SUMMARY

Cellular degeneration and necrosis were studied in the interdigital areas of developing hind limb buds of normal chick embryos by means of enzyme-specific tetrazolium salts and electron microscopy.

Using succinic acid as a substrate and the tetrazolium salt, nitro blue tetrazolium, succinate dehydrogenase was specifically demonstrated via a colour reaction in which degenerating and necrotic cells—those with no enzyme activity—did not stain, while those with enzyme activity stained deeply blue-black. The interdigital cells exhibited near absent levels of succinate dehydrogenase as early as stage 26–27, one to two days prior to when morphological evidence of degeneration and necrosis was present. It was postulated that the mechanism of cell death resulted from decreased activity and/or loss of strategic cellular enzymes such as succinate dehydrogenase, with a subsequent fall in the cellular adenosine triphosphate (ATP) level and a resultant compromise in vital cellular processes, eventually leading to cell death. The evidence indicated that ‘biochemical degeneration’ occurred prior to morphological changes in cells.

The ultrastructural events of degenerating and necrotic cells were also studied. Most ‘dying’ cells observed had already been phagocytosed and were observed in various stages of degeneration. The degenerating cells exhibited both nuclear and cytoplasmic changes. There was evidence of active intracellular digestion within the phagocytes. Numerous lysosomes were observed within these cells, and some appeared fused with the digestive vacuole’s membranes. The origin of the phagocytes was not determined.

No definite information was obtained concerning the utility of cellular degeneration and necrosis in the interdigital areas of the hind limb buds. It appeared to be at least partially causal in separation of the digits.

INTRODUCTION

Cellular degeneration and necrosis are conspicuous aspects of most pathological processes. In the embryological development of many vertebrates, however, cellular necrosis appears to be a frequently used mechanism in the differentiation of tissues and organs, in sculpturing of various organs and structures, and also in eliminating vestigial tissues and organs. Glücksmann (1951) has described in considerable detail and has tabulated, according to location and developmental functions, the occurrence of cellular death in a number of normal vertebrate embryos.

Most studies to date have employed vital dyes as a means of demonstrating specific
areas of necrosis. The exact mechanism of the staining of necrotic cells by vital dyes has not been elucidated but probably relates to cellular membrane permeability (Trump & Bulger, 1967). The mechanism of cellular death in embryological development is for the most part also unknown. Most studies to date have indicated that it is under genetic control, but how on a cellular level genetic control of cell death is mediated is completely unknown. Suffice it to say now that it may not be due to intracellular release of acid hydrolases from lysosomes, since some investigators have found no increase in acid phosphatase activity in the dying cell prior to its phagocytosis (Milaire, 1963; Ballard & Holt, 1968; Goldblatt, Trump & Stowell, 1965).

Since most studies to date have employed various vital dyes as a means of demonstrating cellular necrosis and since vital dyes often demonstrate the necrotic cells during or after phagocytosis, it seemed useful and important to approach the problem in a more basic and direct manner, namely the evaluation of the activity of an enzyme involved in energy metabolism. It was decided to use the tetrazolium salt technique of demonstrating a specific dehydrogenase enzyme, namely, succinate dehydrogenase (SDH). This enzyme is a constituent of all living cells and is known to be located in the mitochondrion (Siekevitz & Watson, 1956; Novikoff, 1961). It serves to catalyse a reaction in the terminal oxidation of glucose in the Krebs cycle which ultimately produces energy for the cell in the form of adenosine triphosphate (ATP). Its function is therefore well known and it is in a key position in energy production in the cell. The tetrazolium salt nitro blue tetrazolium (NBT) is extremely suitable for demonstrating SDH activity. With succinic acid as substrate, NBT is specific for SDH. This technique has been used to demonstrate experimentally produced (Nachlas & Shnitka, 1963) and human myocardial infarctions (Morales & Fine, 1966). In these instances SDH is lost from the necrotic muscle cell as early as 4 to 6 h after infarction, and as a result there is no reduction of NBT to a blue-black formazan. Thus, the intact tissue stains intensely blue-black, while the degenerating and necrotic area is pale and outlined rather vividly. Mouse liver cells, cultured in vitro, have also been demonstrated to be necrotic by this method (Goldblatt et al. 1965).

Thus, in the course of cellular degeneration in embryonic development, it is logical to assume that mitochondrial and other cellular enzymes are lost from the dying cells or are changed in such a way as to be enzymically inactive and, as a result, these areas of cell degeneration should be demonstrable, in a negative sense, by the NBT-SDH technique.

The ultrastructural morphologic events of cell death in the interdigital areas of the chick hind limb bud, as well as other areas of cell necrosis in various embryos, have received little study. Jurand (1964) examined the ultrastructural aspects of early development in the forelimb buds of the chick and mouse embryo. He observed that numerous cytoplasmic changes occurred prior to any profound nuclear changes in the degenerating cells. Jurand (1966) also examined the electron-microscopic changes in limb buds treated with thalidomide and found again that cytoplasmic changes—predominantly mitochondrial swelling and fragmentation—preceded the nuclear changes of pyknosis and karyorrhexis. Farbman (1968) studied the ultrastructural events of palate fusion in mouse embryos. He found widespread evidence of cell death
Cellular degeneration and necrosis immediately following palate fusion. Morphologically these cells appeared as dense bodies in which degenerating organelles were seen. Bellairs (1961) has observed the electron-microscopic changes occurring in degenerating cells of chick blastoderms and has observed cytoplasmic changes as well as nuclear changes. Manasck (1969) has observed degenerating myocardial cells in the embryonic chick ventricle and has observed both nuclear and cytoplasmic changes, some of which were similar to those seen by Bellairs (1961). Maruyama & D’Agostino (1967) have described the electron-microscopic morphology of degenerating cells in the telencephalic roof plate of normal rat embryos. They observed mainly nuclear changes. Since the interdigital area of the chick embryo hind limb bud was relatively easily accessible, it seemed important to study these areas by means of the electron microscope.

The purpose of this paper then is threefold: (1) to demonstrate areas of cellular degeneration and necrosis in the interdigital zones of the chick hind limb bud by means of the tetrazolium salt technique; (2) to seek insight into the mechanism of cellular degeneration and necrosis; and (3) to study the ultrastructural aspects of cellular degeneration and necrosis within the interdigital areas of stages 31 to 33 chick hind limb buds.

**MATERIAL AND METHODS**

The hind limb buds of chick embryos, stages 24–34 according to Hamburger & Hamilton (1951), were used for this study. Fertile eggs from White Leghorn stock were incubated at 38°C for 4 to 9 days (stages 24–35). The embryos were removed at the appropriate time and, using fine steel dissecting knives, the hind limb buds were excised and deposited in a beaker of Earle’s solution. They were washed in 3 separate beakers of cold Earle’s solution and then transferred to the incubation medium containing 1 part NBT (0.5 mg/ml), 1 part 0.2 M sodium succinate, and 1 part pH 7.6 phosphate buffer. The limb buds were incubated in this medium for 60–90 min. They were then removed from the medium, examined grossly, fixed in neutral buffered formalin for 24 h, dehydrated and embedded in paraffin. Serial sections, 6 μm thick, were made of each limb bud and the individual section was processed in one of 3 ways: (1) cleared and dehydrated in xylene and ethanol; (2) cleared and dehydrated in xylene and ethanol, then stained lightly with eosin; or (3) cleared and dehydrated in xylene and ethanol, then stained with haematoxylin and eosin.

Because of the possibility of poor or unequal penetration of the incubation medium, 5 limb buds of each stage were incubated and examined grossly to verify the consistency of the results.

The interdigital areas of chick hind limb buds of stages 31–33 were examined under the electron microscope. The buds were fixed in a modification of Karnovsky’s fixative (Mottet & Jensen, 1968) for 4 h and then processed in the usual manner prior to embedding in Epon. They were sectioned at 1–1.5 μm thick, stained with toluidine blue (Trump, Smuckler & Benditt, 1961), and then grossly oriented prior to thin sectioning at 0.05 to 0.1 μm. Photographs of appropriate interdigital cells were made at various magnifications.

**RESULTS**

By stage 24 (4 days incubation) when experimentation started in this study, the hind limb bud has already undergone a considerable amount of growth. What began as a condensation of the mesoderm in the lateral body wall has now become a recognizable appendage which is longer than it is wide. It is during this stage that the limb paddle or foot plate forms. It appears as the broad, flattened portion of the limb bud
from which the autopodium will develop. It is the cellular changes which occur in this part of the limb bud, particularly the interdigital areas, that will be considered.

At stage 24, NBT incubation resulted in a hind limb bud which was near homogeneously blue-black in appearance, with some tendency towards more intense staining in the post-axial portion of the bud (Fig. 1). Unfortunately, the anterior and posterior necrotic zones described by Saunders, Gasseling & Saunders (1962) were not grossly visible. This may have been due to their small size and location or to the characteristics of light passing through the limb. Consistently seen at this stage was a slight decrease in staining in an area which will become an interdigital zone. On microscopic section (Figs. 2, 3), most of the cells showed formazan within their cytoplasm.

During stages 25 and 26 (4.5-5 days incubation), a characteristic distribution of enzymic activity was observed (Fig. 4). The most active areas were located in the precartilaginous area of the developing toe (Fig. 5). The interdigital regions showed a conspicuous absence of enzyme activity (Fig. 6). The cells of the interdigital area, however, did not appear abnormal at this time. No degenerating nuclei were seen.

During stages 27 and 28 (5-5.5 days incubation), the developing toes were more clearly elucidated by their high SDH activity (Fig. 7). The cells of the interdigital areas were almost completely devoid of any enzymic activity (Figs. 7, 8) but were not morphologically abnormal. The neural elements displayed a high level of SDH activity. They were seen (Fig. 8) coursing into the limb bud at an oblique angle. The epithelium overlying the developing toes also exhibited SDH activity, although not as much as in the precartilaginous toes. An area of high SDH activity was also observed where the limb was excised from the body of the embryo. This may have been an inherent region of high enzyme activity or it may have been due to tissue destruction, with the release of SDH from areas which the medium usually did not penetrate. The cells of the central portion of the limb (Fig. 8) appeared to have no enzymic activity. At higher power, a few formazan granules were usually visible within each cell's cytoplasm. Some of the most interior cells, however, did not show SDH activity. This was probably due to poor and/or uneven penetration by the incubation medium. Fig. 9 shows a higher power of the limb paddle seen in Fig. 8. Again the high SDH activity is readily visible in the precartilaginous areas. The cells in these areas were arranged in a longitudinal plane. Most conspicuous and interesting was the almost total absence of interdigital cell enzyme activity. No morphologic changes of these cells could be observed at this time.

During stages 29 and 30 (6-6.5 days incubation), 4 toes were visible in the limb paddle and were high in SDH activity (Fig. 10). The developing toes are clearly visible in the low-power micrograph (Fig. 11) and are high in SDH activity as noted in the gross photograph. The interdigital zones separating the toes exhibited no staining activity (Figs. 11, 12). Besides showing lack of enzyme activity, the interdigital cells were seen to be more widely separated than at earlier stages of development. Most significant during this period was the observation of an occasional degenerating cell within the interdigital zone, which had a pyknotic nucleus and surrounding clear zone of cytoplasm.
Cellular degeneration and necrosis

In stages 31 and 32 (7–7.5 days incubation) (Fig. 13), the enzyme distribution was essentially the same as in the previous stages. The developing joints were readily visible within the toes (Fig. 14). The chondroblasts had a high SDH activity which was to be expected in view of their active metabolic function during this stage of development (Fig. 14). Necrosis was a common feature in the interdigital areas during these stages. The morphological changes observed in the dying cells were predominantly nuclear. In Fig. 15, many stages of degenerating nuclei can be seen. Separation of the chromatic from the non-chromatic nuclear material is visible in at least 3 of the cells. Necrosis which occurs in these areas may account in part for the observed separation of the interdigital cells.

During stages 33 and 34, cellular necrosis in the interdigital areas was massive. Figs. 16 and 17 are a gross and low-power microscopic view, respectively, of the limb bud at that time showing the absence of interdigital cell enzyme reaction. In Fig. 18, an intense region of cellular degeneration and necrosis is obvious. Numerous phagocytes, some with engulfed cellular debris, are present within the area.

This portion of the study shows that succinate dehydrogenase activity is lost at an early stage from the interdigital cells. Interdigital necrosis was not recognized in this study until stage 29–30, at which time the degenerating cells were sparsely distributed. By stage 31–32, it was a prominent feature, and during stages 33 and 34 (7.75–8 days incubation) necrosis could be described as massive.

It was noted that the SDH activity in the interdigital cells decreased rapidly after the development of the limb paddle. It may have been that these cells were in a stage of degeneration that Bessis (1964) described as ‘death agony’ in which ‘biochemical degeneration’ was a prominent feature, prior to their actual necrosis between stages 30 and 34.

The ultrastructural morphology of degenerating cells within the interdigital areas of the chick hind limb paddle (stages 31–33) was examined. Since little work has been done in this area, this investigation seemed of particular importance.

Fig. 19 reveals an interdigital cell of a stage 31–32 (7–7.5 days incubation) embryo. The nucleus of the cell is undergoing condensation as exhibited by its increased electron opacity. There is no evidence yet of separation of the chromatic form from the non-chromatic nuclear material. The cytoplasm appears morphologically normal, and there are several mitochondria present which appear normal. It may be that this represents an early nuclear change in a degenerating cell or possibly a cell in early mitosis.

Within the interdigital area of a stage 31–32 embryo, shown in Fig. 20, a phagocyte is seen containing an engulfed cell. Within one of the digestive vacuoles of the phagocyte an obvious dying cell is seen. It is characterized by its degenerating nucleus in which the chromatic and non-chromatic material have separated, by the fat droplets and by other cytoplasmic debris. The origin and nature of the contents of the other digestive vacuole present within the phagocytic cell are not evident. It may be that the intracellular material is in a later stage of degradation. The other interdigital cells within the photograph appear morphologically normal. They appear to contain in their cytoplasm abundant rough endoplasmic reticulum. The nucleus of the phagocyte
appears small compared to the nuclei of the interdigital cells but this is probably due to sectioning.

Fig. 21 is an electron micrograph of an isolated phagocyte with an ingested necrotic cell within its digestive vacuole. The cellular membrane of the engulfed cell is readily apparent, as is the lining membrane of the digestive vacuole. Inside the vacuole a degenerating nucleus is obvious. There also appears to be a degenerating mitochondrion, but it is rather large compared to the mitochondria of the phagocyte. This is not unusual, however, since it is known that mitochondrial swelling and fragmentation are early changes in cellular degeneration (Jurand, 1964). Several small, black vesicular bodies seen within the cytoplasm of the phagocyte are probably lysosomes. Three of these lysosomes are very close to the membrane of the digestive vacuole, and one appears to be touching it (possibly fused with it). Numerous clear, small, membrane-bound vesicles are also obvious just inside the degenerating cell’s membrane; their nature and function are unknown. They may be lysosomes that had ‘injected’ their hydrolytic enzymes into the digestive vacuole and consequently appeared colourless.

Fig. 22 represents another single phagocyte within the interdigital area of a stage 32–33 embryo (7-7.75 days incubation). It contains 3, possibly 4, ‘inclusion structures’ whose contents cannot be identified. These inclusion structures would be classified as residual bodies according to de Duve & Wattiaux (1966). The large residual body in the left portion of the cell can be seen to have 2 closely adherent membranes, thus implying that its content is that of a necrotic cell. The contents of the residual bodies appear to be in various stages of ‘digestion’. Lysosomes are again present within the cytoplasm of the phagocyte.

Fig. 23 is an interdigital phagocyte of a stage 32–33 embryo which contains a large single residual body. Its contents are not discernible but are probably digested cellular material. The ‘ordered’ structure of some of the engulfed material is of interest, since this may be due to changes in a particular component of the cell, perhaps the endoplasmic reticulum.

The interdigital phagocyte pictured in Fig. 24 is unique among those observed in this study in that its residual body is almost completely devoid of inclusion material and that its cytoplasm contains numerous electron-lucent vacuoles. The residual body is seen to be separated from the other cytoplasmic material by a single lining membrane. There appear to be several small vacuoles near the residual body. These have a similar appearance to the vesicles shown in Fig. 21. It is conceivable that the clear vacuoles are derived from the residual body and may function as a migrating type of pinocytotic vesicle, carrying the digested material from the residual body to the extracellular space. There is no proof of this, however, for no vacuole is seen opening into the extracellular space, and there is no information as to the previous size of the residual body. Another possibility is that they are lysosomal vesicles which have emptied their enzymes into the now residual body.

The morphology of dying cells in many stages of degeneration was observed. Most of these cells had already been phagocytosed when observed. It was hoped that this study might have revealed some early morphological changes in the interdigital cells
Cellular degeneration and necrosis

Prior to their engulfment. The early changes may have been missed as a result of sampling error (only stages 31-33 were studied), or it may be that morphological changes are a late occurrence in the overall degenerative process that occurs in the interdigital cells.

DISCUSSION

When considering cellular degeneration in limb morphogenesis, one is immediately confronted with the question of the mechanism of cell death. The information to date has shown that in the majority of cases, cellular degeneration in embryological development is under genetic control. In mutants of various species of vertebrate embryos, cell death has been found to be abnormal in extent and location. Zwilling (1942) has shown that rumplessness in the fowl is due to an increase in cell death occurring in a normal location. Hinchcliffe & Ede (1967) have observed a markedly decreased amount of necrosis in the superficial mesenchyme and interdigital areas of the developing limb of the polydactylous mutant of the fowl known as talpid. Exactly how, on a cellular level, genetic control of cell death is mediated is unknown. At least 2 possible genetic mechanisms may exist a priori, a direct and an indirect. Cell death could conceivably be due to the specific programming of the genetic code in which operator or regulator genes would ‘turn off’ an essential cellular function resulting in necrobiosis. Alternatively the genome could be such that the milieu internae is more or less inhospitable to the survival of cells in a particular region as a result of altered nutritional supply, excessive metabolites, etc.

From this study it was possible to postulate a theory concerning the mechanism of cell death. It was shown that within the developing hind limb of the normal chick embryo there was a variable distribution of succinate dehydrogenase activity. The greatest areas of activity were located in the precartilaginous blastemic areas of the developing toes in the limb paddle. Of special interest were those areas in the limb paddle which exhibited little or no enzyme activity; most obvious of these were the interdigital areas. As early as stage 25, interdigital area number 3 showed less enzyme activity than its immediately adjacent precartilaginous area. Interdigital SDH activity decreased rapidly and progressively, and by stage 27-28 many of the interdigital cells exhibited no enzyme activity. This is not to say that these cells were necrotic at this stage, for on histological examination they displayed no obvious morphologic change. One can assume, however, from this information, that the interdigital cells were less active metabolically than many of their neighbouring cells, and it is plausible that this may signify the initial process in the eventual necrosis that is so prevalent between stages 30 and 34. Since it is known that SDH is involved in aerobic cellular metabolism, which ultimately provides the cell with energy in the form of ATP, a decrease in SDH activity implies a decrease in the amount of ATP available to the cell, which in turn implies a decrease in many cellular activities. Such an effect has been observed in senescent red blood cells (Prankerd, 1961; Simon, Giblett & Finch, 1966). In the ageing red blood cells, decreased activities of enzymes of the pentose shunt (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and Embden
Meyerhof pathway (hexokinase, aldolase, pyruvate kinase and phosphofructokinase) result in a decrease in the amount of ATP made available to the cell. In erythrocytes it is known that ATP is essential for maintaining the cellular membrane intact, cation transport, membrane potential, and other energy-requiring processes. It is postulated (Prankerd, 1961; Simon et al. 1966) that in the ageing red blood cell activities of many enzymes decrease. This results in a fall in ATP with subsequent compromise of many vital cellular processes which ultimately leads to cell death. Thus, in the case of the interdigital areas of the chick embryo hind limb, a fall in SDH activity implies a fall in ATP activity. One can postulate that as the level of SDH activity decreases in the interdigital cells, the level of ATP must also decrease and eventually a point is reached where the cell is no longer capable of carrying on its vital functions and thus it dies. The mechanism by which the SDH activity of the interdigital area decreases is unknown. It most likely occurs at the DNA-RNA protein synthesis level. This mechanism of cell death correlates nicely with the data of Saunders & Fallon (1966) concerning DNA synthesis in the posterior necrotic zone of the chick embryo limb bud. They injected tritiated thymidine into chick embryos of stages 19 through 23 and evaluated the percentage of necrotic cells with radioactive label as a function of the stage of the embryo. Their results indicated that the cells in the PNZ area had become ‘biochemically moribund’, showing a marked decrease in DNA synthesis, before the actual necrotic process was observed.

Whereas little is known concerning the mechanism of cell death, a great deal has been postulated concerning the usefulness of cell death in embryological development. Saunders et al. (1962) using the vital dye Nile blue sulphate and standard histological techniques demonstrated localized areas of cell death in the chick embryo wing bud which they concluded contributed to the ultimate form of the avian wing. Hinchcliffe & Ede (1967), also using a vital dye technique, demonstrated areas of necrosis in the hind limb buds of the chick embryo and postulated that their absence may play a role in the production of a polydactylous mutant of the fowl known as talpid. Glücksman (1965) studied the development of the eye in Rana temporaria and postulated that the cell death occurs in this developmental process and is a regulatory mechanism to cope with the overproduction of cells that takes place. Maruyama & D'Agostino (1967) observed cellular degeneration and necrosis in the telencephalic roof plate of normal rat embryos but were unable to explain its function apart from postulating that it may represent an attempt at removing a vestigial organ, the paraphysis. Farbman (1968) was likewise unable to explain the significance of the cellular necrosis occurring immediately after palate fusion in the mouse embryo.

Saunders et al. (1962) have implied that cell death is also a prominent feature in embryological skeletal formation, particularly with respect to the formation and differentiation of the joints. They cite observations by Fell & Canti (1934) and have themselves observed that the location of the prospective knee joint in the fowl is a region of intensive cellular death, being so transilluminable in vitro as to be called an opaque patch. Fell (1964) has demonstrated, however, that the necrotic area is actually located in the potential femur region and plays no role in joint formation. Moreover, Henrikson & Cohen (1965), studying the developing chick interphalangeal joint by
Cellular degeneration and necrosis

light and electron microscopy, have found no evidence of cellular necrosis in the developing joint region. Drachman & Sokoloff (1966), using neuromuscular blocking agents and spinal cord transection, have shown that embryonic joint development in the chick embryo hind limb seems to be related to the effects of muscular movement in the limb rather than to cell death.

Cellular necrosis has also been implicated as an important process in the separation of the digits of many vertebrates, including man. Milaire (1962), using the Una-Brachet technique has demonstrated interdigital necrosis in normal mole and mouse embryos. Forthoefel (1959) and Ballard & Holt (1968) have also observed interdigital necrosis in normal mouse embryos. Menkes & Deleanu (1964), using the Nile blue sulphate technique, have demonstrated interdigital necrosis in the chick embryo hind limb which they feel plays a part in the separation of the digits. Indeed, in this study, interdigital necrosis appeared to be a regularly occurring process. It is difficult to assess exactly what role interdigital necrosis plays in separation of the digits. At the same time that necrosis was occurring, there was a great deal of differential growth of the toes, with resultant increase in length of the toes without an increase in the length of the interdigital tissue. Menkes & Deleanu (1964) found that intra-amniotic injection of Janus green at stage 27 consistently resulted in soft tissue syndactyly and hypophalangy in the developing hind limb of the chick embryo. They concluded that the most important factor leading to syndactyly was reduction and/or suppression of the necrosis and macrophage reaction within the interdigital areas. Saunders (1966) points out, however, that treatment with Janus green also retards growth of the limb and in general causes numerous other anomalies. He therefore concluded that the action of the dye was not directly related primarily to degenerating cells and that absence of interdigital necrosis was a coincidental happening. Thus, one is still left in a quandary in many respects concerning the utility of cell death during development. The hypothesis of Glücksmann (1965) that cellular necrosis in eye development reflects a regulatory mechanism to cope with the overproduction of cells as a result of imprecision of stimuli for proliferation may well be applicable to the cell death that occurs in this area.

The morphology of degenerating cells in vertebrate embryos has been studied by light and electron microscopy. Under the light microscope, cell degeneration involves a series of nuclear changes. The details of the cytoplasmic changes have not been recorded, mainly because of technical difficulties. The nuclear changes which occur have been described by Glücksmann (1951) in some detail; they may occur before or after phagocytosis. In this study, degenerating cells were observed in the interdigital areas that appeared most often in the stage of hyperchromatosis of the nuclear membrane. Degenerating cells located within macrophages were also quite evident in the interdigital areas under light microscopy.

The electron-microscopic aspects of cellular degeneration occurring in embryonic development have been described only in a few instances. Bellairs (1961) studied cell death in chick blastoderms and found both nuclear and cytoplasmic changes. The nuclear changes corresponded quite closely to those observed under the light microscope, namely, condensation and separation of the chromatic nuclear material from
the non-chromatic material. The cytoplasmic changes observed were numerous and consisted of increased density of the cytoplasm, banding of electron-opaque cytoplasmic granules into parallel rows, increased accumulation of fat droplets within the cytoplasm, and increased electron opacity and vacuolization of the mitochondria. She postulated that the rows of cytoplasmic granules were most likely ribosome clusters appearing as they did because of dehydration. She also observed that a great deal of cellular degeneration had occurred prior to any changes in the cell membrane. Jurand (1964) observed both nuclear and cytoplasmic changes in degenerating cells of the chick limb bud. The cytoplasmic changes consisted of mitochondrial swelling and fragmentation as well as dilatation of the endoplasmic reticulum. He did not observe the rows of cytoplasmic granules observed by Bellairs (1961). Maruyama & D'Agostino (1967) observed similar nuclear changes in degenerating cells of the telencephalic roof plate of normal 11-day rat foetuses.

In this study most of the degenerating cells observed had already been phagocytosed by macrophages. Within the macrophages, the cells were in various stages of degeneration. The nuclear changes were similar to those described by Bellairs (1961), Maruyama & D'Agostino (1967), and Jurand (1964). We did not, however, observe any banding of cytoplasmic granules as described by Bellairs. Mitochondrial changes were observed only after phagocytosis.

The exact role of acid hydrolases—released from lysosomes—in embryonic cellular degeneration and necrosis is controversial. Zwilling (1964) has implied that necrobiosis is most likely due to the intracellular release of acid hydrolases from lysosomes. Jurand (1964) also implied that the acid phosphatase activity is closely associated with the cellular degeneration and necrosis in the apical ectodermal ridge cells of the chick embryo. Milaire (1963), however, does not associate the increased acid phosphatase activity with necrosis in this region. Ballard & Holt (1968), studying degenerating cells within the interdigital zones of the mouse embryo, found no increase in acid phosphatase activity of the degenerating cells prior to the onset of phagocytosis. Goldblatt et al. (1965), studying necrosis of mouse hepatic parenchymal cells in vitro, concluded that although acid phosphatase activity was demonstrable histochemically within the degenerating cells, the lysosomes per se did not initiate the changes of cellular disintegration but rather the acid phosphatase activity reflected an increased fragility and rupture of the lysosomes during biochemical processing of the degenerating cells. Thus, future work seems necessary to elucidate this controversy.

In this study no lysosomal activity was observed in the interdigital cells prior to phagocytosis of the degenerating cells. Once phagocytosis occurred, however, intracellular digestion appeared very active. Obvious degenerating mitochondria were seen within the digestive vacuoles of the phagocytes. Much of the membrane-lined degen-erating material within the macrophage could not be identified. Thus, the whole structure would probably be classified as a 'residual body' according to de Duve & Wattiaux (1966). This may not have been correct since it appeared that the 'residual bodies' were becoming smaller with less concentrated material within, thus implying continual digestion, and hence they would have been classified as digestive vacuoles (de Duve & Wattiaux, 1966). Nevertheless, cell-laden phagocytes were clearly visible
within the interdigital areas of the hind limb paddle of the 6.5-7.5 day old chick embryo and appeared to be physiologically active.

No information was obtained concerning the origin of the phagocytes or their subsequent fate. Apparently almost any cell type can become phagocytic. Farbman (1968) noted in palate fusion in mouse embryos that even epithelial cells can act as phagocytes. In the interdigital areas of the chicks, no definite cell type could be identified as phagocyte. The chemotaxic stimulus for phagocytosis is still unknown, as is the subsequent ‘life history’ of the phagocytic cells.

In conclusion, one can still only speculate concerning the overall process of cellular degeneration that occurs in the chick embryo limb paddle. From this study, however, it appears that the known morphological changes of cell degeneration are preceded by biochemical changes, specifically the decrease then loss of SDH activity, a crucial step in the cellular production of energy.

The data reported herein represent, in part, research conducted in the fulfilment for the award of thesis honours at the University of Washington School of Medicine. This investigation was supported by U.S. Public Health Service Research Grant AM 68-727 and Pathology Training Grant GM 100-09.

We are indebted to Dr A. Glücksmann for his critical reading of the manuscript. We are also indebted to Mr John Namkung for taking the photographs and to Mrs Viola Musselman for typing the manuscript.

REFERENCES


S. P. Hammar and N. K. Mottet


(Received 17 March 1970—Revised 6 August 1970)
Cellular degeneration and necrosis

Figs. 1–6. For legend see following page.
Fig. 1. Gross view of stage 24 (4 days incubation) hind limb bud. Note the slight area of decreased staining in the distal portion of the paddle: probable interdigital area, ia, ×100.

Fig. 2. Microscopic view of stage 24 hind limb bud. Note the formazan pigment within the cell. Haematoxylin and eosin, ×40.

Fig. 3. Stage 24 limb bud showing intracellular formazan pigment. Haematoxylin and eosin, ×250.

Fig. 4. Gross view of stage 25-26 (4.5-5 days incubation) hind limb bud. Decreased staining is evident in interdigital area (ia). ×100.

Fig. 5. Microscopic section of stage 25-26 limb bud. The areas of high SDH activity correspond to the developing toes (dt). Observe the lack of staining in the interdigital area (ia). Haematoxylin and eosin, ×40.

Fig. 6. Cells in the developing toe (dt) area and adjacent interdigital area (ia) of stage 25-26 limb bud. There is conspicuous absence of staining in the interdigital cells. Eosin, ×250.

Fig. 7. Gross view of limb paddle of stage 27-28 embryo (5-5.5 days incubation). The high enzyme activity of the developing toes (dt) is contrasted by the near absence of enzyme activity in the interdigital area (ia). ×100.

Fig. 8. Section of limb paddle in stage 27-28 embryo. Three areas of high SDH activity correspond to the developing toes (dt). Interdigital areas (ia) exhibit no enzyme activity. Neural elements (ne) are of high enzyme activity. Eosin, ×40.

Fig. 9. Distal end of stage 27-28 limb paddle. The developing toes with their high enzyme activity are apparent. The interdigital area (ia) shows more clearly its lack of enzyme activity. Eosin, ×100.

Fig. 10. Gross view of stage 29-30 (6-6.5 days incubation) hind limb bud. Again the high concentration of SDH in the developing toes (dt) and lack of enzyme activity in the interdigital areas (ia) is apparent. ×40.

Fig. 11. Stage 29-30 hind limb bud. The high concentration of SDH in the developing toes (dt) and lack of enzyme activity in the interdigital area (ia) is obvious. Eosin, ×100.

Fig. 12. Limb paddle of stage 29-30 embryo showing portions of 2 developing toes (dt) and interdigital area (ia). Eosin, ×250.
Fig. 13. Gross view of limb paddle stage 31–32 (7–7.5 days incubation). Note developing toes (dt) and interdigital areas (ia). × 40.

Fig. 14. Developing limb paddle showing developing toes (dt) with high SDH activity and interdigital areas (ia) showing no enzyme activity. The developing joint is also visible within one toe. Eosin, × 100.

Fig. 15. Microscopic view of interdigital area of stage 31–32 (7–7.5 days incubation) limb bud. Note the several degenerating cells (dc) with their pyknotic nuclei. Eosin, × 250.

Fig. 16. Gross view of stage 33–34 (7.5–8 days incubation) limb bud. The developing toes (dt) continue to show high enzyme activity, which is especially high in their distal portion. The near absence of enzyme activity in the interdigital areas (ia) is apparent. × 40.

Fig. 17. Microscopic view of two of the developing toes (dt) and accompanying interdigital area. Note the developing joint in the toe. Note the absence of enzyme activity in the interdigital area (ia). Eosin, × 100.

Fig. 18. Interdigital area of stage 33–34 (7.5–8 days incubation) hind limb bud. Note the massive area of cellular necrosis (cn). Necrosis involves both the ectoderm (e) and mesoderm. Eosin, × 250.
Cellular degeneration and necrosis
Fig. 19. Interdigital cell of stage 31–32 (7–7.5 days incubation). Observe the condensation of the nuclear material of the degenerating nucleus (dn). The cytoplasm appears normal. There are numerous mitochondria (m) and desmosomes (d) present. × 10000.

Fig. 20. Interdigital phagocyte of stage 31–32 embryo. The phagocyte contains 2 digestive vacuoles (dv). Within the digestive vacuoles are 2 obvious degenerating cells in which degenerating nuclei (dn) and fat droplets (fd) are apparent. The phagocyte nucleus (pn) and the normal interdigital cell and its nucleus (n) and long profiles of endoplasmic reticulum (er) are distinct. × 6250.
Fig. 21. Isolated interdigital phagocyte of stage 31–32 embryo. The digestive vacuole contains a degenerating cell. The degenerating nucleus (dn) and degenerating mitochondria (dm) are apparent. The cell membrane (cm) of the phagocytosed cell and the lining membrane (lm) of the digestive vacuoles are apparent. Small clear vesicles (v) are seen on the inner aspect of the degenerating cell’s membrane. Several lysosomes (l) are prominent. ×10000.

Fig. 22. Interdigital phagocyte of stage 32–33 (7.5–8 days incubation) embryo. Three, possibly 4, residual bodies (rb) are present within the cytoplasm of the macrophage. The phagocyte nucleus (pn) is distinct. The arrow points to a double membrane which implies a phagocytosed cell. Lysosomes (l) are again seen. ×12500.
Cellular degeneration and necrosis

21

22

249
Fig. 23. Another example of interdigital phagocyte containing a single residual body (rb). The inclusion material is somewhat fibrillar but no definite structures are visible. Double membranes can again be seen. (l, lysosomes.) × 9500.

Fig. 24. Interdigital phagocyte of stage 32–33. The residual body (rb) contains a scanty amount of material and appears to be at the end stage of 'digestion'. The phagocyte cytoplasm contains numerous vacuoles (v), the content of which is unknown; the nucleus (pn) is again obvious. Lysosomes are also present. × 12500.
Cellular degeneration and necrosis