FINE STRUCTURAL CHANGES IN THE INTESTINAL EPITHELIUM OF THE BULLFROG DURING METAMORPHOSIS

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ABSTRACT

The fine structural changes occurring in the columnar absorbing cells of the intestinal epithelium during metamorphosis of the bullfrog, *Rana catesbeiana*, have been examined by phase contrast and electron microscopy. Tissue samples taken just posterior to the entrance of the hepatopancreatic duct were fixed in veronal acetate-buffered osmium tetroxide and embedded in methacrylate. Under the action of the metamorphic stimulus (thyroid hormone), specific and characteristic responses were given by differentiated larval cells and undifferentiated basal cells within the same epithelium. The functional larval cells underwent degenerative changes and were retained for a time within the metamorphosing epithelium. Dense bodies appeared and increased in number in association with the loss of normal cell structure. Because of their morphology and time of formation, these bodies have been tentatively identified as lysosomes. Early in metamorphosis the basal cells did not change, but they subsequently proliferated to form a new cell layer beneath the remaining degenerating cells that lined the lumen. After the dying cells were sloughed into the gut, the new epithelium differentiated to form the adult tissue. The columnar epithelial cells of the mature animal differed in their fine structural organization from their larval precursors. Therefore, their adult configuration was molded by the action of the metamorphic stimulus.

INTRODUCTION

Amphibian metamorphosis is a phenomenon that is especially attractive to those interested in understanding morphogenetic processes. It is obvious that the sudden onset of metamorphic changes that progress rapidly and dramatically allows easy collection of developmental stages. But more than this, the period of final maturation that transforms larva into froglet occurs under the influence of the thyroid secretion. The first indication of this hormonal control of differentiation came in the work of Gudernatsch (1). His observations were extended and confirmed in a variety of experiments by many different investigators. Etkin (2) has provided a review of the body of this work, which led to widespread recognition of the thyroid hormone as a causative agent—probably the unique one—in amphibian metamorphosis.

As the structure of several physiologically active components of the thyroid gland hormone became known, investigators produced structural changes through the use of known chemical agents. Exploitation of this method has led to more detailed

understanding of the interaction between the hormone and responding tissue, and it is now well established that each tissue and even individual cell types within tissues respond specifically to the metamorphic stimulus. This point was well illustrated on the anatomical level by the experiment of Schwind (3), on the histological level by the work of Weiss and Rossetti (4), and more recently on a biochemical level in investigations such as that of Finamore and Frieden (5). Therefore it seems reasonable to expect that equally specific cytological changes occur both as a basis for the grosser, well known events of metamorphosis and as a reflection of the subtle, well ordered biochemical changes more recently described. As yet, however, fine structural changes have not been observed. Although formerly it was, of course, impossible to record the effects of the metamorphic stimulus upon the fine structure of cells or to follow with certainty the fate of individual cells, now study by electron microscopy can provide unequivocal identification of cell types and detection of cytological changes within them. Indeed, this developmental problem seems especially suited to this particular method of investigation. The short, dramatic nature of events allows the complete process to be observed easily and tissue samples to be taken at frequent intervals. Furthermore, the changes already revealed by other means hold promise that cytological changes seen under the electron microscope will be clear-cut and easy to define. With these considerations in mind, an investigation of fine structural changes in the intestinal epithelium of the bullfrog during metamorphosis was undertaken.

The intestinal epithelium promised to be a suitable object for such an investigation primarily because previous studies had established that striking metamorphic changes occur in this organ. The general shortening of the tract, changes in size and position of the stomach, and rearrangement of the coils of the intestine were described many years ago (6–10). In addition, histological and cytological observations of the epithelial changes in the anuran intestine (7, 8, 11–13) demonstrated that cell degeneration and regeneration were important in reshaping the tissue during metamorphosis. Furthermore, in spite of complicated tissue changes, there seemed little doubt that comparable samples of epithelium could easily be located throughout metamorphosis by removing the area just posterior to the entrance of the hepatopancreatic duct into the intestine.

The observations made here will be concerned with the specific responses of the functional larval columnar epithelial cells, which are destroyed and removed from the epithelium. Their behavior will be contrasted with that of the basal or germinative cells, which are stimulated to divide and to undergo cytodifferentiation preparatory to the formation of the adult epithelium. The differential response of these two cell types, which is to be examined here in detail, is an early event in the metamorphic process and prepares the way for development of the adult intestinal epithelium. Finally, it will be shown that the differentiation of basal cells follows a new course under the influence of the metamorphic stimulus, producing cells which differ in fine structure from their larval precursors.

MATERIALS AND METHODS

Animals

All observations to be reported here were carried out on bullfrogs, *Rana catesbeiana*, which were collected from the Connecticut Valley region of Massachusetts. During the summer months, premetamorphic tadpoles hatched in the current year are found together with metamorphosing forms hatched in the previous year. In addition to the large adult bullfrog, a group of animals was selected for the study of natural metamorphosis. Those used in the present work will be described briefly below. Fig. 1 includes sketches of their general form as well as certain body measurements.

LARVAL STAGE: A long fin-bearing tail and a globular body shape characterize the vigorously swimming herbivorous tadpole. During the spring, short legs appear on the year-old tadpole, but they remain small, and for a time further maturational changes are not observed.

HINDLIMB GROWTH STAGE: The onset of metamorphosis is heralded by noticeable rapid growth of the hindlimbs. Body form, tail shape, and feeding habits remain of the larval type.

FORELIMB STAGE: Although tail length is almost unchanged, the shape of the body and head are markedly froglike by the time that both forelimbs have emerged. Continued growth produces long, well developed hindlimbs.

EARLY TAIL REDUCTION STAGE: At this stage, the larva has become a froglet with a long tail. Reduction of the tail fin indicates that this organ is beginning to disappear. By this time the animal has stopped feeding and does not take food again until

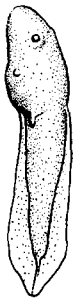
STAGE	LARVAL	HINDLIMB GROWTH	FORELIMB GROWTH	EARLY TAIL REDUCTION	LATER TAIL REDUCTION
GROSS ANATOMICAL APPEARANCE					
POSITION OF STOMACH	RIGHT	RIGHT	RIGHT	CENTER	LEFT
BODY LENGTH	35mm	38	40	33	37
TAIL LENGTH	52 mm	56	64	57	29
HINDLIMB LENGTH	6 mm	14	50	43	40

FIGURE 1 This figure describes the naturally occurring stages of bullfrog metamorphosis observed in the present investigation. Each stage was given a name, which is shown in the top line. All the sketches of the animals were made at the same magnification by tracing projected photographs taken just prior to fixation. The position of the stomach in the body cavity is recorded below each drawing. In the lower part of the figure are listed the lengths (in millimeters) of the body, tail, and hindlimbs of the animals described.

after metamorphosis is completed. Internally the stomach is found in the center of the body cavity rather than on the right side, the characteristic larval position.

LATER TAIL REDUCTION STAGE: Tail reduction is rapid during this late metamorphic stage. The stomach now lies on the animal's left side as it does in the adult.

Preparation of Material for Light and Electron Microscopy

Fixation of material for all histological and cytological observations in this work was carried out in a veronal acetate-buffered 1 per cent solution of osmium tetroxide, prepared in the manner of Palade (14), to which a final concentration of 4.9 per cent sucrose was added (15). The fixative was injected into the intestine just above the entrance of the hepatopancreatic duct, so that it spread into the duct region and 1 cm or more posterior to it. Care was taken not to damage the tissue below the duct during the injection, since this region was to be used for all observations. After removal from the animal, the tissue was placed in a drop of fixative, cleared of food material, cut into transverse slices, and transferred into a jar of fixative, which was cooled in an ice bath. Fixation times varied from 15 to 40 minutes. Tissues were then placed for 15 to 30 minutes in 70 per cent ethyl alcohol, then 30 minutes in 95 per cent. Dehydration in several changes of absolute ethanol was carried out over a period of 2 hours. Specimens were then infiltrated and embedded in methacrylate. The embedding medium was composed of methyl and butyl monomers of the plastic in the ratio of 20:80. This mixture contained 0.25 per cent uranyl nitrate. To bring about polymerization, 1 per cent Luperco was used as a catalyst. All sectioning was done with a glass knife on a Porter-Blum microtome. For phase contrast microscopy, sections 2 μ thick were placed on glass slides and mounted in paraffin oil. For electron microscopy, some of the thin sections were stained for 15 minutes in a saturated solution of uranyl acetate dissolved in 40 per cent ethanol. Electron micrographs

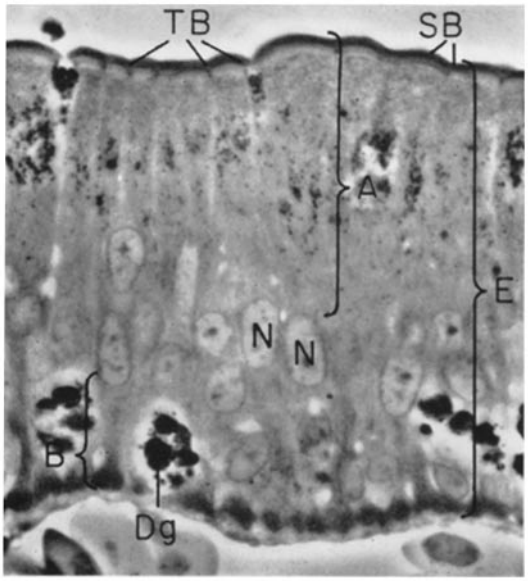


FIGURE 2 Phase contrast photomicrograph of a cross-section through the intestinal epithelium (*E*) of the bullfrog larva. Striated borders (*SB*) line the lumen of the gut. Terminal bars (*TB*) separate each cell from its neighbors. Granular supranuclear cytoplasm (*A*), a zone of nuclei (*N*), and a subnuclear area (*B*) may be observed. Dense granular areas (*Dg*) probably represent degenerating cells. × 1000.

were taken on RCA microscopes, models EMU 2 and 3C, at magnifications ranging from 1000 to 27,000, and thereafter enlarged photographically.

OBSERVATIONS

The Structure of the Larval Intestinal Epithelium

Several important structural features of the intestinal epithelium of the bullfrog larva (Fig. 1) may be observed immediately upon inspection of a phase contrast photomicrograph of a cross-section through the wall of the gut (Fig. 2). The most prominent units are the columnar absorbing cells, which are as high (50 μ) as the epithelium itself (*E*). The striated borders (*SB*) of these cells are seen lining the intestinal lumen, and terminal bars (*TB*) demarcate each cell from its neighbors. The extensive supranuclear region (*A*) is filled with granular cytoplasm. Oval nuclear profiles (*N*) separate the latter region from the less extensive subnuclear area (*B*). Other differentiated cell types, such as goblet cells, may be present, but they are seen much less frequently; it is, then, with the columnar absorbing cells that this description is chiefly concerned.

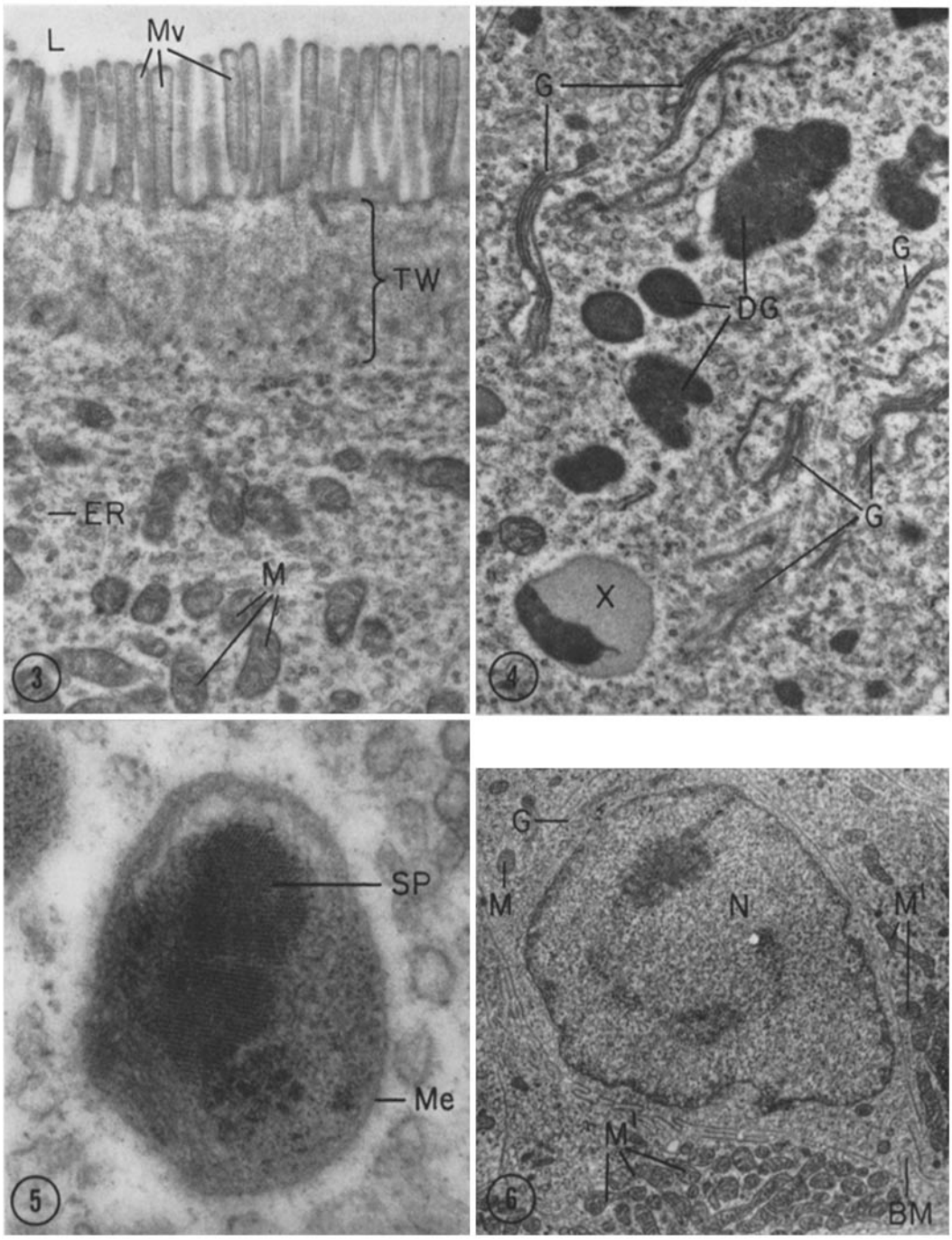
Although it is apparent from light microscopy that differentiation within the larval intestinal

FIGURE 3 Electron micrograph of the apical region of a columnar absorbing cell within the intestine of a larval bullfrog. Microvilli (*Mv*) project into the lumen (*L*) of the gut. Fibrillar material within them is similar to that found in the terminal web (*TW*). Deeper in the apical cytoplasm, mitochondria (*M*) and small vesicular components of the endoplasmic reticulum (*ER*) are frequently seen. × 17,000.

FIGURE 4 Electron micrograph through the supranuclear cytoplasm of a columnar absorbing cell of the larval intestinal epithelium. An extensive Golgi region (*G*) is characteristically found near the nucleus. Near by, dense, membrane-bounded granules (*DG*) are abundant. Their size and form (*X*) may vary. ×17,000.

FIGURE 5 Dense granule typical of those abundant in the supranuclear region of a larval columnar absorbing cell. Such granules are found in association with an extensive Golgi region. They are membrane-bounded (*Me*) and contain a matrix of medium density within which lie small dense particles. The latter may be regularly arrayed (*SP*) or randomly scattered within the matrix. × 80,000.

FIGURE 6 Electron micrograph of a basal cell lying near the basement membrane (*BM*) of the intestinal epithelium of the larval bullfrog. Surrounding the relatively large nucleus (*N*) is a rim of cytoplasm containing a few mitochondrial profiles (*M*) and a juxtanuclear Golgi region (*G*). No evidence of cytodifferentiation is observed. In contrast, the subnuclear cytoplasm of the columnar absorbing cells is crowded with mitochondria (*M'*) in the region adjacent to the basement membrane. × 6500.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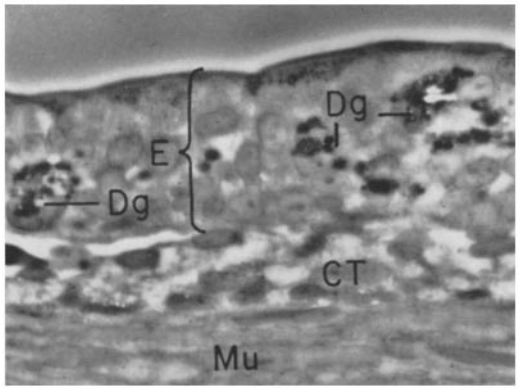


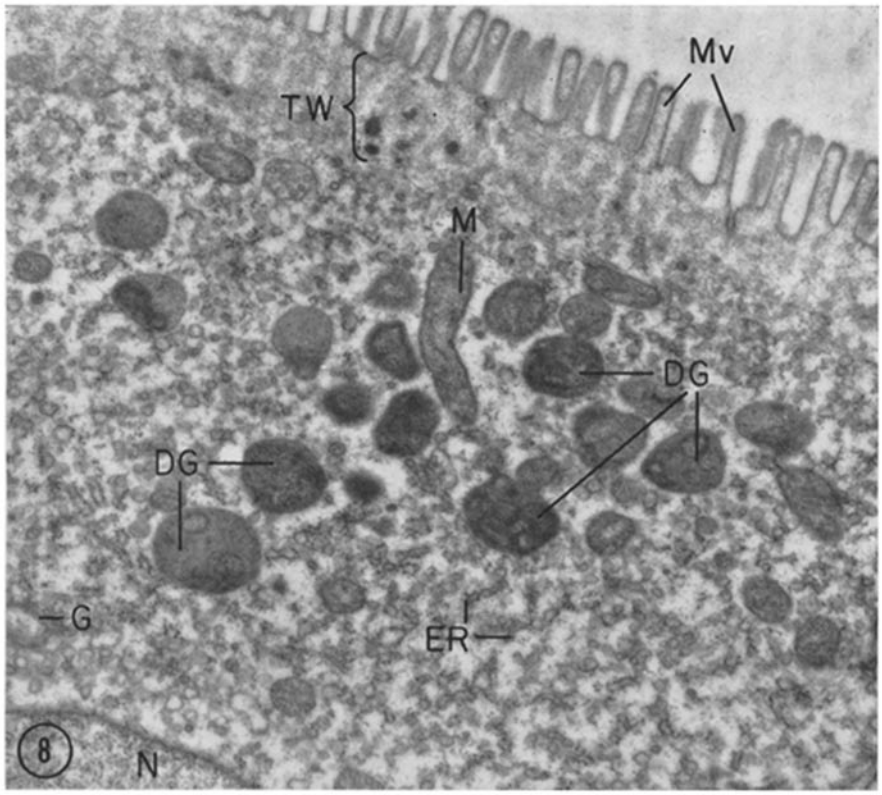
FIGURE 7 Phase contrast photomicrograph showing a cross-section through the wall of the gut taken from a bullfrog during an early stage of metamorphosis (hindlimb growth stage). The epithelium (*E*) is only one-third as high as that found in the larva (Fig. 2, *E*) and contains many dense granular areas within it (*Dg*). Connective tissue (*CT*) and smooth muscle (*Mu*) of the intestinal wall may also be observed. × 900.

epithelium gives rise to absorbing cells with highly organized and complex structure, this fact is demonstrated more clearly by electron microscopy. Thus in thin sections of material comparable to that shown in Fig. 2, the striated border is resolved into a series of fingerlike projections, the microvilli (Fig. 3, *Mv*), which extend into the lumen (*L*) of the gut. They average about 1.2 μ in height and 150 mμ in diameter. Fine fibrillar material within the microvilli is continuous with that found in the underlying ectoplasmic zone (terminal web, *TW*), from which most of the commonly occurring cytoplasmic organelles are excluded. Basal to this cortical zone, mitochondria (*M*) are found in fairly dense array among small vesicular components of the endoplasmic reticulum, several of which may be seen near *ER* in Fig. 3. Between the mitochondrial zone and the nucleus, the supranuclear cytoplasm is typified by a surprisingly extensive Golgi region (Fig. 4, *G*) and the presence of many granules filled with dense particulate material (*DG*). These often have oval profiles, measuring about 0.6 μ along their long axes, but they may be irregular in shape and show wide variation in size. More rarely, dense particulate material is observed within a larger granule containing a homogeneous matrix of low density (*X*). Closer inspection of a typical granule (Fig. 5) reveals that it is a membrane-bounded structure (*Me*), within which are small dense particles (*SP*). These may be irregularly distributed or regularly arrayed. Each measures about 70 A in diameter and lies in a matrix of medium density. The presence of the dense granules within the supranuclear cytoplasm doubtless accounts for the granularity of this region when it is observed under the phase contrast microscope.

This brief, though incomplete, description illustrates the fact that the columnar absorbing cell of the larval intestinal epithelium is a highly organized and differentiated unit. This complexity

FIGURE 8 Electron micrograph showing the supranuclear cytoplasm of a columnar absorbing cell during an early stage of metamorphosis (hindlimb growth). Although both structures are less extensive than at the larval stage, microvilli (*Mv*) and the terminal web (*TW*) are still present. Normal cytoplasmic organelles—mitochondria (*M*), elements of the endoplasmic reticulum (*ER*), and a Golgi region (*G*) lying near the nucleus (*N*)—occur in the attenuated supranuclear area. In addition, dense granules (*DG*) are characteristic of the columnar cells at this stage. × 14,500.

FIGURE 9 Electron micrograph of an abnormal, degenerating cell, a type frequently encountered within the intestinal epithelium of a bullfrog during the early stages of metamorphosis. It is surrounded by a plasma membrane (*PM*) and contains normal mitochondria (*M*). However, its most striking feature is an array of dense granules (*DG*) of various sizes. The irregularly clumped material at right may perhaps represent remnants of the nucleus. The degenerating cell is covered by portions of two cells (*A* and *B*) which possess microvilli (*Mv*) and a terminal web region (*TW*); but in contrast to the normal larval cell structure, the microvilli may be absent from certain parts of the cell surface (*Z*) or project at an acute (rather than right) angle (*Mv*) into the lumen of the gut (*L*). Both in the cell that is highly abnormal and in the cells covering it, dense granules of similar size and structure (compare *X* and *X'*) may be found. These are presumed to give rise to the large dense granules (*DG*) formed during cell degeneration. × 10,000.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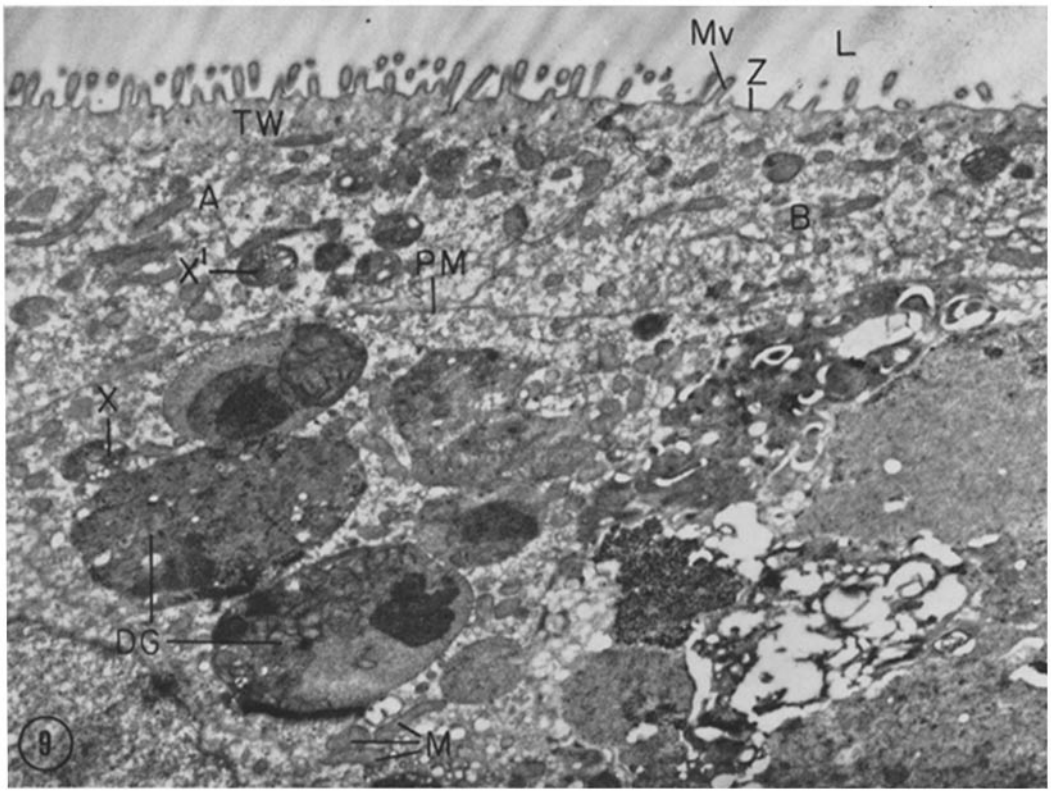
Mv
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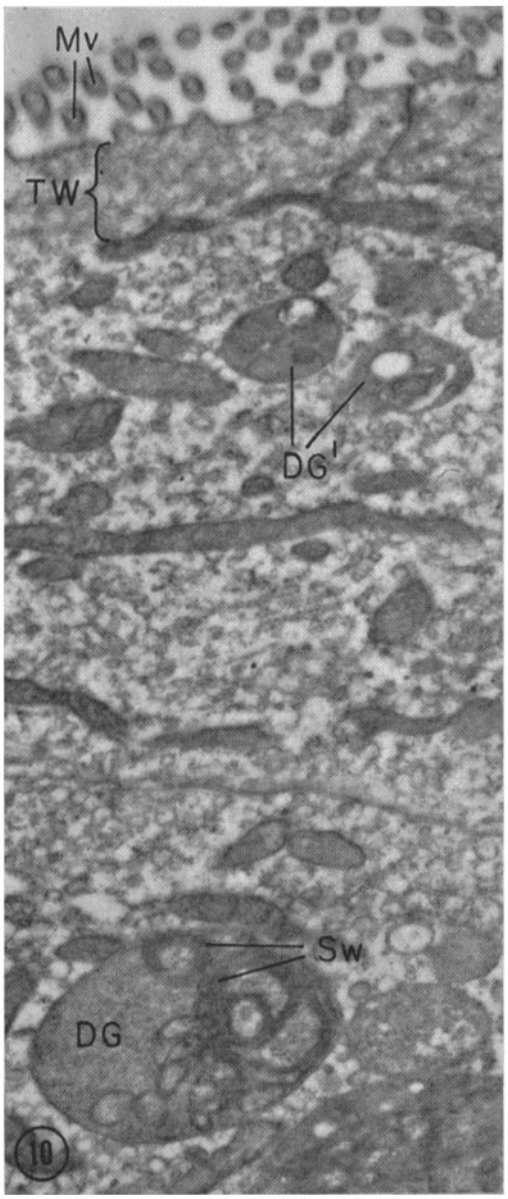
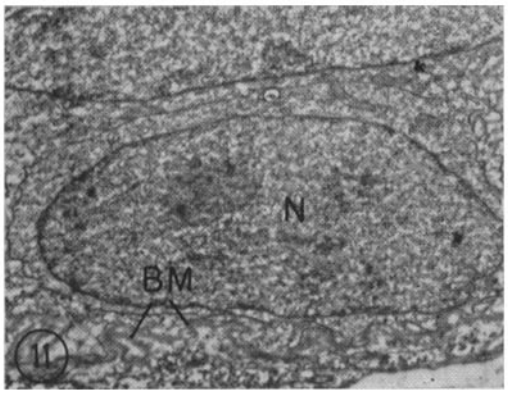




is in marked contrast to the structure of the basal cells, which are seen infrequently but regularly within the epithelium. In Fig. 6, a cell of this type is shown lying near the basement membrane (*BM*). The nucleus (*N*) is large in relation to the amount of surrounding cytoplasm. The latter contains a Golgi region (*G*) near the nucleus and a few mitochondria (*M*) in contrast to the large number (*M'*) found in the basal region of adjacent columnar absorbing cells. The ground substance of the cytoplasm is crowded with small particles, presumably ribonucleoprotein. The particles are not associated with the small vesicular components of the endoplasmic reticulum that are also present. In short, the basal cell exhibits features of cytological organization associated with undifferentiated cells generally (16). This point will be considered again in the Discussion.

Early Metamorphic Changes in the Intestinal Epithelium

The metamorphic process interrupts larval life in a sudden and dramatic fashion. The rapid growth of the hindlimbs is particularly striking (Fig. 1, hindlimb growth stage). Examination by phase contrast microscopy of a cross-section through the intestinal epithelium taken from such an animal reveals that histological changes are already occurring. The epithelium (Fig. 7, *E*) is now approximately 16 μ in height, or about one-third of its former height. Furthermore, it is characterized by the frequent occurrence of coarse, dense granular areas (*Dg*).

A more detailed description of these early metamorphic changes is derived from examination of electron micrographs taken of comparable tissue samples. For example, Fig. 8, which shows the

FIGURE 10 Electron micrograph of the intestinal epithelium of the bullfrog fixed early in the metamorphic period (hindlimb growth stage). In the upper part of the picture, a part of a larval columnar cell is shown. It possesses microvilli (*Mv*) and a terminal web (*TW*), and it has within it dense granules (*DG'*) that are generally similar in structure to larger granules (*DG*) within abnormal cells lying in the lower part of the epithelium. In the larger granule, swirls of internal membranes (*Sw*) are quite clearly seen. × 17,000.

FIGURE 11 Electron micrograph of a basal cell lying near the basement membrane (*BM*) within the intestinal epithelium early in the metamorphic period (hindlimb growth). The nucleus (*N*) is surrounded by a slender ring of undifferentiated cytoplasm. The cell shown here is similar to the basal cell found within the larval epithelium (Fig. 6). × 7000.

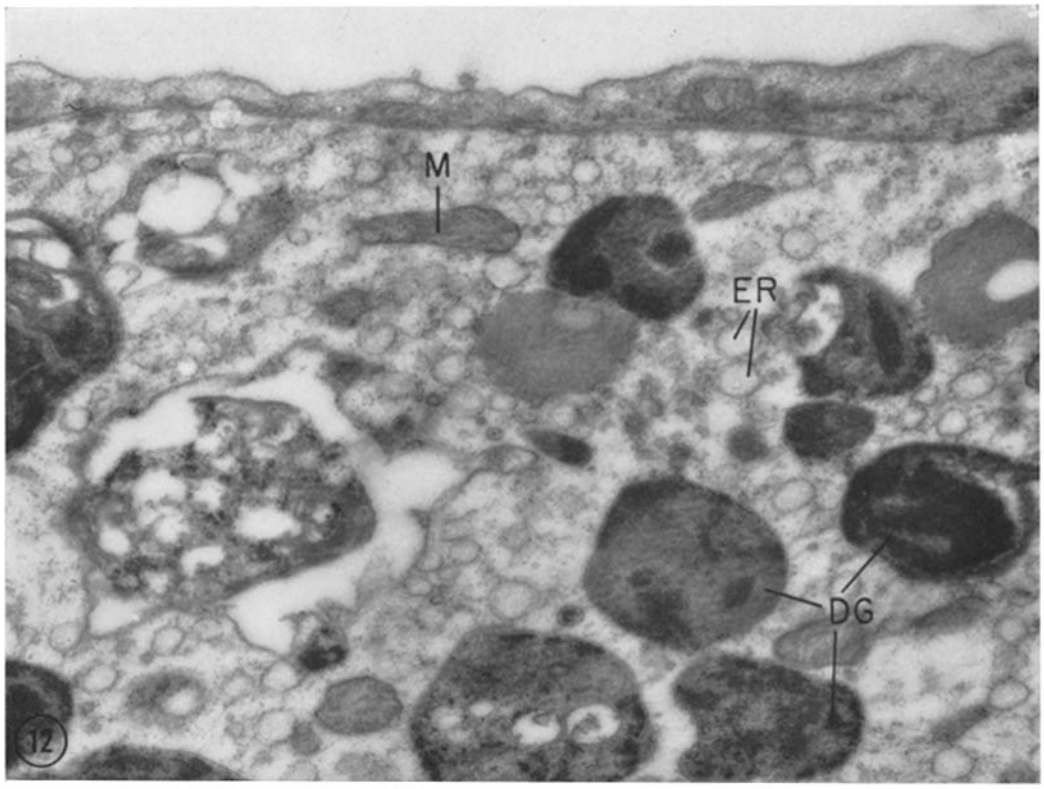
apical region of an absorbing cell, demonstrates that the complex organization of the larval cell has, to a certain extent, been lost. Microvilli (*Mv*) are shorter; in some instances they are fewer (Fig. 9, *Z*) and are not always perpendicular to the cell surface (Fig. 9, *Mv*). Beneath the terminal web (Fig. 8, *TW*) the supranuclear cytoplasm is less extensive and lacks the zonation that is so striking in the larva. It does contain, however, recognizable mitochondria (*M*), elements of the endoplasmic reticulum (*ER*), and Golgi material (*G*). In addition, oval membrane-bounded granules (*DG*) are frequently observed. As in the case of the dense granules occurring in the cells of premetamorphic animals, these bodies often contain dense particles, measuring about 70 A in diameter, together with membranous material within a homogeneous matrix of medium density. However, in comparison with the granules of the larval stage, the membranous element is more prominent. Furthermore, the number of granules per cell seems on the whole to be less than in the premetamorphic period. Therefore, the relationship between the dense granules found at the two stages so far examined is not immediately clear; for it is not certain that granules seen in metamorphosing tissue arose from those existing in the larval cell.

In addition to these many changes in their fine structure, the number of intestinal epithelial cells has been reduced. Counts made of the numbers of nuclei for a standard length of epithelium (50 μ) show that whereas in the larva there are 12 to 13, the early metamorphic stage has only 8 to 9. The distortion of the cell shape and reduction in height, apparent in both light and electron micrographs, probably results from stretching of the cells to cover temporarily a larger area of the gut lumen as units are lost. Yet the transformed larval columnar epithelial cells continue to line the gut. Since the tadpole still feeds, it is presumed that they also function to some extent in absorption, even in their changed condition.

The nature of the densely staining granular areas, easily visible in phase contrast photomicrographs (Fig. 7, *Dg*), is readily determined by examination with the electron microscope. In Fig. 9 one of these unusual areas is illustrated. Beneath the cytoplasmic covering provided by two more normal cells (*A* and *B*) lies what appears to be an abnormal cell. It is bounded by a plasma membrane (*PM*) and has within it recognizable cell organelles, such as mitochondria (*M*); but it also contains dense spherical to oblate masses of various sizes (*DG*). More detailed examination of representative granules from another such cell (Fig. 10, *DG*) shows that within the outer limiting membrane, swirls of membranes (*Sw*) and very dense particles, measuring about 70 A in diameter, lie in a homogeneous matrix of medium density. Small particles of similar size and density are also observed lying free in the cytoplasm. In spite of their lack of uniform internal arrangement, the granules are thought to constitute a single class of organelle, characteristic of the abnormal cytoplasm. The structure of cells such as these seems well beyond the limit of variations found in normal cells and is believed to be related to the progress of irreversible degenerative changes, which are destroying normal cell organelles.

The origin of the abnormal cells, or degenerating cells as they will now be called, though difficult to identify with complete certainty, is in all likelihood the larval columnar absorbing cells. The latter, as already noted, possess dense granules, which are similar in size to the smaller granules found in the degenerating cells. In Fig. 9, for example, the granule (*X*) lying in the abnormal cytoplasm is very similar in size (and structure) to that (*X'*) found in a cell having a free surface typical of columnar absorbing cells at this early stage of metamorphosis. Further examination confirms the structural similarity between the smaller granules (Fig. 10, *DG'*) in the more normal cells, in which the microvilli (*Mv*) and terminal web (*TW*) are still present, and the larger granules lying in degenerating cells (*DG*). Dense particulate material and swirls of membranes lie within a limiting membrane in the granules of both cells. Therefore, the dense granules observed in larval columnar epithelial cells early in metamorphosis presumably increase in size and prominence and are associated with degenerative processes that transform a large part of the cytoplasm of the larval epithelial cells into masses of autolysing protoplasm. Yet in spite of their catabolic state, the altered cells are retained within the epithelium, enclosed within the more normal, presumably functioning units (Fig. 9). The retention of the degenerating cells in the basal region of the epithelium suggests that during metamorphosis material from them may be reabsorbed and thereby conserved.

In contrast to the marked changes in the configuration of the columnar absorbing cells, the structure of the basal cells is unchanged in the early stages of metamorphosis. In Fig. 11 an oval nucleus (*N*) of a basal cell is shown surrounded by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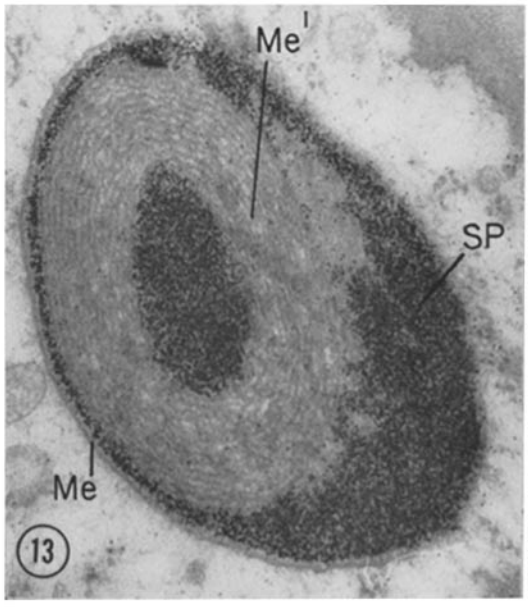
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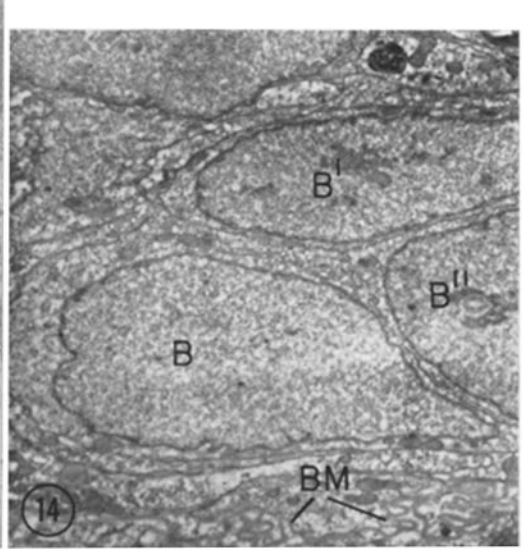
B'
B''
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BM
14

cytoplasm that is undistinguished by any special cytodifferentiation. In structure and in its position near the basement membrane (*BM*) this cell is in every respect like the basal cell of the larval epithelium, illustrated in Fig. 6, and it further resembles the larval cell in its infrequent occurrence.

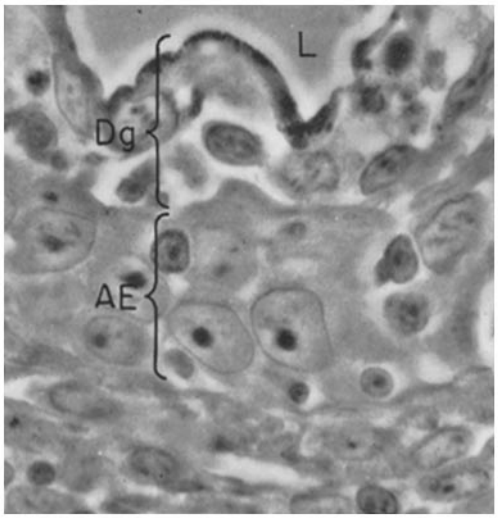


FIGURE 15 Phase contrast photomicrograph of a cross-section through the intestinal epithelium of the bullfrog during an advanced stage of metamorphosis (early tail reduction). A layer of degenerating cells (*Dg*) lines the gut lumen (*L*) and covers a new cell layer, which is forming the adult epithelium (*AE*). × 900.

Since in the metamorphosing epithelium as in the larval tissue very few mitotic figures were observed, there seems to be no evidence that this cell type is yet responding to the metamorphic stimulus.

Progressive Degeneration of Differentiated Larval Epithelial Cells

As metamorphosis proceeds to the forelimb stage (Fig. 1), very few cells indeed can be found that resemble in any way the functional epithelial cells of the larval intestine. Rather, the epithelium is a mass of granular areas similar to those identified just above as degenerating cells. Observation by electron microscopy reveals that they have undergone changes that are similar to those found in abnormal cells at the hindlimb growth stage and that presumably foretell their imminent death. In some instances, remnants of normal cell structure can still be observed. Some cells possess a differentiated free surface with microvilli extending from a fibrillar ectoplasmic zone, although dense granules frequently occur in the underlying cytoplasm. However, in most of the cells degeneration is more advanced and normal cell configuration is completely lost. The cytoplasm is often crowded with granules and bodies of varying size and form (Fig. 12, *DG*). The granules are commonly made up of small dense particles (Fig. 13, *SP*) associated with membranous material (*Me'*), the whole surrounded by a membrane (*Me*). Their structure is therefore similar to that of granules already described within degenerating cells at the hind-

FIGURE 12 Electron micrograph of an intestinal epithelial cell of the bullfrog. During metamorphosis, just after emergence of both forelimbs, the typical cell contains profiles of normal mitochondria (*M*) and vesicular components of the endoplasmic reticulum (*ER*); but the outstanding feature of its cytoplasm is the presence of a large number of dense granules (*DG*). The structures characteristic of larval columnar absorbing cells are rarely seen. × 27,000.

FIGURE 13 Electron micrograph of a dense granule, typical of those found in abundance within degenerating columnar epithelial cells of the bullfrog intestine during metamorphosis (forelimb stage). Within an outer limiting membrane (*Me*), lamellae of membranous material (*Me'*) are found together with small dense particles about 70 A in diameter (*SP*). × 67,000.

FIGURE 14 Electron micrograph of the intestinal epithelium of the bullfrog during metamorphosis. A nest of undifferentiated basal cells (*B*, *B'*, *B''*) lies near the basement membrane (*BM*). The basal cells have apparently undergone division at a time when larval columnar cells are in an advanced stage of degeneration (Fig. 12). Their structure remains similar to that of basal cells found within the larval epithelium (Fig. 6) and at an early period during metamorphosis (Fig. 11). × 5800.

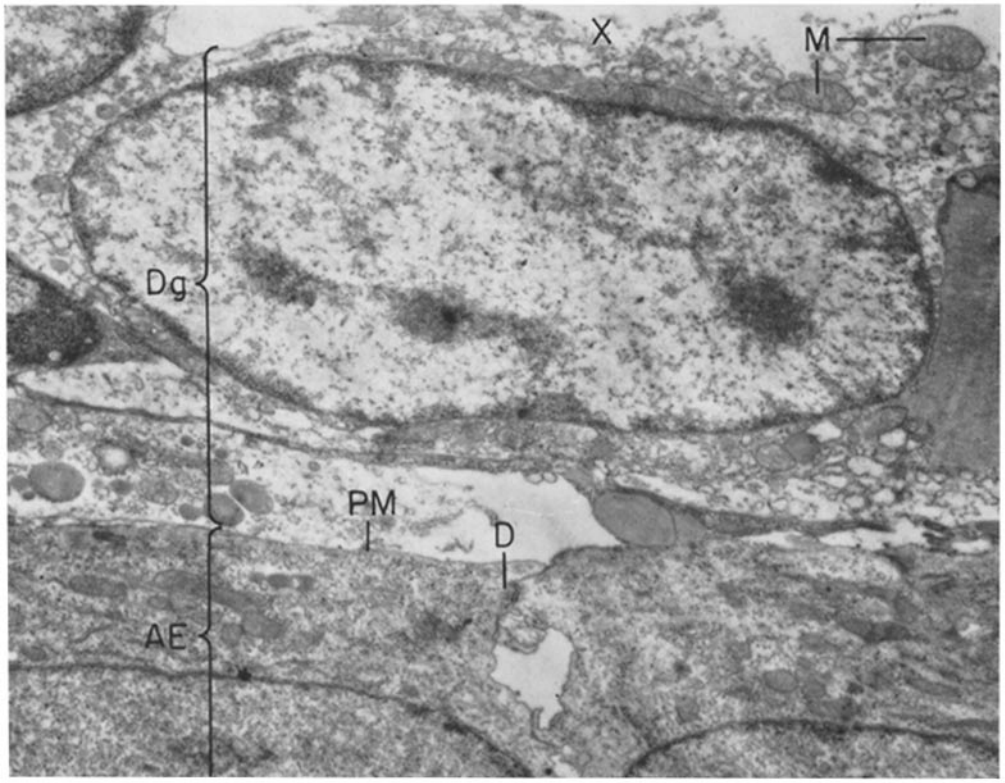
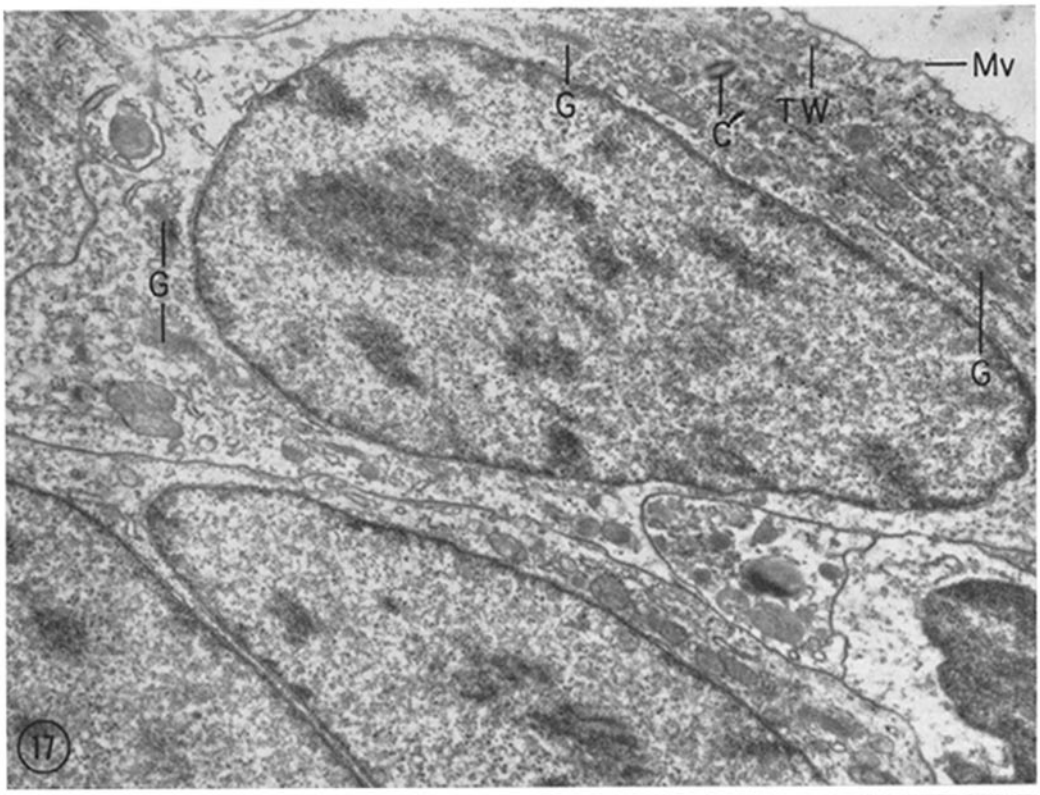


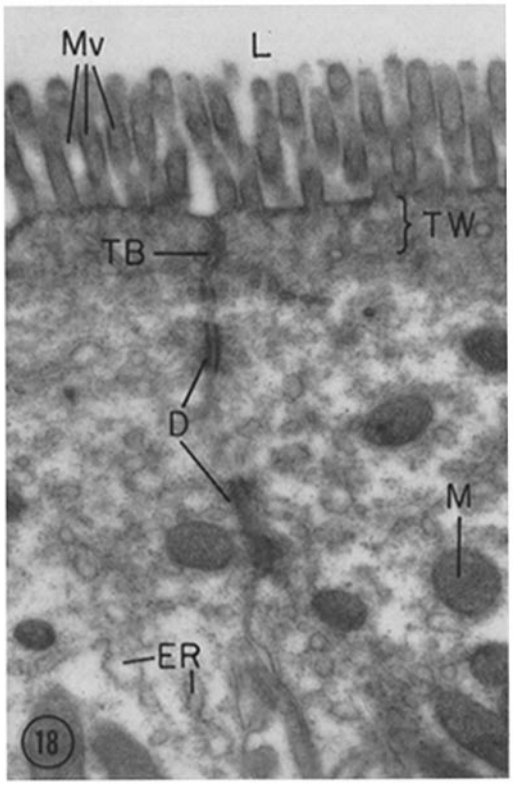
FIGURE 16 Electron micrograph of the bullfrog intestinal epithelium, showing the bilayered structure present during metamorphosis (early tail reduction). A degenerating cell (*Dg*) lines the gut. Although its plasma membrane is disrupted (*X*), some of its cytoplasmic organelles (*i.e.*, mitochondria, *M*) retain normal structure. Beneath the degenerating cell a new epithelial layer (*AE*) is seen. The lateral surfaces of the developing cells are attached by a desmosome (*D*), but as yet their apical plasma membranes (*PM*) remain undifferentiated. × 8700.

FIGURE 17 Electron micrograph of the intestinal epithelium of the bullfrog late in the metamorphic period. The gut lumen is lined by differentiating cells, which will form functional units of the adult tissue. Early development of the microvilli (*Mv*) and terminal web region (*TW*) may be observed. Note also the presence of a centriole (*C*) and an extensive Golgi region (*G*). × 10,000.

FIGURE 18 Electron micrograph of a columnar absorbing cell of an adult bullfrog. Microvilli (*Mv*) project into the intestinal lumen (*L*). A terminal web (*TW*) can also be seen. The lateral cell borders of two adjacent cells are joined near their apices by a terminal bar (*TB*) and a series of desmosomes (*D*). Mitochondria (*M*) and vesicular elements of the endoplasmic reticulum (*ER*) are present beneath the specialized cell border. The general disposition of cell structure is similar to that in the larval columnar absorbing cell; however, in the adult the microvilli are shorter and the terminal web less extensive. × 24,000.

FIGURE 19 Electron micrograph of a columnar absorbing cell taken from the intestinal epithelium of the adult bullfrog. An extensive Golgi region (*G*) lies near the nucleus (*N*). A group of flattened, rough surfaced cisternae of the endoplasmic reticulum (*ER*) is assembled in the supranuclear region. Mitochondria (*M*) are present, but no dense granules occur in the adult cell. × 20,000.


Mv
G
C
TW
G
G
17


Mv
L
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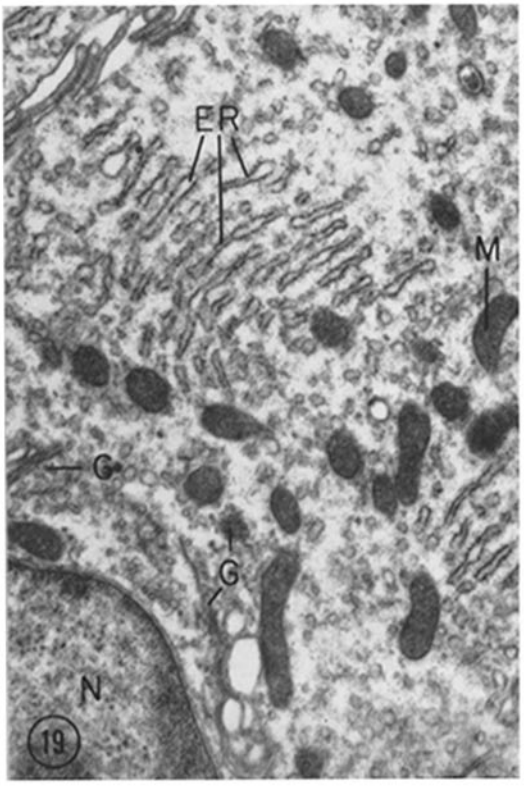
ER
M
G
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N
19

limb growth stage (*DG*, Figs. 9 and 10). At this stage of metamorphosis, however, the particulate component of the granules is more conspicuous. Their smaller size (70 A in diameter) and higher density distinguish them from RNP particles and allow the identification of individual units lying free in the cytoplasm. The extensive production of these small particles both within granules and free in the cytoplasm, as well as the increase in the number of dense granules, is apparently related to processes of progressive cell degeneration.

Since the animal no longer feeds, it seems unlikely that the degenerating cells retain any absorptive function. Some may be sloughed off into the gut; but many remain, forming a coherent intestinal lining and a covering for groups of basal cells.

Electron micrographs reveal that the basal cells (Fig. 14, *B*, *B'*, *B''*) have the undifferentiated structure previously observed in the larval epithelium (Fig. 6) as well as at the early stage of metamorphosis (Fig. 11). However, they are now seen frequently, and their aggregation into groups suggests that divisions within the basal cell population are occurring as an early step in the formation of a new epithelium. At any rate, it is to be noted that their response to the metamorphic stimulus is completely unlike that of the larval columnar absorbing cells.

Formation of a Bilayered Epithelium

As metamorphosis progresses to the stage of early tail reduction (Fig. 1), the degenerating and growing components of the intestinal epithelium become segregated from each other, with the result that the epithelium is bilayered. A thin layer of degenerating cells lines the gut (Fig. 15, *Dg*) and covers the developing adult epithelium beneath it (Fig. 15, *AE*). Often a single dying cell forms the superficial lining of the gut (Fig. 16, *Dg*); and it is still attached to the underlying cells, although its plasma membrane is disrupted (*X*) and cytoplasmic elements lie free in the intestinal lumen.[1]

The cells of the inner epithelial layer, on the other hand, give evidence that differentiative processes have begun in the new epithelium. These healthy looking cells (Fig. 16, *AE*) are joined laterally by desmosomes (*D*) and form a coherent layer. Their cytoplasm is more extensive than that seen previously in basal cells, from which they have apparently arisen. However, the smooth plasma membrane on their luminal surface (*PM*) as yet exhibits no special differentiation.

Loss of Degenerating Larval Cells

By the time of later tail degeneration (Fig. 1), the intestinal epithelium has completely lost the layer of degenerating cells and consists only of the developing adult epithelium. When observed under the electron microscope, the epithelial cells lining the gut show some differentiation of their free surfaces. Microvilli (Fig. 17, *Mv*) and an ectoplasmic zone (*TW*) are forming. In deeper lying cells, the active proliferation is demonstrated by mitotic figures, which may be seen in almost every section. Thus both growth and differentiation are progressing under the influence of the metamorphic stimulus.

Maturation of the Adult Epithelium

Progressive differentiation of the new epithelium, which first appears during metamorphosis, leads to the formation of columnar absorbing cells. These share with their larval counterparts cytological features generally associated with this cell type. However, an examination of the fine structure of the apical cytoplasm brings to light details of their structure in which they exhibit differences from functional larval cells. Thus, although microvilli (Fig. 18, *Mv*) filled with fibrillar material project into the gut lumen (*L*), their height averages about 0.7 μ, whereas those of the larval cell average about 1.2 μ. Furthermore, the terminal web or fibrillar ectoplasmic zone, common to larval and adult cells, is 3 times as high in the young form (Fig. 3, *TW*) as it is in the mature animal (Fig. 18, *TW*). The deeper supranuclear cytoplasm of the adult (Fig. 19) adjacent to the nucleus also differs from that found in the larva. The granules so characteristic of the larval cell (Fig. 4, *DG*) were not observed in the adult. Rather, in the mature form, rough surfaced cisternae of the endoplasmic reticulum assemble (Fig. 19, *ER*) near the Golgi material (*G*), their long axes parallel to the long axis of the cell. Thus the adult epithelial cells, developing under the influence of the metamorphic stimulus, show con-

[1] It is worth noting that the structure of the mitochondria (Fig. 16, *M*) can remain normal in spite of cell disruption. If the metamorphic stimulus (*i.e.*, the thyroid hormone) has any primary or direct action upon these organelles during metamorphosis, it does not obviously and unequivocally affect their cytological structure.

stant, though not extreme, differences from larval cells that mature in absence of this stimulus.

DISCUSSION

Observations of the fine structural changes in the intestinal epithelium of the bullfrog during metamorphosis entirely confirm and extend a view previously held concerning the nature of tissue responses to the morphogenetic stimulus (thyroid hormone); namely, that this response is specific for each tissue and for individual cell types within tissues. Thus the highly differentiated larval columnar epithelial cells degenerate and are lost, while undifferentiated basal cells in the same tissue proliferate and differentiate into the adult epithelium. But in addition, study with the electron microscope has shown that the course of differentiation of the basal cells is definitely influenced by the action of the metamorphic stimulus. The functional larval cells are not replaced by identical units, but by cells that exhibit measurable differences in their fine structural organization from that observed in their larval counterparts.

The Specificity of Cytological Responses during Metamorphosis

At the beginning of this century, by means of investigations using the light microscope, several workers described events during the metamorphosis of the anuran intestinal epithelium as being essentially similar to those found in the present study (7, 8, 11). However, attempts to give more detailed descriptions (12, 13) could not demonstrate clearly whether or not at least some of the differentiated larval cells contributed to the formation of the adult epithelium. This question can now be reexamined, since the resolution provided by electron microscopy makes possible more certain identification of cell types within the larval epithelium as well as observation of their fate during metamorphosis.

The complex fine structure of the functional larval columnar epithelial cells makes their identification a simple matter. The specializations at the free cell surface (Fig. 3)—microvilli, fibrillar cortical zone, terminal bars—are similar to those found commonly in the absorbing cells of other vertebrates (17–24). In the early stages of metamorphosis, these structures serve as markers, identifying the changing columnar cells while the characteristic zoning of the cytoplasm is lost (Fig. 8). There appear in the larval absorbing cells dense granules (Fig. 8, *DG*), which in size and structure form a part of a continuous spectrum that also includes granules found in highly abnormal cells (Figs. 9 and 10, *DG*). Apparently, then, the abnormal cells arise from functional larval cells and represent a state into which the latter are transformed in response to the metamorphic stimulus. Their configuration is so far beyond the limit of normal cell structure that it is considered to be degenerative in nature. This interpretation is strengthened by the observation that the abnormal cells do not take part in the formation of the new epithelium. Although retained during the early stages of metamorphosis (Fig. 9), they are later segregated into a separate layer, which for a time lines the gut (Figs. 15 and 16, *Dg*), and are finally sloughed off into the intestinal lumen, leaving the new single layered epithelium.

In contrast, the basal cell in the larva (Fig. 6) has no obvious cytoplasmic specializations, and it is not subject to confusion with the columnar absorbing cell. Basal cell cytoplasm, which is scanty in relation to the size of the nucleus (Fig. 6, *N*), contains few mitochondria and is characterized by the prominence of small particles that are usually free and only occasionally associated with the sparsely occurring membranous components of the endoplasmic reticulum. In the mammalian intestine, the cell population apparently is renewed by crypt cells (25). In the rat jejunum, the configuration of the cytoplasm of the crypt cells (26) is comparable to that of basal cells examined in the present study. In both cases—in basal cells and crypt cells—the cytoplasmic fine structure is that typical of undifferentiated cells generally (16). This immature state is maintained by basal cells of the anuran intestinal epithelium at the time when the columnar cells are already affected by the metamorphic stimulus. Proliferation produces nests of basal cells (Fig. 14), but not until their segregation into a cell layer separate from the degenerating cells do they begin to differentiate (Fig. 16, *AE*).

Therefore, all the observations reported here not only demonstrate, as previously stated, that each cell type responds specifically to the metamorphic stimulus, but suggest also that the fate of each cell type is separate from that of the other and that, in the anuran, metamorphosis of the intestinal epithelium does not involve the partici-

pation of differentiated larval cells in the formation of the adult epithelium. Thus degeneration, proliferation, and differentiation are the processes involved in the transformation from the larval into the adult epithelium.

The prominence of cell degeneration warrants further discussion, since the nature of this process suggests a possible mechanism by which it is effected. Cells that are to be eliminated from the cytoplasm are characterized by the presence of large dense granules. Though the structure of these granules is not uniform (Fig. 8, *DG*; Fig. 9, *DG*, *X*, *X'*; Fig. 10, *DG*, *DG'*), yet all have, within an outer limiting membrane, small dense particles measuring 70 A in diameter, swirls of membranous material, and a homogeneous matrix of medium density. It is possible to observe a spectrum of granule size: smaller ones (0.6 μ in diameter) occur in absorbing cells showing only moderate structural changes (Fig. 8, *DG*), whereas highly abnormal cells contain many large granules together with smaller examples (Figs. 9 and 10). The dense particulate component (Fig. 13, *SP*) becomes more abundant in the large granules of highly abnormal cells, which also have these small particles lying free in their cytoplasm. Though similar granules or bodies have been noted in the columnar absorbing cells of the rat jejunum (19), it is only during anuran metamorphosis that their occurrence clearly coincides with degenerative changes in the absorbing cells and is apparently involved with catabolic processes taking place within these cells.[2]

The association of dense bodies or granules with cell destruction suggests that these structures may be a form of lysosome. Both de Duve (27) and Novikoff (28) propose that particles, called lysosomes, which have been identified in biochemical experiments as sites within which certain enzymes are segregated, have as one of their functions the destruction of cells during normal development. This hypothesis is based on observations that in a number of cases physiological autolysis is associated with the activities of certain enzymes located in lysosome particles (28). Of special interest in relation to the present report are the findings of Weber (29, 30) that the specific activity of cathepsin, a lysosome enzyme (31), increases during anuran tail resorption, reaching up to 22 times its initial level in the final stages of metamorphosis. Furthermore, by staining procedures, Novikoff (28) has shown that there is a high level of acid phosphatase activity in regressing *Rana* tadpole tail. Thus two enzymatic activities characteristic of lysosomes are present when large amounts of tissue are being removed. These observations suggest that lysosomes play a role in muscle degeneration during metamorphosis.[3] It therefore seems reasonable to ask whether lysosomes are also involved in degeneration of the intestinal epithelium. Since the appearance of dense granules is correlated with cell degeneration, it might be expected also that at least some of the granules are lysosomes and future histochemical tests will demonstrate the presence of characteristic lysosome enzymes within them.

The positive identification of lysosome particles within tissue sections has presented difficulties; however, the structure of the dense granules seen in degenerating intestinal epithelial cells is reminiscent of those bodies identified as lysosomes in liver tissue, upon which both biochemical and histochemical studies have been performed. Examination of acid phosphatase-rich fractions isolated from rat liver (33) revealed the presence of many membrane-bounded bodies containing a large number of electron-opaque particles that resembled ferritin molecules. In addition they occasionally possessed internal vacuoles. Thus they were similar in structure to the pericanalicular dense bodies of the liver (33). Subsequently, evidence from studies in which histochemical techniques were adapted to electron microscopy indicated that the dense bodies may contain the lysosome enzyme acid phosphatase (34, 35). During hemoglobin reabsorption in the mouse kidney, droplets arise in the cells of the proximal convoluted tubules. They have, within an outer limiting membrane, membranous material together with

[2] It is not possible to determine whether or not dense granules found early in the metamorphic period are derived from those occurring in cells at the larval stage. Differences in structure and frequency of occurrence exist between granules at the two stages (compare Fig. 4, *DG*, and Fig. 8, *DG*) and intermediate stages were not observed. It seems likely, therefore, that dense granules involved in cell destruction arise during metamorphosis, whereas those occurring in premetamorphic cells disappear after performing a quite different function.

[3] Recent examination of fine structural changes in tadpole tail muscle during metamorphosis (32) has shown that many bodies appear within degenerating muscle fibers. It remains to be shown whether or not these bodies contain lysosome enzymes.

ferritin particles lying in a matrix of moderate density (36). Subsequent investigation (37) has revealed that such bodies have acid phosphatase associated with them and can therefore be considered a form of lysosome. Thus, in the present study, the inner structure of the dense membrane-bounded bodies—membranous material in a matrix of medium density together with small particles of the density and size of ferritin—might be identified tentatively as a form of lysosome upon consideration of their morphology alone.

Observations of metamorphic changes have not as yet yielded any direct evidence concerning the way in which the dense granules (lysosomes) function in cell destruction. Since cell organelles lying near the granules can appear quite normal (Fig. 8, *M*) and since no degenerative stages of individual organelles lying free in the cytoplasm have yet been seen, further detailed examination may support the evidence of Ashford and Porter (38) and also Hruban *et al.* (39) that cell structures are "segregated" or "sequestered" into bodies within which they are finally destroyed.

The Influence of the Metamorphic Stimulus upon Differentiation of the Adult Epithelium

The fine structure of the columnar epithelial cell of the adult frog differs in certain respects from that of its larval counterpart. In the adult form (Fig. 18) the microvilli are shorter (Mv), the ectoplasmic zone is lower (*TW*), and rough surfaced elements of the endoplasmic reticulum are more plentiful in the supranuclear region (Fig. 19, *ER*); but dense granules commonly found in the larva are lacking. Therefore, the presence of the metamorphic stimulus changes in a subtle but constant way the course of differentiation of the basal cells so that they no longer produce typical larval units, but rather adult cell types. This pattern of response is believed to be essentially similar to that observed in other epithelial transformations occurring under the influence of extraneous agents. Fell (40) has emphasized that the change of one differentiated cell type into another is difficult, perhaps impossible, to demonstrate; but, on the other hand, there is good evidence that certain agents can stimulate the basal cell population to give rise to an epithelium that is functionally different from that which it formerly produced. This description fits both the case in which the transformation of the keratinizing epithelium of embryonic chick skin into a mucus-secreting tissue occurs under the influence of excess vitamin A (41) and that in which the vaginal epithelium of the mouse loses its ability to secrete mucus and forms keratin in response to estrogen treatment (42). Although the functional significance of the fine structural changes in the intestinal epithelium is not known, it is presumably related to the change from herbivorous to carnivorous diet occurring during metamorphosis. In any case, it is clear that the hormonal stimulus exerts a precise control over developmental processes.

More detailed observations of the cellular responses to hormone stimulation can undoubtedly increase our knowledge of the nature of cytodifferentiation. With such a goal in mind, the development of the array of uniformly spaced microvilli has been studied more extensively and is the subject of a manuscript now in preparation.

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