Death in Embryonic Systems

Death of cells is the usual accompaniment of embryonic growth and differentiation.

John W. Saunders, Jr.

One confronts less than comfortably the notion that cellular death has a place in embryonic development; for why should the embryo, progressing towards an ever more improbable state, squander in death those resources of energy and information which it has laboriously won from a less ordered environment? Nevertheless, abundant death, often cataclysmic in its onslaught, is a part of early development in many animals; it is the usual method of eliminating organs and tissues that are useful only during embryonic or larval life or that are but phylogenetic vestiges (phylogenetic death), for example, the pronephros and mesonephros of the higher vertebrates, the anuran tail and gills, and larval organs of holometabolous insects; it plays a role in the differentiation of organs and tissues, as exemplified by the histogenesis and remodeling of cartilage and bone (histogenetic death); and it accompanies the formation of folds and the confluence of anlagen (morphogenetic death) (*1*). It occurs frequently in the early blastoderm of the chick embryo, more or less at random, and dead cells are scattered throughout the tissues of the older embryo (*2*,*3*). Indeed, degeneration of cells and tissues is a very prominent part of development, and it is unfortunate that little has been done to analyze its significance in the processes with which it is associated, its embryonic control, the biochemical events in the onset and realization of necrosis, and the developmental role of the products of degeneration. These are the topics of the discussion which follows, and it is hoped that it will contribute to the further recognition and fruitful formulation of problems concerning cellular death during development.

The Mechanical Utility of Morphogenetic Death

The "utility" of cellular death in eliminating transient and obsolete organs and tissues is clear, and deaths involved in morphogenetic movements and histogenesis may function as integrative mechanisms in the acquisition of form. But, one must not accept without experimentation that, for a particular morphogenetic or histogenetic process, those deaths accompanying it, regardless of their prominence, are requisite for its achievement. Thus cell deaths almost invariably appear when tissues fold or invaginate; but would the fold or invagination fail to occur if death were prevented?

This kind of question can be asked about several morphogenetic processes accompanied by extensive necrosis. In these cases, cells in a localized area die in great numbers and are engulfed by macrophages, which are then easily recognized in living tissue by their whitish opacity and the affinity of included cell debris for various basic dyes (Fig. 1, a and b). Resorption of the cellular debris in these areas is completed in a matter of a few hours.

Such widespread necrosis in the midventral tissue between fusing elements of the bilaterally derived components of the avian sternum has been described by Fell (*4*). Presumably shrinkage of this tissue, caused by cellular death and resorption, could draw the sternal rudiments together. Apparently, however, they provide their own traction: isolated from intervening tissues and grown in their normal relationship in organ culture on a plasma clot—that is, medial sides facing—they move together; in the reverse relationship, they move apart. The movements of fusion are thus accomplished by factors intrinsic to the isolates, such as the ameboid crawling of the cells of their ventral borders, and not through traction forces exerted by shrinking tissues. What would occur in vivo if there were no cell death in the midline tissue is not known.

Sculpturing of the limb in the 3- to 9-day-old chick embryo follows closely upon waves of necrosis that sweep proximodistally along the mesoderm of the anterior and posterior margins of the limb and finally appear in the interdigital tissues (Fig. 2). One zone of degeneration, named for convenience the posterior necrotic zone (PNZ), occurs at the posterior junction of wing bud and body wall at stage 24 (Figs. 1c and 2a). It undergoes degeneration as dramatic morphogenetic movements begin to separate the materials of the limb-bud base, which form the distal part of the scapula and associated structures of the body wall, from those contributing the elbow and posterior portion of the upper arm (*5*).

In the egg, the PNZ of the right wing bud is readily accessible to surgery, and it has been excised from embryos before or during the visible occurrence of necrosis. Not unexpectedly, the wing develops normally; apparently surgery simply eliminates tissue in which most of the cells would normally die. But, normal development likewise ensues if the cells of the PNZ remain *in situ* without dying, as they do when a graft of dorsal wing tissue is implanted between the mesoderm of the PNZ and the ectoderm overlying it posteriorly (Fig. 3) (*6*). Cell death in the PNZ is thus a dispensible adjunct to the processes which shape the upper arm.

The PNZ of the chick may be unique or, at least, unusual among vertebrate embryos, for a similar zone is not found in embryos of the mouse, duck (*3*), or Japanese quail (*7*); but necrosis of the interdigital tissue reportedly occurs in many higher vertebrates, including the duck, mouse, rat, mole, and man (*8*), and is perhaps causally related to the separation of the digits. Others (*9*) have reported, and John Fallon in my laboratory has

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confirmed, that injecting the amniotic cavity of the 6½-day-old chick embryo with Janus green results in the absence of necrosis in the interdigital spaces of the foot and in the development of soft-tissue syndactyly. The interpretation that necrosis has an essential causal role in the formation of the interdigital clefts retains, however, an element of uncertainty, for treatment with Janus green also retards the growth of the limbs and causes anomalies, particularly of the head, integument, and body wall. Moreover, administration of the dye to embryos at stage 18 (3 days of incubation) does not eliminate necrosis in the PNZ at stage 24 or in other sites where deaths normally occur during morphogenesis of the wing and leg buds. Thus the effect of the dye is probably not exercised directly on prospectively degenerating cells, and the failure of interdigital clefts to form could issue from altered patterns of limb growth which, only coincidentally, also prevent the occurrence of interdigital necrosis.

Nevertheless, the idea that cellular death is a necessary adjunct to morphogenetic processes carving the contours of the toes remains somewhat attractive, and it is reinforced by the observation that during development of the webbed foot of the duck, necrosis involves the entire interzone between digits I and II, which are not webbed, but is restricted to the marginal regions of the interzone between the other digits, which are connected by webbing. Moreover, during development of chimeric legs composed of chick mesoderm and duck ectoderm, interdigital necrosis, as determined by direct observation of vitally stained limbs at appropriate stages, is greatly reduced, and all the toes usually remain connected by a narrow web (*11*). This effect is possibly not caused by specific action of the duck ectoderm, however, for similar combinations of chick tissues sometimes give the same result.

Interdigital erosion should be studied in other normal and mutant vertebrates having webbing or soft-tissue syndactyly. Of course, the presumed mechanical role of degeneration in systems other than the limb should also be clarified, but most cases are not readily amenable to experimentation. Is it, for example, necessary that the mandibular mesoblast degenerate in order that the invasion of myogenic

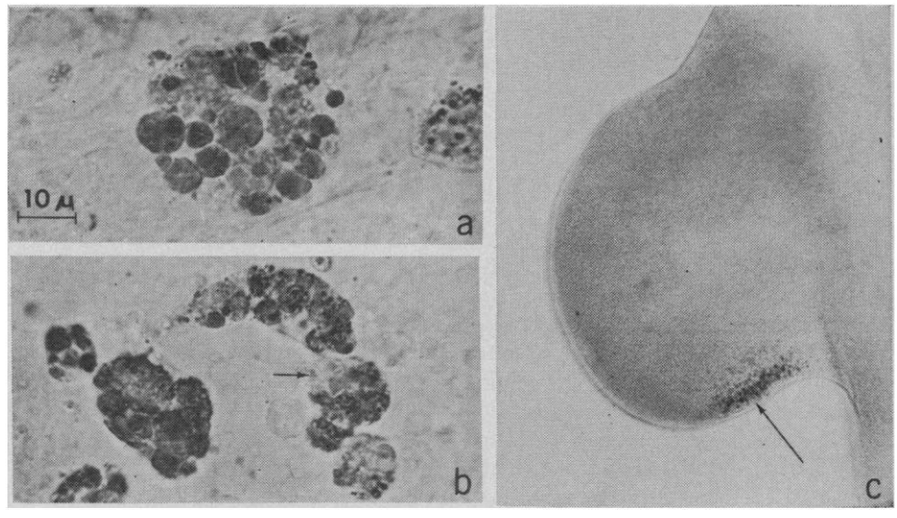

Fig. 1. (a and b) Living macrophages in a squash of fresh tissue from a zone of massive necrosis in the leg bud of a 4-day-old chick embryo (stage 24) stained with neutral red and photographed at equal magnifications. These cells are filled with large, red-staining masses (varying dark shades in the reproductions) of cellular debris from ingested moribund cells. The nucleus, usually obscured, is seen in a cell in b, (arrow). Various tiny granules are seen around the phagocytic vacuoles; they give a strong positive reaction in the Gomori method for acid phosphatase, and it is tempting to suggest that some represent lysosomal sources of hydrolases, aggregated about the phagocytic vacuoles. (c) Whole mount of the left wing bud of a 4-day-old chick embryo fixed and cleared after vital staining with neutral red. The posterior necrotic zone (arrow) is densely populated with debris-laden macrophages and necrotic cells (photograph courtesy of J. Fallon).

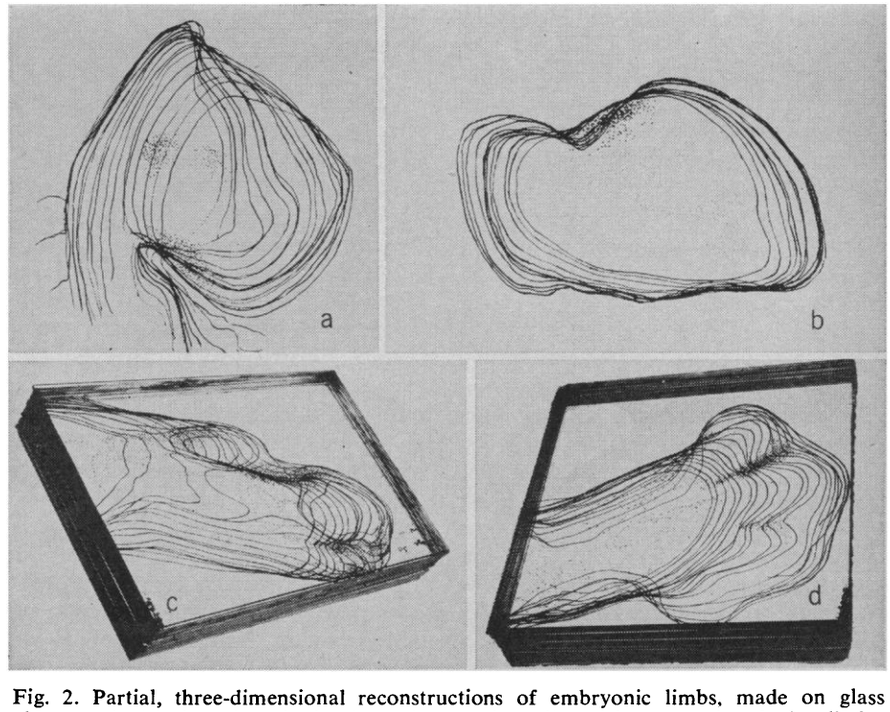

Fig. 2. Partial, three-dimensional reconstructions of embryonic limbs, made on glass plates and showing the distribution of necrotic cells during growth of the limbs. The transparent models were transilluminated and photographed from directly above (a and b) or from an angle above (c and d) [modified from Saunders *et al.* (*5*)]. (a) The wing bud at stage 24 with necrosis in the PNZ, in the glenoid region, and in the central portion of the precartilaginous mass (see Fig. 1c). (b) A massive zone of necrosis sweeps the entire length of the anterior edge of the wing in the superficial mesoderm. As it passes, the curvature of the radial margin of the wing appears, as shown in this model of the wing bud at stage 25. (c and d) The distribution of degeneration figures in interdigital tissues of the wing bud (c, stage 30) and leg bud (d, stage 29).

mesenchyme can occur (*12*)? Would the cloacal membrane perforate in the absence of cell death; the Bursa of Fabricius arise from the roof of the hind gut (*13*)?

Control of the Patterns of Developmental Degeneration

Tissue-environmental control. The now-classic studies of Hamburger and Levi-Montalcini (*14*) on the chick embryo focused interest on the role of cellular degeneration in differentiation of the nervous system. Failure of pioneering fibers of neurons of spinal ganglia and of the somatic motor columns to find an adequate periphery leads to their death. Also, cell death in certain brain centers is affected by input from peripheral sense organs. Thus, when the otocyst (*15*) or the eye (*16*) is excised from the 50-hour-old chick embryo, the corresponding brain centers develop normally at first, but then degenerate, losing great numbers of cells. On the other hand, prolonged synaptic association with appropriate afferent neurons may stabilize central nuclei against cellular loss, for, whereas excision of the mouse eye at birth is followed in 4 to 7 days by massive loss of cells in the superior colliculus, its removal from adult mammals commonly leads only to progressive atrophy in the corresponding central area, possibly without significant cell death (*17*). It would be of interest to examine effects of variation in the quantity and source of sensory input on the stability of cells in particular brain centers.

The posterior necrotic zone of the wing bud provides a simple system for use in the analysis of the environmental control of cell degeneration during development. Cells of the prospective PNZ of a stage-17 donor, grafted to the somite region of a host embryo of the same age, or older or younger, die and are obviously ingested by macrophages at the time the donor embryo is at stage 24. This is the stage at which PNZ cells normally are recognizably necrotic (control tissues from the dorsal side of the wing bud do not die when grafted to the somites). Evidently, by stage 17, a "death clock" intrinsic to the cells of the PNZ is set (*5*). These cells should not, however, be looked upon as undergoing an accelerated senescence or premature aging, or as suffering from inherent obsolescence (*18*) for, until stage 22, they can be diverted to a new developmental fate simply by being grafted to the dorsal side of the wing bud, where they may form extra feathers and cartilage, or by having dorsal tissue placed adjacent to them *in situ* (Fig. 3). Thus the "death sentence" of PNZ cells is revocable in the environment of the dorsal side of the wing bud until stage 22; thereafter it is irreversibly programmed. As will be shown later, the cells which one finds engulfed by macrophages at stage 24 were, in fact, probably moribund at stage 22.

Prospective PNZ tissue isolated in organ culture on a variety of media also has the typical pattern of necrosis (*10*); at 38°C, necrosis occurs in vitro at the same time it occurs in vivo in the contralateral wing bud of the donor embryo. Associated in similar organ cultures with mesoderm from the dorsal region of the wing-bud prior to stage 22, however, the PNZ shows no degeneration, even when the association is across Millipore filters, which bar cytoplasmic contact. In contact with somite tissue or separated from it by a filter, the PNZ degenerates on schedule, regardless when the culture is made. Clearly, this effect is mediated by diffusible materials. Although the critical diffusing factors have not been identified and their role in normal development is still obscure, the demonstration that they exist suggests many new approaches to analysis of the epigenetic control of morphogenetic death.

Hormonal control. Striking though they may be, the morphogenetic deaths considered in the foregoing are less dramatic and have less extensive developmental consequence than a number of death-borne ontogenetic upheavals that are mediated by recognized hormonal factors. Notable among these are the metamorphic changes which occur in amphibians and insects and in the differentiation of some accessory sexual structures in vertebrates—events which are characterized by the destruction of entire populations of cells, and even of organs and organ systems. The hormonal control of degeneration, however, follows the same general principles as those which apply to hormonal regulation of growth and differentiation.

The hormones that initiate cell death and those that stimulate growth and differentiation are the same, the selectivity of effect being the property of the target organ. In the amphibian, thyroid hormone stimulates the widespread breakdown of structures such as gills, tail, intestinal epithelium, operculum and larval head muscles, but it likewise causes the limbs to grow and the entire organism to develop the adult form. In the silkmoth (*19*), under the influence of ecdysone, a cataclysmic wave of destruction sweeps over the internal organs shortly after the pupal molt, eliminating larval organs but leaving untouched the imaginal discs; after diapause, the latter respond with spectacular growth to a renewed secretion of ecdysone. Under appropriate conditions, Wolffian and Müllerian ducts excised from the chick embryo prior to the stage of sexual commitment and cultured side by side in vitro continue to grow. In the presence of male hormone, the Müllerian ducts promptly regress, disappearing almost completely in 24 hours; but the Wolffian ducts, produced in the same coelomic environment, are completely unaffected (*20*). This list of examples could be greatly expanded, but the cases cited make it clear that the competence of some cells and tissues to respond to hormonal stimulus by degenerating and dying is programmed in advance, just as for others there is programmed the competence to respond by growing and differentiating.

Different tissues and organs scheduled for hormonally controlled regression may differentiate this competence at different times in development. Moser has shown that tissues from tadpoles of *Rana temporaria* become competent to respond to thyroxine in a definite sequence; the axial tissue of the tail, for example, becomes competent 7 hours later than the ventral tail fin; the opercular fenestration, 6 hours after that (*21*). Unfortunately, it is not always possible to determine whether a tissue has not yet differentiated the ability to respond to hormones, or merely has a high threshold for response. Likewise there are problems concerning the time during which the hormone must act and the rate at which the target organ responds. These matters have confused understanding of the control of histolysis and other metamorphic events in insects and have also clouded the interpretation of metamorphosis in the Amphibia (*22*). Kollros, however, has been able to measure absolute threshold requirements of thyroxine for metamorphosis in *Rana pipiens* by using hypophysectomized tadpoles exposed for prolonged periods to very low concentrations of

the hormone. With this treatment, individual thresholds for each of several metamorphic events, whether regressive or progressive, may be determined. Indeed, this method is so sensitive that it has revealed what appears to be an asymmetry in the thresholds for left and right opercula to degenerate and thereby form the fenestrae through which the forelimbs emerge *(22)*.

In the Amphibia, hormones appear to bring about degeneration, for the most part, by acting directly on appropriately programmed cells. For example, isolated tails of *Xenopus* regress dramatically when exposed to appropriate concentrations of thyroxine in tissue culture *(23)*. Fenestration of the operculum *(24)* and regression of Mauthner's cells *(25)* also may be direct responses to thyroxine, for these events occur apart from metamorphosis in response to local action of thyroxine released from an implant.

Insect hormones can also act directly on a target organ, programming its death for some time in the future when the hormone is no longer present. In silkmoths, secretion of ecdysone in the absence of juvenile hormone is resumed at the break of pupal diapause, provoking growth and differentiation of the adult musculature and the rapid breakdown of the remaining larval organs, including the source of ecdysone, the prothoracic gland. Certain intersegmental muscles of the abdomen survive, however, and, upon completion of the imaginal molt three weeks later, they participate in unfurling the wings by contracting and compressing the hemolymph. Thereafter they promptly degenerate and, within 48 hours, disappear *(26)*. Lockshin and Williams have shown that degeneration in *Antherea pernyi* is potentiated by the initiation of adult development in the presence of ecdysone and in the absence of juvenile hormone; breakdown occurs upon completion of adult development, regardless of hormonal conditions *(26)*.

Administration of hormones can cause extension of normal foci of necrosis into adjoining regions which would not normally regress or, conversely, in some situations hormones may delay degeneration or prevent it. Kollros *(22)* has shown that an appropriately low concentration of thyroxine, provided in the medium to hypophysectomized tadpoles of *R. pipiens* for a prolonged period, causes

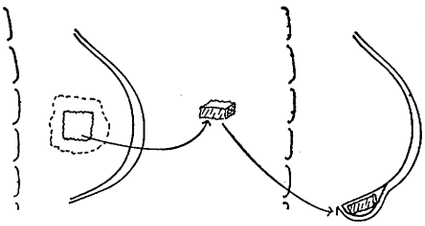

Fig. 3. Scheme of operation in which a block of dorsal mesoderm of a wing bud from a donor embryo is grafted subjacent to the ectodermal covering of the PNZ of a host wing bud, both at stage 21.

opercular fenestration, as usual; but as treatment is prolonged, erosion spreads progressively, extending the window to involve as much as the entire operculum, sometimes leaving the gills completely exposed. Zwilling, who administered insulin to chick embryos on the 5th day of incubation, observed considerable shortening of the long bones of their legs, especially of the tibia, and progressive extension into the epiphyses of a zone of degeneration that normally appears in the region of the future knee joint (the "opaque patch") on the 6th day. In extreme cases, the entire central portion of the tibial epiphysis was destroyed by the 8th day. Tibial rudiments cultured in vitro in the presence of insulin develop short squat bones whose swollen ends are filled with irregularly oriented small-celled cartilage, but they show no signs of degeneration. Apparently, therefore, the extension of degenerative changes that occur in the embryo results from factors related to the conditions of growth in the chondrogenic masses and not to direct effects of the hormone *(27)*.

Appropriate hormonal treatment of insects may prevent or delay degeneration of tissues that normally do not persist after the adult molt. In *Rhodnius prolixus* the prothoracic gland, active in production of ecdysone throughout the five larval instars, is found in an advanced stage of degeneration 24 hours after the adult molt. Degeneration is irreversibly programmed in the prothoracic gland when molting occurs in the absence of juvenile hormone from the corpus allatum. This hormone is absent or in very low titer at the time of the adult molt, but is abundant throughout the larval period and in adult life. Degeneration of the fifth-instar prothoracic gland is prevented if the gland is grafted into a mature adult whose corpus allatum is active, or to a fourth- or fifth-instar larva in which juvenile hormone is in high titer. In the latter instance, the glands survive until the adult molt of the host and are then irreversibly committed to death by going through that molt in the absence of juvenile hormone. In the first case, they persist indefinitely *(28)*.

Genetic control. It is abundantly clear that regressive phenomena during embryonic development and larval metamorphosis are programmed to occur in a highly predictable manner. Thus degenerative changes in development would seem to proceed under genetic control, even as the progressive ones do; and as might be expected, there are mutant genes that achieve phenotypic expression through extending or contracting the normal pattern of cellular death, or by bringing about extensive death of tissues or organs in which degradative changes normally do not occur.

In fowl carrying the gene for "dominant rumplessness," which is inherited as a simple dominant, the mutant phenotype results from extension of a normal zone of cell death and lacks caudal vertebrae and associated tail structures. Zwilling showed that, in embryos issued from matings between heterozygotes (the homozygote fails to hatch), there are various degrees of necrosis in the end bud and tail bud during the 2nd, 3rd, and 4th days of incubation, the degree corresponding to that degree of rumplessness expected in the normal (nonrumpless), heterozygous (intermediate rumpless condition), and homozygous (extreme rumplessness) offspring of the cross *(29)*.

Increased cellular degeneration in the early notochord, neural tube, and somites is among the first signs of the mutant phenotype caused by a number of genes (*Brachyury, vestigial tail, crooked tail, Danforth's short tail, truncate)* affecting the structure of the tail in mice *(30)*. These genes have various effects, but all cause reduction of the tail beginning 8 to 10 days after fertilization. It has been shown that the onset and degree of pathologic involvement, and consequently the morphological expression of the mutant phenotype, varies with the gene dosage and genetic background. In mice homozygous for *Danforth's short tail,* the tail is completely eliminated or reduced to a filament, but it is present at various lengths in heterozygotes *(31)*. By controlling the genetic background through appropriate back crosses to inbred stocks, Dunn and

Glüecksohn-Schoenheimer were able to raise heterozygotes either lacking a tail altogether or having a normal one (the homozygote dies and is completely tailless regardless of background) (*32*). Comparing homozygotes and heterozygotes having the same genetic background, Grüneberg (*31*) pointed out that necrosis begins earlier in the notochord and neural tube of the homozygote than it does in the heterozygote, and it proceeds more rapidly (*33*).

A number of genetically controlled pathological degenerations act later in development, particularly in organs, such as the eye and the ear, which complete their histological differentiation after birth. In the mouse, the basic adult pattern of the retina is differentiated by 12 days after birth, but, in animals homozygous for *retinal dystrophy*, pathological changes set in at this time, and the rods degenerate almost completely in 2 weeks (*34*). The waltzing, head-shaking behavior in mice is brought about by several different genes, all of which cause extensive degeneration in the cochlea and vestibular apparatus. Details of the pathological development of many of these have been recorded by Deol and by Grüneberg (*35*); without reviewing these details, suffice it to say that the data show that identical areas of cellular destruction may be controlled by different genes acting at the same or different times in development and causing the same or different sequences of regressive events. Deol (*35*) has noted, however, that current information is insufficient to allow one to determine to what extent the different patterns of pathological development reflect differences between actions of specific genes and differences in the genetic backgrounds against which they were examined.

Genetic factors may determine whether normally scheduled degenerations actually occur, and they also may alter the time at which these degenerations take place, thereby causing abnormal development. A number of congenital anomalies in the human being, such as persistence of the cardinal veins or of the embryonic tail may be attributable, conceivably, to a failure of normal degenerative processes to occur (*36*).

Advancing the time at which degenerations occur may, likewise, have serious consequences. This happens in several mutants of *Drosophila*, one of many forms in which nurse cells degenerate during advanced stages of oogenesis and contribute their substance to the developing oocyte. In a number of female sterile mutants such as *deep orange, female-sterile 2.1, singed 36a, raspberry^{1}, apterous^{1}, tiny, diminutive*, and others (*37*), the nurse cells break down prematurely, and the oocyte usually degenerates.

Additional material related to the realization of the phenotype through genetic control of patterns of degeneration could be mentioned, but, hopefully, the foregoing examples are sufficient to implement our thesis, namely that the concepts of classical genetics and epigenetics find application in the phenomenology of ontogenetic cellular death equally as well as they do in that of the progressive events of development.

Suicide or Assassination?

For some time the view enjoyed ready acceptance that many or most cells are capable of effecting their own demise, and of simultaneously arranging for the elimination of their own remains by releasing autolytic enzymes, acid hydrolases, from "suicide bags," the lysosomes. Alternatively, cells scheduled for death might be "slain" by extrinsic agents which would trigger the discharge of the lysosomal contents in the cell marked for destruction.

Intriguing though these notions may be, most of the evidence suggests that the release or activation of acid hydrolases is probably a manifestation, rather than a cause, of cell death (*38*). Thus, mitochondrial damage is morphologically visible in ischaemic liver or that treated with carbon tetrachloride before there is any appreciable release of lysosomal enzymes (*39*), and, likewise, changes in the activities of oxidative enzymes (*38, 40*) and nonlysosomal hydrolases (*37*) precede increases in free lysosomal hydrolases in involuting mammary tissue and liver.

It is, moreover, quite evident that increased synthesis and release of lysosomal hydrolases are not necessarily followed by necrosis. In the presence of excess Vitamin A, cartilage cells release considerable quantities of acid protease, while remaining quite viable and synthesizing additional quantities of enzyme (*41*). Also, it is evident that focal cytoplasmic degradation and phagocytosis (*42*) may be accompanied by increases in the number of lysosomal bodies (cytolysomes and phagosomes) (*43*) and in the activities of hydrolytic enzymes in cells destined for survival.

In the intersegmental abdominal muscles of the silkworm, the activities of acid hydrolases, cathepsin, and acid phosphatase increase progressively after the break of pupal diapause until, when adult development is completed, frank necrosis is triggered by cessation of nervous stimuli (*26*). Thus, the muscles seem to prepare for their own death and destruction. In the PNZ of the chick wing, and in other necrotic areas of the embryo, however, there is no detectable increase in acid phosphatase until stage 24 or late in stage 23, whereupon a greatly enhanced activity is found associated with the debris-laden macrophages; in other situations, too—for example, in resorption of the amphibian tail and in that of the mammalian foetus—increases in the activities of hydrolytic enzymes in zones of massive cell death are associated with the invasion of the site by phagocytes or their differentiation *in situ*. Both Weber and Tata have presented clear evidence that regression of the tadpole tail requires synthesis of proteins—apparently hydrolytic enzymes—by macrophages (*44*).

Because they are not usually seen until cytolysis has begun, phagocytes are generally not held to be the cause of the developmental death of cells; sometimes tissue breakdown occurs without their participation at all (*45*). Studies by light microscopy of the PNZ and other centers of early morphogenetic death in the chick have revealed no sign of cell degeneration prior to the onset of phagocytosis. Mrs. Marilyn Ricklin, in my laboratory, has been studying, by means of the electron microscope, the PNZ area of wing buds before and during phagocytosis. Distinctive cells have been found in which there is increased electron opacity of the nucleus and decay of polyribosome clusters; their identification is not certain as yet, but some of them, at least, may be the precursors of the large phagosomes found packed within macrophages as regression of the PNZ proceeds during stage 24 (Fig. 4). One would certainly expect to find some distinctive cells in the PNZ as early as stage 22, for it is then that the "death sentence" becomes irrevocable (as determined by

transplantation experiments), and it is also then that those PNZ cells which are subsequently ingested by macrophages show a radical decrease in their ability to take up tritiated thymidine (*46*).

These considerations, in sum, suggest that for the PNZ, at least, and probably for other areas of morphogenetic degeneration in the chick, death does not result from the triggering of a cell's own "biological booby traps" (*47*), but from other processes which render it morbid and ready for engulfment by macrophages. The failure of DNA to replicate is possibly accompanied by cessation of RNA transcription. Whatever the factors that cause death of embryonic cells, they may very well operate at the level of genetic copying and be entirely independent of the synthesis or importation of hydrolases.

Disposition of the Products of Cellular Death

Of what significance are the chemical by-products of necrosis during an organism's development? Are they reused? In what form and by what pathways? Is their content of specific information lost? There is, of course, abundant evidence that cellular fractions, protein digests, nucleic acids, exudates from injured tissues, degenerating lymphocytes, and so forth, may stimulate growth and cell division in vivo and in vitro (*48*), and it is not unthinkable that important contributions to the organismic economy might be made by degenerating cells. This is certainly so in holometabolous insects where, within the almost-closed system comprising the pupa, larval tissues break down and imaginal tissues grow, or in the starving colony of *Perophora* (*49*), which advances its stolon distally and buds new zooids at the expense of regressing stolons and zooids proximally. But, in the absence of direct evidence, one must regard as gratuitous the suggestions of a number of students of vertebrate development (*50*) that cell deaths provide "mitotic metabolites," energy sources, inductive substances, and so forth, essential to subsequent developmental events.

For the most part, injury and death of cells would release a spectrum of cellular components ranging from low-molecular-weight building blocks to macromolecules, the former probably directly contributing to pools of metabolites, coenzymes, and other compounds, the macromolecules conceivably being incorporated into the machinery of other cells or, more likely, being first broken down to diffusible mole-

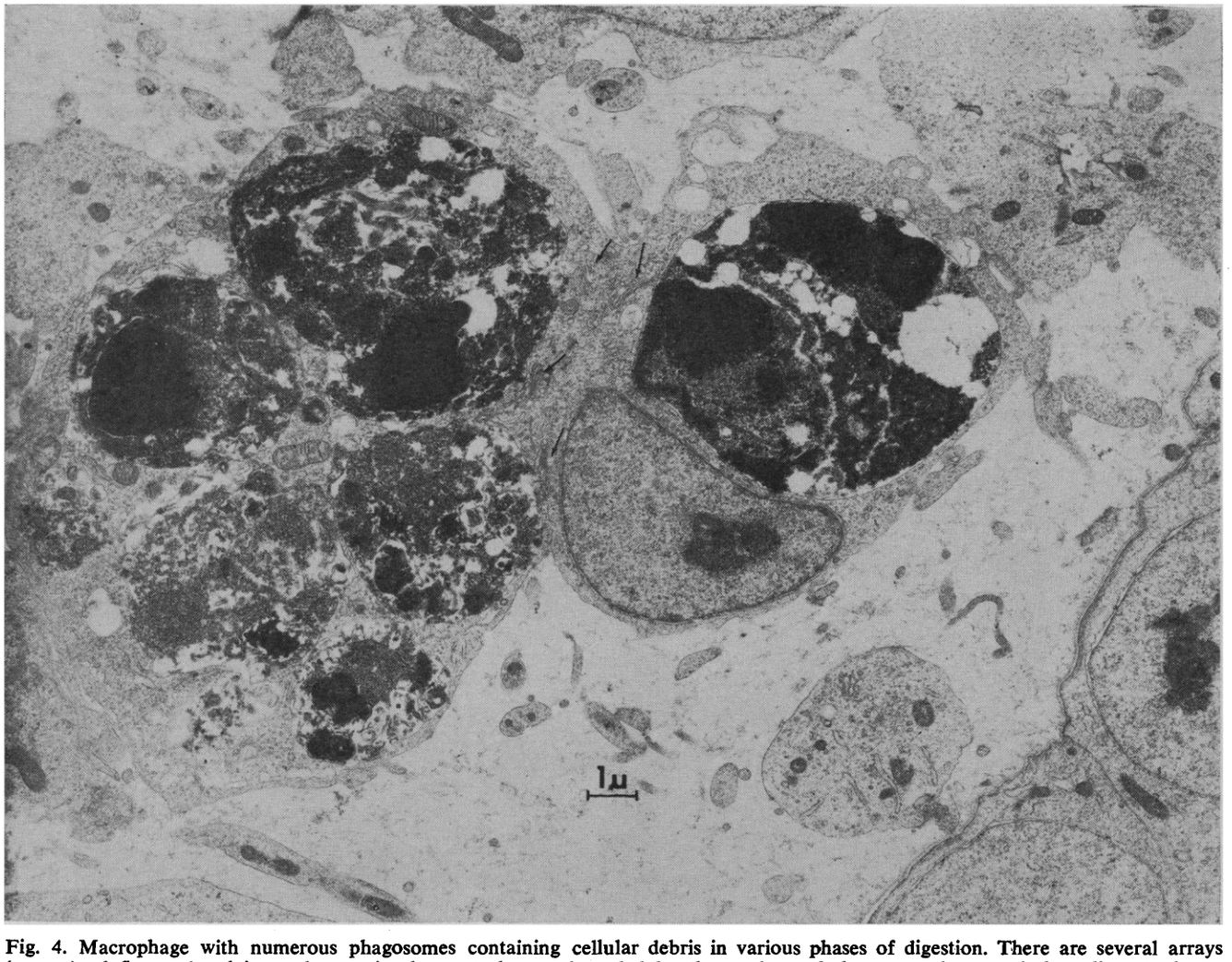

Fig. 4. Macrophage with numerous phagosomes containing cellular debris in various phases of digestion. There are several arrays (arrows) of flattened golgi membranes in the central zone bounded by the nucleus of the macrophage and the adjacent phagosomes; cytoplasm containing normal mitochondria and numerous polyribosomes ramifies around and between the phagocytic vacuoles (photograph courtesy of D. Heinkel). The tissues were fixed in glutaraldehyde, then in osmic acid, embedded in Vestopal, and stained with potassium permanganate and lead hydroxide.

cules and then added to pools. What effects the increased availability of metabolites might have would, of course, be highly variable, depending on the relative availability of similar materials to the recipient cells and on their possessing the machinery to handle the compounds. But, depending on their physiological state, one might expect to find such phenomena as mitotic stimulation and enhanced protein synthesis in embryonic cells in the vicinity of an area of necrosis. Wounds and implants of necrotic tissue do, indeed, stimulate mitosis and growth under some circumstances, but the mechanism is generally unknown. Do amino acids released in necrotic tissues stimulate in adjacent or phagocytizing cells the synthesis of their own activating enzymes (*51*)? Do differentiating tissues show enzymatic induction in response to certain substrates? There is generally no clear evidence that, during embryogenesis, genetic transcription for any enzyme protein is initiated by its substrate, although the rate of synthesis of the enzyme may be greatly enhanced by the presence of substrate in some instances (*52*). It is conceivable, nevertheless, that local enhanced availability of metabolites as a result of necrosis could have an effect on differentiating tissues. Wilde, for example, has offered evidence that the amino acid phenylalanine elicits in ectoderm of the urodele belly the differentiation of melanocytes, cells usually derived from the neural crest (*53*). The same amino acid will inhibit synthesis of melanin in melanocytes of embryonic chicks (*54*), presumably by competing with tyrosine, which enhances the process.

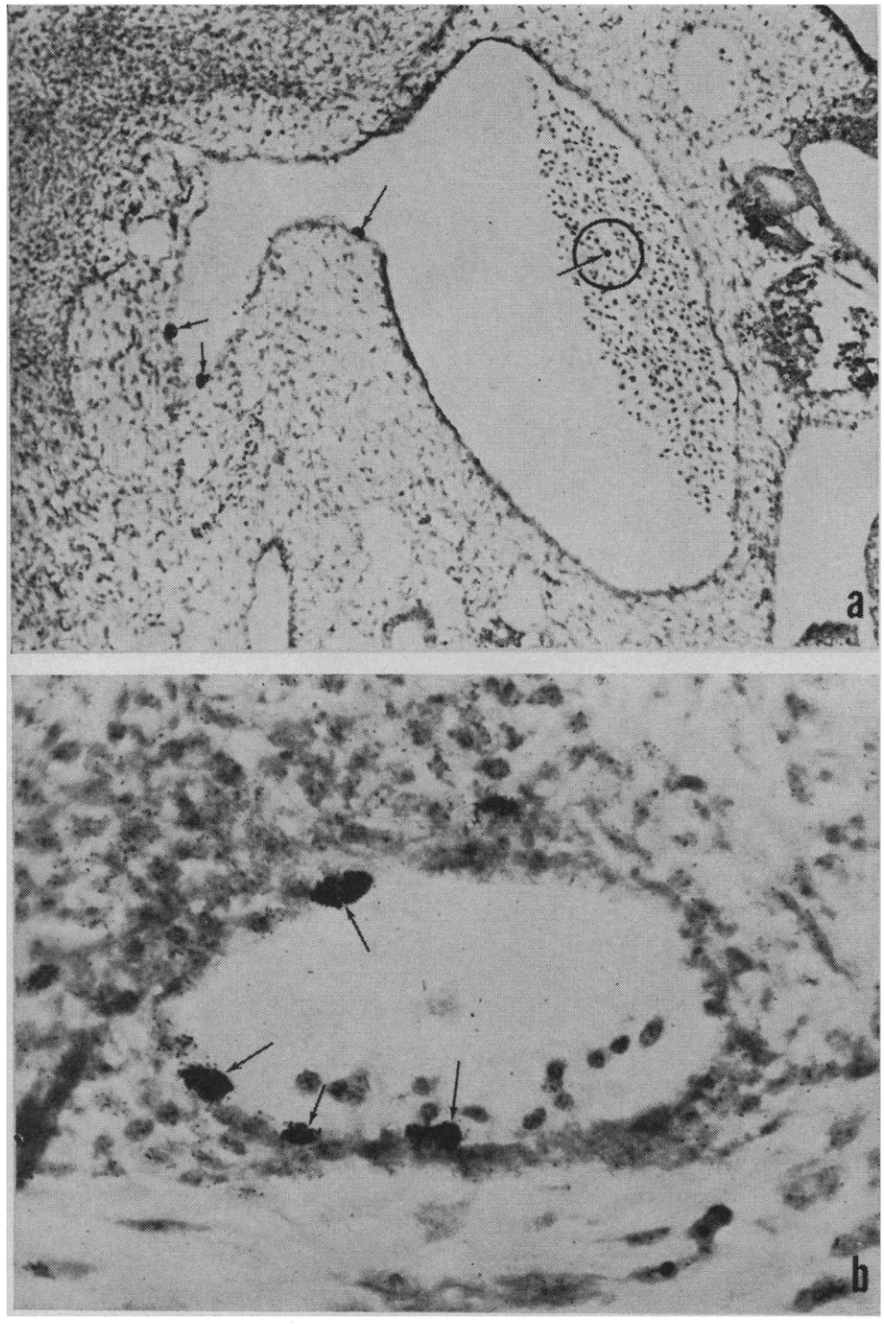

Fig. 5. Phagocytes laden with radioactive debris from a graft of limb bud. They are found in the subclavian artery and aorta of the host, which had not been labeled. a, Low-power view of the junction of the subclavian artery and aorta, showing macrophages (arrows) in the endothelium of the former and in the aortic contents. b, Another section at higher magnification showing labeled macrophages in and on the endothelium of the subclavian artery some distance after it leaves the aorta. The graft is outside the picture to the upper left. Labeling of host cells apparently results from the uptake of diffusible breakdown products from the degenerating graft.

What of macromolecules which might be made available during cellular breakdown? Are they ever utilized as such? Is their information of any significance to the cells which take them in by phagocytosis or micropinocytosis? Some cultured cells or cells of regenerating liver may incorporate into their genetic fine structure unchanged blocks of highly polymerized DNA (*55*) from heterologous or homologous sources. But of what significance to cells of a normally developing tissue would be the acquisition from necrotic cells of genetic information of a kind already possessed?

A number of reports indicate that uptake of specific RNA's can result in specific morphogenetic effects (*56*), in synthesis of tissue-specific enzymes or other protein molecules (*57*), and in induction of the synthesis of specific antibodies (*58*). Although results of these and similar studies must be approached with reservations (*59*), they, nevertheless, do suggest the possibility that more or less stable informational RNA might survive the death of the cell in which it was synthesized and contribute to the developmental performance of a neighboring phagocytic or structural cell. It seems unlikely, however, that the macromolecule would convey information that the neighbor could not transcribe from its own genome.

One may pose a final question: What is the role of the phagocytic cell where necrosis occurs during embryogenesis? Many tissues may be resorbed slowly, or even very rapidly in the apparent absence of phagocytes. Yet, phagocytes are most avid "eaters" of necrotic cells and often contain many times their original volume in the form of cell debris. Do they use the breakdown products for their own growth and reproduction? Release them into the extracellular matrix? Channel them to other parts of the body? One aspect of the problem is illustrated by Fig. 5 which shows macrophages, laden with radioactive debris from a necrotic graft of wing bud labeled with H^3-thymidine, in the subclavian artery and aorta of a 4-day-old chick embryo. *Quo vadistis?*

General Conclusions

The principal conclusion to be drawn from the foregoing discussion is that the death of cells and the destruction of tissues, organs, and organ systems are programmed as normal morphogenetic events in the development of multicellular organisms. Death in embryonic systems may thus be explored within the same conceptual framework as growth and differentiation.

The present exploration has revealed that death during embryogenesis serves utilitarian goals in some instances, at least, that its occurrence is subject to control by factors of the immediate cellular and humoral environment, and that aberrations in its normal pattern of expression provide the mechanism for realization of many mutant phenotypes. Hopefully, it has also pointed toward the appropriate formulation of some of the problems that confront us in understanding the control of death at the level of genetic transcription, the biochemical events which determine and accompany its occurrence, and the pathways of disposition and the developmental significance of disassembled cellular building blocks.

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Oases for the Future

New trends in gaining, using, and conserving fresh water are here or on the horizon.

Kenneth Hickman

We are repeatedly told there is a water problem. What is it, and where? In the broadest, average sense, there is none at present. World population stands at about 3×10^9 persons. Under the most extravagant conditions of use—agricultural, industrial, and domestic—the amounts used add up to about 2.2×10^5 gallons (8.3×10^6 liters) per year per capita (*1*), or a world total of 6.6×10^{14} gallons. The known world supply (*2*) of "fresh" fresh water is about 1.4×10^{16} gallons per year, or 50 times as much as anybody can need. Even by the year 2000, when the population may have doubled, the ratio of available water to needed water will be approximately 25 to 1.

These are gross average figures; the regional situation is very different. Most rain falls on wasteland, and much of this rain evaporates; perhaps 30 percent finds its way to rivers, and of this some 15 percent flows unused into the ocean (*3*). Thus the amount of available fresh water is some 2.0×10^{15} gallons per day, or about twice the total needed and enough, on the average, to meet the needs of the year 2000 but not those of the year 2010! The average is, of course, a fiction. Water, like air or money, is fantastically plentiful, but some people never have enough and some have none at all. These people and situations are our concern *now*.

Narrowing the inquiry to the terrestrial United States, we find that the gross daily precipitation is about 4.3×10^{12} gallons (*4*), the net yield from rivers and lakes is about 6.5×10^{11}, and the gross average demand is about $(185 \times 10^6 \times 2.2 \times 10^5)/365$—say 1.12×10^{11} gallons. Thus the ratio of available to needed water is roughly 5 to 1. But this is an average. Actually, an area of a million square miles (2½ million square kilometers) west and south of the Rockies is essentially without water, and the Great Lakes region has a surfeit. The situation in New York State may be evaluated (*5*) in terms of reservoirs and time rather than gallons and people. During the period of 1959 to 1965 the rainfall was less than the average 42 inches (107 centimeters) per annum in every year except 1960, the deficit totaling about 80 percent of one year's fall. The reservoirs, as of December 1965, were 33 percent full, but they are now (September 1966) 67 percent filled (*6*). Will swings of the rain cycle completely make up the deficiency? For short periods, undoubtedly yes, but not, I think, over the long pull. Our use of fuel appears to be increasing more rapidly than world climate is changing. The energy received from sunlight is of the order of 3.2×10^{16} British thermal units per 10^6 square miles per day (*7*). In North America, the energy released by human activity has reached a total of about 6×10^{13} British thermal units per day, adding 0.2 percent to the energy received from the sun, enough to raise the temperature by 0.6 degree Celsius. But this energy release is concentrated in three regions—the Eastern Seaboard, the Chicago–St. Louis central area, and the Los Angeles basin. In the first and third of these areas, in particular, the ratio of heat from fuel to heat from the sun is much higher than the average value. In these regions, too, carbon dioxide and other atmospheric pollution foster development of a radiation-heat trap which may increase the dry-bulb temperature by 3.6 or more degrees Celsius over areas of 1000 square miles. Civilization creates its own drought in proportion to its concentration of people and thus of water needs —and the situation will get worse. The

The author is professor of experimental research, Rochester Institute of Technology, Rochester, New York. This article is based on a talk given at the joint meeting of the French Engineers in the United States and the American Section of the Société de Chimie Industrielle, held in New York in February 1966. The text of the address appeared in the August 1966 issue of *Chimie et Industrie*.