hind feet of litter-mates were divided into three groups according to the stage of foot development as judged by the external contour (Fig. 2).

On day 18 (Fig. 1d) the digits are no longer webbed. Sometimes traces of the interdigital degenerating zones can be seen as small patches of dead cells.
Observations on 5–10-μ sections stained by classical methods

These observations were all made on degenerating interdigital mesenchyme taken from hind feet on day 17 unless specified otherwise.

The tissue is formed by loosely packed mesenchymal cells. Many of them are

Fig. 1. Diagram representing the distribution of dead cells (stippled areas) at the periphery of the foetal rat hind foot: (a) day 15; (b) day 16; (c) day 17; (d) day 18. (ect, ectoderm; ms, marginal sinus.)

Fig. 2. Diagram representing the contours of the foetal rat hind foot on day 17, showing three stages of development: (a) stage 1; (b) stage 2; (c) stage 3.
obviously dead, but a considerable proportion show no degenerative changes and look exactly like loose mesenchymal cells in parts of the foot where no cell death occurs.

Compared with neighbouring viable-looking cells, the dead cells are shrunken and easily seen in sections extracted with pyridine and stained with Baker's acid haematein (Fig. 3). They vary from 2 to 5 μ in diameter, those larger than 4 μ usually being smooth and nearly circular in profile. These are occasionally found in isolation but usually occur in small groups or clusters which sometimes seem to be bound by a common membrane. However, the cryostat sections could not be cut thin enough to establish this with certainty. Smaller dead cells 2-4 μ in diameter usually have an irregular profile and are most often found among large clusters. The cytoplasm of dead cells is possibly represented by a pale uneven rim seen around pyknotic nuclei (Figs. 3, 4), some of which are fragmented (Figs. 8, 12).

In sections stained with Baker's acid haematein without pyridine extraction, and counterstained with neutral red (not illustrated), the pyknotic nuclei of large dead cells stain dark red and are partly enveloped by black-stained material not seen in neighbouring viable-looking cells nor in dead cells in pyridine-extracted control sections. Small dead cells stain only faintly with neutral red and show little black stain. Black-stained granules measuring 0.5–2.5 μ in diameter are often seen between dead cells in large clusters and are most numerous in those clusters consisting mainly of the small dead cells that stain little with neutral red. Similar granules are also seen in sections stained with Heidenhain's iron haematoxylin (Fig. 4).

The degenerating zone contains a large number of fat droplets which stain with oil red O. They measure up to 2 μ in diameter and many lie close to dead cells.

In sections stained with methyl green and pyronin the viable loose mesenchymal cells have light green nuclei, dark green chromatin and pink cytoplasm. The dead mesenchymal cells are readily distinguished by their uniformly blue-green pyknotic nuclei and red condensed cytoplasm. They lie in clusters which contain a few small pyknotic nuclei that stain with pyronin but not with methyl green. The clusters sometimes appear to contain a light-green viable-looking nucleus and a continuous red-stained cytoplasm seems to surround the dead cells. However, the tissue could not be sectioned thinly enough and the quality of preservation was not good enough to confirm these last two observations.

The use of the periodic acid/Schiff stain showed that the material surrounding clustered dead cells stains with uniformly moderate intensity. In sections counterstained with haemalum, large dead cells stain dark blue and small ones dark blue with a red border, or red only.

Acid hydrolase survival in fixed tissues

Measurements of hydrolase survival in foetal rat liver preserved by formaldehyde fixatives showed that the enzymes suffer a high degree of inactivation (Table 2). Visual assessment of the amount of stain produced by incubating sections in acid phosphatase media using standardized conditions, showed that tissues fixed in glutaraldehyde for 4 h are considerably more active than those fixed in formaldehyde for
20 h. With respect to esterase activity, the glutaraldehyde-fixed tissues seemed to be only slightly more active than formaldehyde-fixed ones.

Observations on 10-μ sections stained to show enzyme activity

Cryostat sections of fixed 17-day hind foot stained by the Gomori method for acid phosphatase show a striking result (Fig. 5). The degenerating interdigital regions are stained much more heavily than are other regions. Similar staining patterns were obtained by using the naphthol AS-BI method for acid phosphatase and by both esterase methods. No staining was seen in control sections incubated in Gomori medium lacking substrate, in complete Gomori or naphthol AS-BI acid phosphatase media containing 0·01 m sodium fluoride or in esterase media containing 4% formaldehyde. Exposure of sections to the vapour of 40% formaldehyde at 50 °C for 10 min before incubation also prevented all staining. However, substrate-free media containing pararosanilin imparts a pale red-brown colour to the dead cells but this was easily distinguished from the bright red stain produced by enzyme activity in the presence of substrate.

Attempts to distinguish between acid phosphatases demonstrated by the two staining methods were unsuccessful (Table 3). Four inhibitors reacted with the substrate media and their effects could therefore not be properly assessed. Furthermore, sodium citrate and sodium tartrate were found to reduce the precipitation of lead phosphate in the Gomori medium when soluble phosphate was added. In those cases where true enzyme inhibition is believed to have been demonstrated—that is, in both media containing cupric acetate and in naphthol AS-BI medium containing citrate, tartrate or arsenate—the degree of inhibition was the same in normal and degenerating tissue.

Evidence for enzyme latency in sites of acid phosphatase activity was provided by the fact that sections of unfixed tissue incubated in Gomori medium show no staining in viable cells and very little or virtually none in degenerating tissue. Unfixed sections incubated in naphthol AS-BI acid phosphatase medium again show no staining in viable cells but degenerating tissue is stained with about half the usual intensity.

In sections of fixed tissue stained by the Gomori method, regions free from cell death show discrete sites of acid phosphatase activity about 1 μ in diameter (Fig. 6). They are present in all cell types except erythrocytes. Viable loose mesenchymal cells possess about seven sites. As far as can be judged, the same or very similar sites are stained by the naphthol AS-BI method and by both esterase methods. The naphthol AS-BI method reveals diffuse cytoplasmic acid phosphatase activity in addition to the discrete sites already mentioned. Granules with esterase activity are present in erythrocytes.

In degenerating interdigital tissue, sites of activity 0·5–1 μ in diameter are stained by all four methods, but when both acid phosphatase methods and the naphthol AS esterase methods are used, much of the stain is in larger sites 1·5–3 μ in diameter. These sites frequently lie close together forming dense patches surrounded by diffuse stain (Figs. 9, 11–13). The amount of diffuse stain is least in sections stained by the Gomori method. In sections stained by the indoxyl esterase method, high activity is
Cell death in foetal rat foot

represented by groups of closely packed 0.5–1 μ sites surrounded by diffuse, lightly stained areas (Fig. 10).

The intense hydrolase activity in degenerating tissue is closely associated with the dead cell clusters, in particular with the large clusters. This was clearly demonstrated in sections stained by the Gomori and indoxyl esterase methods and counterstained with haemalum and picric acid in both cases. Detailed observations were made on preparations in which the enzyme stain was not partially obscured by counterstain and in which dead cells could easily be recognized by their size and shape. It became evident that dead cell clusters can be divided into three broad classes on the basis of their hydrolase activity demonstrated by any of the four staining methods.

Class 1 (Fig. 7). These clusters possess the same staining characteristics as neighbouring, viable-looking mesenchymal cells, mainly localized in sites measuring up to 1 μ in diameter. There are about 6 such sites of acid phosphatase activity situated very close to the surface of each dead cell. The dead cells are 4–5 μ in diameter, having a dense nucleus and smooth circular profile.

Class 2 (Figs. 8–10, 12). These clusters possess a relatively high level of enzyme activity which varies from one dead cell to another. Dead cells with high activity are 4 μ or less in diameter and have an irregular profile. Those with low activity are larger and have a smooth circular profile; each has about 6 sites of acid phosphatase activity, 0.5–1 μ in diameter, close to its surface. Much of the enzyme activity is localized outside the dead cells, particularly when the naphthol AS substrates are used.

Class 3 (Figs. 9, 11, 13). These clusters possess high activity localized in sites up to 3 μ in diameter and associated with all or nearly all the dead cells, which are mainly 2–3 μ in diameter, less dense than those in class 1 clusters, and often irregular in profile. A few large dead cells with smooth profiles and low activity may also be present. Some 3-μ sites of enzyme activity appear to be intensely stained, small, dead cells. Much of the activity is localized outside the dead cells, particularly when the naphthol AS substrates are used. The clusters sometimes measure as much as 25 μ in diameter.

All classes may be found together in the interdigital tissue of one foot but usually two classes predominate. When limbs were divided into three groups according to their developmental stage (Fig. 2), it was found that limbs at the first two stages contain clusters mainly of classes 1 and 2, whereas limbs at the third stage contain some clusters of class 2 but most are of class 3.

The possibility that these results were influenced by variations in technique causing different degrees of enzyme inhibition is discounted because clusters of both high and low activity were often found close together in the same section. In addition, the examination of serial sections showed that these differences in activity are not related to the level at which the clusters are sectioned.

Isolated dead cells measuring 5 μ in diameter and showing no evidence of being digested possess about seven discrete 0.5–1 μ sites of acid phosphatase activity. The diffuse cytoplasmic stain produced by incubation in naphthol AS-BI phosphate medium is more intense in these cells than in viable cells.

Information concerning the possible involvement of macrophages in the degenera-
tive process could not be obtained from the material described above. Even in sections 5 \( \mu \) thick there is too much superimposition of structures to obtain the degree of resolution necessary to demonstrate whether one cell is inside another. For this purpose 1–2 \( \mu \) sections of the Epon-embedded tissue were used.

**Observations on Epon sections**

Although the Feulgen method cannot generally be used on glutaraldehyde-fixed tissue, because many cell components become Schiff-positive without hydrolysis, this effect was not met with foetal tissues in the present case. In serial sections cut 1–2 \( \mu \) thick and stained by the Feulgen method and with toluidine blue, dead cells are readily distinguished by their dense, Feulgen-positive pyknotic nuclei (Fig. 14) and rounded-up cytoplasm (Fig. 15). They lie scattered between viable-looking cells, most of which possess large nuclei with a thin marginal rim of chromatin, one or two small nucleoli, a pale granular ground substance and relatively sparse cytoplasm forming several processes extending into the intercellular matrix (Figs. 15, 16). Dead cells are not often found lying free and display no further signs of degeneration apart from the general condensation just described. In some sections, mitotic figures were seen in non-phagocytic cells close to dead cells (Fig. 16).

Most dead cells lie within the cytoplasm of another cell. Such phagocytic cells containing one large dead cell in a section (Fig. 16) are usually very similar in appearance to neighbouring, viable-looking mesenchymal cells; that is, their nuclei are not very darkly stained, have a thin marginal rim of chromatin, small nucleoli and the cytoplasm is sparse. The engulfed dead cells are completely rounded up and measure 4–5 \( \mu \) in diameter, often showing no other signs of degeneration. They are more condensed than those lying free.

Phagocytic cells containing three to four dead cells in one section (Fig. 17) differ from neighbouring, viable-looking mesenchymal cells in that their nuclei have a very dense marginal rim of chromatin, a large amount of scattered, densely staining chromatin and relatively large nucleoli. The cytoplasm is increased in volume.

Large phagocytes are often found containing numerous dead cells, most of which measure 2–4 \( \mu \) in diameter. The macrophage nuclei may adopt a contorted shape and regularly stain more darkly than the nuclei of most neighbouring, viable-looking mesenchymal cells (Fig. 18). The cytoplasm is relatively abundant and rounded up, but short cytoplasmic processes can be seen. The cytoplasm of the macrophages contains many structures measuring about 0.5–1 \( \mu \) in diameter which stain with toluidine blue.

An important finding was that macrophages containing dead cells were often seen in mitosis (Figs. 19, 20), thus providing evidence of their viability.

The engulfed dead cells are usually closely surrounded by macrophage cytoplasm and sometimes by a light halo which might be a shrinkage artefact. In a few cases they lie within a definite vacuole (Fig. 18). The cytoplasm of the engulfed dead cells is seldom recognizable and they present a range of appearances (Figs. 14, 17, 18). In sections their size ranges from 1.5 to 5 \( \mu \) and they vary in their response to toluidine blue and Feulgen stain. Many have pitted surfaces suggestive of enzymic digestion.
DISCUSSION

The validity of the staining methods for demonstrating enzyme activity was established by the use of enzyme inhibitors before and during incubation and by the omission of substrate from the Gomori medium. The absence of staining in these experiments is proof that the staining observed in routinely incubated sections is wholly related to enzyme activity. The fact that sites of acid phosphatase activity measuring 0.5-1 μ in diameter also appear to possess esterase activity, and exhibit enzyme latency in sections of unfixed tissue incubated in naphthol acid phosphatase medium, is taken as evidence that these sites are lysosomes.

The high degree of inactivation suffered by the enzymes of foetal rat liver during fixation and subsequent processing makes it likely that enzymes in the developing foot are inactivated to a similar extent by formaldehyde. Glutaraldehyde-fixed tissue was used in all detailed studies and stained more intensely, suggesting that the enzymes survive this fixation somewhat better. Nevertheless, the enzyme-staining results present a consistent pattern and can meaningfully be discussed in conjunction with the cytological observations made here.

Although the difficulty in deducing the correct sequence of events from a series of still photographs is well known, there is little doubt that the loose mesenchymal cells with pyknotic nuclei and rounded up cytoplasm are dead or dying and that they are engulfed by phagocytes. The experiments of Littau, Allfrey, Frenster & Mirsky (1964), showing that dense chromatin is inactive in RNA synthesis, suggest that pyknotic nuclei are wholly inactive and probably dead.

No evidence was found which could throw any light on the mechanisms controlling cell death. It has been suggested that localized death in embryos and foetuses might be due to the deficiency of vital factors (Saunders et al. 1962; Zwilling, 1959), or the presence of inhibitory substances (Zwilling, 1959) but the nature of these hypothetical factors is unknown; in the case of the tadpole tail and chick Mullerian duct, regression is known to be controlled by hormones (Dodd & Matty, 1964; Wolff, 1953). Limb development is believed to depend upon a series of sequential interactions between ectoderm and mesoderm (Zwilling, 1961), although the evidence for this hypothesis has been criticized as being inconclusive (Amprino, 1965). Milaire (1962, 1963, 1965) has suggested, as an extension of the hypothesis, that interdigital cell death is related to a localized lack of an apical ectodermal ridge maintenance factor in the mesoderm between the digits. However, this is not consistent with the present findings in the rat, for dead cells occur all around the limb periphery, including the tips of the digits, both in the ectoderm and to a small extent in the mesoderm.

The mesenchymal cells of the interdigital zones must have differing sensitivities to whatever 'message' induces death, for many cells are apparently unaffected and some even enter mitosis. The nature of the target sites in the affected cells also remains a subject for speculation. In the rat foot, no evidence was found to support the original hypothesis of de Duve (1959) that cell death might be mediated by the intracellular release of enzymes from lysosomes, which could perhaps be target organelles. Isolated dead cells showing no evidence of digestion and presumed to be not yet engulfed, or
to be recently engulfed, seem to have the same acid phosphatase and esterase activities as neighbouring, viable-looking mesenchymal cells, and the staining patterns are equally discrete. However, it is not yet certain whether this discrete hydrolase activity is within the dead cells, although serial thin sections studied by electron microscopy could elucidate this.

The relatively high intensity of the diffuse cytoplasmic stain present in the isolated dead cells in sections incubated in naphthol AS-BI acid phosphatase medium may not be interpreted as representing released lysosomal enzyme, for it could well be accounted for by concentration of diffuse cytoplasmic activity during the shrinkage that dying cells undergo. The results also exclude the possibility that the cells differentiate toward death by synthesizing increased amounts of acid hydrolases (Fell, 1964), although an example of such differentiation is now believed to occur in larval muscles of the silkmoth (Lockshin & Williams, 1965).

The range of size and shape displayed by the dead cells is interpreted as reflecting stages in the degenerative process. The observation that dead cells within clusters are generally smaller and more irregular in shape than those occurring in isolation indicates that degeneration takes place mainly in the clusters. Further evidence for this is found in sections stained with methyl green and pyronin, in which many dead cells in clusters stain faintly or not at all with methyl green, indicating loss of DNA. The variety of appearances shown by dead cells in clusters stained with periodic acid/Schiff reagents and haemalum are also suggestive of degenerative changes. The presence of many fat droplets close to the dead cells is not unusual, for fatty change is known to be a common expression of many types of cell injury (Robbins, 1967).

The nature of the dark granules seen between dead cells in large clusters stained with Heidenhain's iron haematoxylin or Baker's acid haematein is not known, but they may represent products of degeneration. Evidence for this is most clear in the preparations stained with acid haematein, in which black-stained material coats the large dead cells but is absent from small ones, suggesting that loss of this material from the cells as they shrink could perhaps account for the occurrence of the granules. It is also possible that the granules may represent lysosomes, as those in rat kidney are known to stain in this way (Holt, 1959). Some granules might be mitochondria but none were stained in neighbouring viable mesenchymal cells.

The possibility that dead cells in the interdigital regions are engulfed by phagocytes, as has been reported in the mouse by Chang (1939) and in several species by Menkes et al. (1965), was strongly suggested by the semblance of a common membrane around some clusters and the presence of a normal-looking nucleus and continuous cytoplasm in others when stained with methyl green and pyronin. The enzyme-staining results provide further strong evidence in support of phagocytic digestion of the dead cells.

When dead cell clusters were divided into three classes according to their hydrolase activity, this was found to be related in a regular way to the size and appearance of the dead cells. This indicates that the differences in enzyme activity are not random and that the three classes of dead cell clusters can be interpreted as stages in an ordered sequence. The clusters with least activity (class 1) must be the first in the series for the
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large dead cells they contain show the least signs of degeneration. They are found most frequently in limbs at stage 1 on day 17. In limbs at stages 2 and 3 on the same day these clusters are less numerous and occur mainly at the lateral periphery of each interdigital zone where the cells die relatively later. The clusters with most hydrolase activity must be the last in the series for they contain dead cells showing the most pronounced signs of degeneration, and occur most frequently in the more advanced limbs at stage 3.

The observation that class 2 clusters consist of a mixed population of dead cells, some with intense hydrolase activity and others with low activity, provides strong evidence that the differences in activity are real and not due to varying degrees of enzyme inhibition within a section. The dead cells with low activity show little evidence of digestion and are presumably the most recent arrivals in the clusters. The development of high enzyme activity is therefore almost certainly a post-mortem event. The possibility that it arises through the activation of pro-enzyme molecules within the dead cells seems unlikely, for much of the activity is localized outside the dead cells. This suggests that the enzymes originate within the cytoplasm of phagocytic cells.

The results obtained by incubating unfixed sections in naphthol AS-BI acid phosphatase medium demonstrated that the enzymes in degenerating tissue are more accessible to substrate than are those in normal tissue, showing that lysosomal membranes in degenerating tissue are relatively more permeable or fragile. This property has previously been correlated with cell damage and digestion (Bitensky, 1963) and is consistent with the idea that digestive vacuoles are present, as these are expected to be more fragile than other types of lysosome (de Duve, 1964). On the other hand, the almost total lack of staining in the sections incubated in Gomori medium throws some doubt on the usefulness of this medium for demonstrating lysosomal fragility in unfixed sections (compare Bitensky, 1963).

All these observations support the hypothesis that dead cells, possessing about the same acid phosphatase and esterase activity as neighbouring viable-looking cells, are engulfed by cells with similarly low hydrolase activity. These phagocytes then differentiate, synthesize more acid hydrolases and digest the dead cells.

Direct evidence for the presence of macrophages in the degenerating interdigital zone was obtained from the thin Epon serial sections cut through this region. When stained by the Feulgen method and toluidine blue, dead cells were clearly seen to lie within the cytoplasm of other cells. The changes seen in the nuclei of these phagocytes, and the increase in their cytoplasm-to-nucleus ratio, can be correlated with the number and size of the engulfed cells, and are interpreted as stages in their differentiation into macrophages. The observed changes are also consistent with the hypothesis that the dead cells are initially engulfed by neighbouring mesenchymal cells which then become metabolically more active and synthesize acid hydrolases during their subsequent stages of differentiation. The fact that the macrophages, particularly those containing dead cells at late stages of digestion, were often found in mitosis, is taken as conclusive evidence that they are living cells.

Stages in the degeneration of dead cells are seen very clearly in the Epon sections but relate mainly to the nuclei, as the cytoplasm can rarely be distinguished. The loss
of staining affinity, first from the periphery of some dead cells, and the incidence of pits at the surface, are evidence of digestive processes proceeding inwards. The loss of Feulgen staining in dead cells before their affinity for toluidine blue disappears suggests that digestion of DNA may be more rapid than that of RNA-containing structures, but treatment with RNase before applying the basic dye would be necessary to establish this sequence. The small basophilic structures measuring up to 1 μ are believed to represent the last fragments of dead cells before digestion is completed.

The subsequent fate of the macrophages after they have completed digestion of dead mesenchymal cells has not yet been established but it appears that occasionally they too may die and be engulfed.

The conclusions concerning the mechanism of removal of dead cells from the interdigital zone in the developing rat foot are substantially the same as those reported by Weber (1964) and Salzmann & Weber (1963) in the regressing tadpole tail, and by Saunders (1966) and Saunders & Fallon (1967) in the chick wing bud. In all cases, tissue regression is the result of cell death and the differentiation of macrophages which digest the dead cells. Weber (1965) has provided more evidence in support of this theory by showing that both cathepsin synthesis and regression in the tadpole tail are suppressed by actinomycin D, a finding consistent with the idea that regression is dependent upon protein synthesis, i.e. acid hydrolase synthesis by macrophages. Similar conclusions have been reached by Tata (1966), who found that actinomycin D, puromycin and cycloheximide all suppressed regression in isolated tadpole tails grown in organ culture.

It appears that in some cases, tissue regression may result from de-differentiation and shrinkage of cells, and not depend entirely on cell death followed by macrophage activity. In chick Mullerian ducts and mesonephros undergoing regression (Scheib, 1965a, b; Salzgeber & Weber, 1966) epithelial cells contain conspicuous autophagic vacuoles that are sites of autodigestion, and the cells shrink. It is not reported whether all these cells die.

The present finding that macrophages differentiate from mesenchymal cells is not unique. According to Gräper (1914), dead cells in embryos are nearly always engulfed by neighbouring sister cells. Maximow (1932) stated that macrophages can differentiate from fixed mesenchymal cells in embryonic and foetal tissues, Glucksmann (1951) reported that degenerating cells may be engulfed by a neighbour, and Franchi & Mandl (1962) concluded that dead oogonia and oocytes in the foetal rat ovary are engulfed by nearby somatic cells. Other studies (Danchakoff, 1921; Chang, 1939; Weber, 1964) have again suggested that macrophages can originate in this way. Weber (1963) has also shown that macrophages in the regressing tadpole tail probably differentiate from cells with lower acid phosphatase activity, and that the increased activity is due to a different enzyme with a lower Michaelis–Menten constant. In the present study, however, no evidence was found by using inhibitors and staining techniques that the macrophage enzymes differ from those of other cells.

Although dead cells generally seem to be digested by the enzymes of macrophages, the possibility cannot be excluded that autodigestion also occurs to some extent in each dead cell.
Questions concerning the nature of the death signal, and of the factors which initiate the differentiation of mesenchymal cells into macrophages and their production of acid hydrolases, remain unanswered. One is led to envisage the latter processes as the result of derepression of previously inactive tracts of the genome, the synthesis of new messenger RNA and the ultimate translation of the new message upon existing, or perhaps newly formed, ribosomes. Evidence of altered genome activity is shown by the increase in dense inactive chromatin (Littau et al. 1964) which accompanies the differentiation of the macrophages. This change can perhaps be interpreted as inactivation of chromatin concerned with general synthesis and the concentration of cell effort on the synthesis of a smaller selected group of proteins, e.g. acid hydrolases. Somewhat conflicting evidence has come from an electron-microscope study of the differentiation of cultured monocytes to macrophages, in which Sutton & Weiss (1966) found that dense chromatin decreased in amount during this process.

The fact that such events occur mainly at the macromolecular level of organization suggests that an electron-microscope study would reveal further details of the process of cytodifferentiation, and the use of combined techniques of cytochemistry and electron microscopy could provide more information about some of the enzymic processes involved in cell death and digestion. Such a study of the developing foetal limb is now in progress.

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REFERENCES


Cell death in foetal rat foot


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All photomicrographs are from longitudinal sections of foetal rat hind feet on day 17. Figure 5 is a section through a whole foot, Fig. 6 illustrates tissue near the tip of a digit and all other Figs. are of interdigital degenerating tissue. Figures 3–13 are of cryostat sections.

Fig. 3. Pyknotic nuclei are dense and occur mainly in clusters (cl1, cl2, cl3). Cluster cl1 appears to lie within a limiting membrane (arrow). Many cells with normal-looking nuclei lie between the stained clusters. Baker’s acid haematein stain following pyridine extraction. × 1600.

Fig. 4. Pyknotic nuclei are dark and lie in clusters which also contain many black stained granules (g). Heidenhain’s iron haematoxylin. × 1600.

Fig. 5. Intense acid phosphatase activity is present in degenerating interdigital tissue and on the outer margin of the digit at lower right. Moderate activity is seen in developing joints (j). The activity in other tissues is not visible at this magnification. Gomori method. × 50.
Fig. 6. The normal acid phosphatase staining pattern in viable mesenchymal cells, such as these at the tip of a digit, consists of numerous small cytoplasmic sites (approximately 1 μ in diameter). Gomori method. × 2200.

Fig. 7. Below the ectoderm (ect) lies a class 1 cluster of dead cells (arrows) possessing about the same acid phosphatase activity as the neighbouring viable cells at the left of the field. Gomori method. × 1400.

Fig. 8. A small cluster of dead cells lies close to a normal-looking nucleus (n). One dead cell (1) is fragmented and two small sites of acid phosphatase activity are seen close to its surface. A second dead cell (2) has several discrete sites of activity, slightly out of focus, at the upper margin. A third dead cell (3) is above the plane of focus and intensely stained. These cells together form an early class 2 cluster. Gomori method. × 1900.

Fig. 9. The left of the field shows mainly viable cells, while at the centre and right is degenerating tissue. A class 2 cluster (2cl) contains dead cells with various degrees of acid phosphatase activity. To the right are several class 3 clusters (3cl) with very high activity. Gomori method. × 1900.
Fig. 10. A class 2 dead cell cluster possesses esterase activity localized in discrete sites packed closely together. Indoxyl method. × 1900.

Fig. 11. At the left are three conspicuous dead cells stained non-specifically by hexazotized pararosaniline but which also show some esterase activity in small sites (arrows). At the right is a cluster of class 3 with intense esterase activity, much of it located outside the dead cells. Naphthol AS acetate method × 1900.

Fig. 12. A cluster of class 2 has high acid phosphatase activity associated with a few of the dead cells; the others possess low activity. Much of the stain is diffuse and lies outside the cells. One dead cell (1) is fragmented. Naphthol AS–BI phosphate method. × 1900.

Fig. 13. A cluster of class 3 has intense acid phosphatase activity around most of the dead cells. Within the diffuse stain are many discretely stained small sites. Naphthol AS–BI phosphate method. × 1900.

Figs. 14–20 are of 1–2 μ Epon sections stained by the Feulgen method and by toluidine blue. Feulgen stain is represented by black areas and toluidine blue by shades of grey. The resolution afforded by Epon sections is far higher than that obtained with the cryostat sections seen in the above figures, or with paraffin sections.

Fig. 14. Below the ectoderm (ect) is degenerating tissue containing both viable cells, and dead cells with dark stained pyknotic nuclei, many in clusters (cl). Dead cells in advanced stages of digestion are present (arrows). × 1600.
Fig. 15. An isolated dead or dying loose mesenchymal cell (dc) has a condensed nucleus and rounded up cytoplasm with increased basophilia. A typical viable loose mesenchymal cell is also present (arrow), together with other cells showing slight nuclear changes. × 1900.

Fig. 16. A condensed and rounded dead cell (dc) lies within the cytoplasm of a phagocyte, the nucleus of which is only slightly darker than those of neighbouring non-phagocytic mesenchymal cells (arrows). Nearby a dividing normal mesenchymal cell is seen in late telophase. × 1900.

Fig. 17. A large macrophage contains 3 dead cells (1, 2, 3) in various stages of digestion. The macrophage has darker-stained heterochromatin and more extensive cytoplasm than neighbouring cells. × 1900.

Fig. 18. Very large macrophages usually contain numerous dead cells, some of which lie within a clearly defined vacuole (v). One dead cell (dc) is Feulgen-positive in the centre only and stained with toluidine blue at the periphery. This and other engulfed dead cells have pitted surfaces, suggestive of localized enzymic digestion. Small structures stained with toluidine blue (arrows) are also visible in the macrophage cytoplasm, which is more basophilic than that of neighbouring cells. The macrophage nucleus (n) is more darkly stained than those of other (viable) cells in the field. × 1900.

Figs. 19, 20. Dividing macrophages containing ingested dead cells (arrows) are seen in metaphase and in telophase respectively. Both × 1900.