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Histological and cytological changes in the small intestine of Hyla versicolor Le Conte during metamorphosis

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HISTOLOGICAL AND CYTOLOGICAL CHANGES IN THE SMALL INTESTINE OF HYLA VERSICOLOR LE CONTE DURING METAMORPHOSIS

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Acknowledgments

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My parents, Mr. and Mrs. H. B. Robinson for their encouragement and financial assistance.

Introduction

The histological changes that occur in the small intestine of anuran larvae during metamorphosis were first studied in 1891 when Ratner described the subepithelial changes in Rana temporia. Subsequently, epithelial changes were noted by Reuter, Duesburg, and Bowers (for review see Bowers, 1909) using Alytes obstetricans, Rana fusca, and Bufo lentiginosus respectively. More recently, Kuntz (1922), Janes (1932), and Lui and Li (1930) discuss both the subepithelial and epithelial changes in Rana pipiens, Rana clamitans, and Rana nigromaculata, respectively. Kaywin (1936) describes the cytological changes that take place in the epithelium of Rana catesbiena and Bonneville (1963) points out some of the fine structural changes in the epithelium of Rana clamitans during metamorphosis.

It is generally agreed, by previous workers, that the intestinal mucosa degenerates during metamorphosis, and that basal cells proliferate to form a new mucosa. However, there is considerable disagreement as to the mechanism involved in both processes.

The object of the present study was to determine the histological and cytological changes that occur, during metamorphosis, in the small intestine of Hyla versicolor Le Conte. It was felt that such a study would clarify and amplify earlier observations.

Materials and Methods

H. versicolor tadpoles were collected in a small pool near the west end of Westhampton Lake in Richmond, Virginia during September and October of 1963. The organisms were maintained in the laboratory at room temperature in aquaria filled with tapwater. Soil from the bottom of the pool from which the tadpoles were collected was placed in the aquaria in an attempt to simulate natural environmental conditions. The animals were fed lettuce and canned spinach. Algae and bacteria growing on the bottom and sides of the aquaria also made up a large part of their diet.

As the forelegs emerged from the gill chambers, the tadpoles were transferred to fingerbowls containing water and tilted so that only half of the bottom was covered. Here, the animals emerged from the water and completed their metamorphosis. The frogs were then placed in a humid aquarium and were maintained on miscellaneous small live insects.

The animals used in this study corresponded to stages used by previous investigators. These stages are as follows:

Stage No. 1 The larval tadpoles are fully developed but the hind limb buds are only slightly differentiated. The length of the bud varies from equal to its diameter to twice its diameter.

Stage No. 2 The hind limb buds are differentiated into a thigh, shank, and foot. The hind limbs trail behind the body.

Stage No. 3 The hind legs are fully developed and are pulled up alongside the body. The walls of the gill chambers at the points where the forelegs later emerge are not thin and transparent.

Stage No. 4 This stage is similar to Stage No. 3 except that the

walls of the gill chambers at the points where the forelegs later protrude are thin and transparent.

Stage No. 5 Both forelegs have emerged from the gill chambers but the tail has not begun to degenerate.

Stage No. 6 The tail has started to degenerate and is one half its original length.

Stage No. 7 Only a stub of the tail remains.

Stage No. 8 The tail is no longer apparent and the completely metamorphosed frogs have begun feeding.

The specimens selected for study were killed and fixed by placing them directly in a FAA fixative (water, 95% ethyl alcohol, formalin, and glacial acetic acid - 30:16:8:1) for 24 hours. After fixation the specimens were stored in 70% ethyl alcohol.

In preparation for histological study, the stomachs and small intestines were removed from the animals with the aid of a binocular stereoscopic microscope. The small intestines were then uncoiled and straightened so that uniform cross sections could be made along their entire length. Serial transverse sections were cut at 7.5 microns and the mounted tissues were stained with Harris' Hematoxalin and counter-stained in Triosin. The prepared slides were examined using phase contrast and light microscopy.

Preliminary cytochemical tests were performed using the 1952 Gomori Method for acid phosphotase (Pearse, 1961).

Observations

In the larval tadpole, the small intestine extends from the pyloric valve, posteriorly to the ilio-coelic valve. It is very long and wound in the form of a double coil. Histologically it may be divided into an anterior duodenum and a posterior ileum with a transition zone between.

During metamorphosis, by Stage 3, the small intestine began to undergo a marked shortening that resulted in the coil progressively unwinding until by Stage 7 the small intestine was approximately 1/8 its original length (Kuntz, 1922) and passed directly from the stomach to the colon.

During this contraction extraordinary histological changes began in the anterior region of the duodenum and progressed posteriorly to the colon. While histological changes in the duodenum and ileum were similar, they were easier to follow in the duodenum which will be described first.

Duodenum

Stage 1 The serosa was composed of only a single layer of mesothelium which was closely applied to the longitudinal muscle fibers. Connective tissue was not apparent between the mesothelium and the longitudinal muscle layer.

The longitudinal muscle layer consisted of a single layer, and the circular muscle layer of two to four layers of smooth muscle fibers. All of the foregoing components of the muscularis were closely applied to each other.

The submucosa was thin and composed of areolar connective tissue (Fig. 2). White fibers, small capillaries, and lymphocytes were dispersed throughout the region.

The mucosa consisted of only an epithelial layer; a tunica propria and muscularis mucosa were not present. The epithelial layer was composed of tall columnar epithelial cells, goblet cells, lymphocytes, and large spherical cells usually in some stage of mitosis (Fig. 2 and 3). The mucosa was not arranged in folds as it was in the postmetamorphic specimens (Fig. 1).

The columnar epithelial cells were tall and contained large oval nuclei in the basal part of the cell. A large basophilic nucleolus and flakes of basophilic chromatin were scattered throughout the nucleus. The cytoplasm was acidophilic and evenly distributed throughout the cell. A few very small granules were present in the cytoplasm but no empty areas were observed. Terminal bars were present at the distal corners of the cells and the free surface was covered by a striated border (Fig. 3).

Goblet cells were relatively abundant among the columnar epithelial cells in the duodenum and the distal part of each goblet cell was filled with non-staining droplets (Fig. 2).

Lymphocytes in the mucosa were between the columnar epithelial cells and along the inner surface of the basement membrane (Fig. 3). These cells occurred in a variety of shapes. Some were dumbbell-shaped, others sickle-shaped, and some were asymmetrical with pseudopodia.

The large spherical cells, of the mucosa are thought to be columnar epithelial cells in a state of cell division (Fig. 3). Their cytoplasm was acidophilic and the nuclei, when in interphase, were

similar to those of the columnar epithelial cells. These cells underwent mitosis throughout the mucosa. No other cell type was observed dividing in the larval tadpole mucosa.

Stage 2 As the larval tadpoles approached Stage 2 the cytoplasm in the columnar epithelial cells and the goblet cells began to vacuolate.

Stage 3 The mucosa was not smooth as it was in Stage 1 but was arranged in low folds (Fig. 4 and 5).

The internal cytoplasm of the columnar epithelial cells was highly vacuolated and large yellow granules were present in the areas formerly occupied by the cytoplasm (Fig. 6). Some of the yellow granules had been eliminated into the lumen and others appeared to be bursting through the top of the cell.

Typically, the nuclei were unchanged; however a few had started to undergo chromatolysis and small areas were acidophilic instead of basophilic.

A new cell type, (the "basal cell" of previous investigators), was present above but adjacent to the basement membrane between the columnar epithelial cells (Fig. 7). The cell was oval with a large, strongly basophilic nucleus and a small amount of cytoplasm that was also basophilic (Fig. 7). Basal cells and nests of two to six cells, surrounded by a continuous membrane, were in active mitosis (Fig. 9).

Stages 4 and 5 The folds in the mucosa, first observed in Stage 3, were taller and projected into the lumen (Fig. 10).

The majority of the yellow granules had been extruded into the lumen and only the cell membranes and the nuclei remained (Fig. 11).

The number of basal cells increased until numerous nests of

approximately 10 to 20 cells were formed (Fig. 12). The cells became radially arranged with their long axes toward the center of the nest forming a hollow sphere of cells (Fig. 14 and 15). The basal cells then assumed a columnar form but continued to divide. They therefore will be referred to, in the remainder of this paper, as generative cells. Division continued until the membrane enclosing the generative cells broke and the sphere opened on the side toward the lumen forming a hollow cup of cells (Fig. 17).

The submucosa was much thicker and contained large numbers of lymphocytes (Fig. 13).

The muscle fibers of both muscle layers lost their compact arrangement, the mesothelium became separated from the longitudinal muscle layer and fibroblast-like cells invaded the empty spaces formed by the rearrangement of the muscle fibers.

Stages 5 and 6 The hollow cups of generative cells, still beneath the tadpole mucosa, enlarged and deepened as the result of continued division of the generative cells. By Stages 5 and 6, the expanding rims of the cups fused forming a continuous layer of new mucosa composed of generative cells. As the cups fused, they appeared to mechanically lift and pinch away the tadpole mucosa from the submucosa and push it into the lumen of the intestine (Fig. 17, 19, and 20). Further, when adjacent cups fused they formed folds in the new mucosa that were forerunners of the folds in the frog mucosa (Fig. 17 and 21).

Stages 6 and 7 The cells of both muscle layers became compactly arranged. The mesothelium became closely applied to the longitudinal muscle layer and the fibroblast-type cells were no longer observed. The generative cells of the new mucosa had not differentiated into goblet and columnar epithelial cells (Fig. 22 and 23).

Stage 8 The frog mucosa was similar to that of the tadpole mucosa except that it was arranged in tall folds that projected into the lumen (Fig. 24). The mitotic form of the columnar epithelial cell was found at the base of the folds.

The submucosa was like that of Stage 1 except that it extended into the folds of the mucosa and may have been slightly thicker (Fig. 24, 25 and 26).

The serosa and the longitudinal muscle layers were the same as those in the tadpole, but the circular muscle layer was one or two fibers thicker.

Ileum

The larval tadpole ileum was similar to the duodenum except that the epithelial cells were short instead of tall columnar and the nuclei were round instead of oval. Goblet cells were rare and a submucosa was not apparent.

Basal cells appeared in the ileum, as they did in the duodenum, and developed into the hollow cell nest stage. However, when the nests of generative cells broke open, they expanded laterally and spread over the surface of the submucosa pinching and squeezing off the tadpole mucosa into the lumen (Fig. 27). rather than lifting it off as in the duodenum.

The frog ileum was histologically similar to the frog duodenum described earlier except that the folds in the mucosa were low instead of tall.

Discussion

Janes (1932), Kaywin (1936), and Bonneville (1963) contend that basal cells are present in the larval tadpole mucosa.

In this study, basal cells were not observed in the larval mucosa but large spherical cells, similar in position and general morphology to basal cells are present. These spherical cells are mitotic stages of the epithelial cells which have rounded up during cell division. They can be easily distinguished from basal cells because their cytoplasm is acidophilic like that of the epithelial cells and not basophilic like that of the basal cells. It is possible that the basal cells that Janes (1932), Kaywin (1936), and Bonneville (1963) called basal cells were the mitotic stages of the epithelial cells (Fig. 3), especially since Kaywin (1936) described a basal cell in the tadpole mucosa as being strongly basophilic.

Although they have not been previously described in this organ, numerous lymphocytes, similar in shape and location to basal cells, are present in the mucosa (Fig. 3). It is possible that some of the previous investigators may have mistaken them for basal cells.

Most earlier workers contend that the free surface of the columnar epithelial cells is covered with cilia that disappear with the onset of metamorphosis. However, the work of Bonneville (1963) shows that the free surface of the columnar epithelial cells is covered by a striated border (Fig. 3) which Bonneville (1963) showed is composed of microvilli. The striated border does not disappear with the onset of metamorphosis but may persist until the mucosa is sloughed off into the lumen.

The first indication of metamorphosis, in the small intestine,

occurs in Stage 2 animals. It is characterized by the vacuolation of the cytoplasm and the formation of small dark granules.

As the cytoplasm of the epithelial cells continues to degenerate during Stage 3, the small dark granules enlarge into characteristic yellow granules (Fig. 6). The number of yellow granules per cell increases as the volume of cytoplasm decreases indicating that there is probably a direct relationship between the degeneration of the cytoplasm and the formation of the yellow granules.

Because of their morphology, chemical composition, and time of formation, the yellow granules have been tentatively identified as lysosomes. Lysosomes are a group of cell organelles in which the enzyme, acid phosphotase and other hydrolases are segregated from the rest of the cytoplasm (Novikoff, 1961). According to de Duve and Novikoff (Novikoff, 1961) the hydrolases in the lysosomes have as one of their functions the destruction of cells during normal development.

Bonneville (1963) has shown that the yellow granules produce images similar to those of lysosomes in electron micrographs and has tentatively identified the granules as lysosomes on this basis. In this study, preliminary cytochemical tests were performed using the Gomori Method for acid phosphotase. This test has been shown to be specific only for lysosomes (Novikoff, 1961). A positive test was obtained for acid phosphotase in the yellow granules indicating that chemically they are similar to lysosomes according to current chemical concepts.

As the cytoplasm continues to degenerate in the epithelial cells, the yellow granules in the cells continue to enlarge. Some of the granules eventually burst out of the cell into the intestinal lumen.

While the plasma membranes of these cells may be ruptured, the remainder of each cell, now composed only of a nucleus and plasma membrane, persists as a discrete unit until the mucosa is sloughed off into the lumen (Fig. 18). The cell membranes were not observed to degenerate and form a syncytium as reported by Janes (1932) and Lui and Li (1930).

The mechanism involved in the degeneration of the tadpole mucosa has been the subject of considerable speculation but still is not understood. It is possible that through the influence of thyroid hormone, known to play an essential role in metamorphosis (Kollros, 1961) the lysosomes may be stimulated to begin digestion of the components within the cell.

In their observations on the degeneration of the tadpole mucosa Reuter, Duesburg, and Bowers (Bowers, 1909) described "round cells" appearing in the degenerating mucosa. They believe that these cells absorb material from and promote the degeneration of the mucosa cells. Similar structures were observed in this study but are thought to be degenerating nuclei of mucosal cells rather than a unique cell type. Although roughly the same size and shape, the "round cells" differ from the typical mucosal cell nuclei in that they show chromatolysis and only certain areas stain with hematoxalin. The other areas are acidophilic.

On the basis of the increased number of phagocytes present during metamorphosis, Kuntz (1922) and Janes (1932) contend that the degeneration of the columnar cells is due to phagocytosis. However, neither Kuntz nor Janes was able to observe phagocytes attacking a columnar epithelial cell. In this study no evidence for phagocytosis was observed even though a large increase in lymphocytes was noted

during Stage 4. However preliminary cytochemical tests indicate that a high level of acid phosphotase activity is present in lymphocytes within the tadpole mucosa but not in the submucosa. This indicates that the lymphocytes in the mucosa may function in the breakdown of the mucosa, possibly by secreting lysolytic enzymes or by engulfing particles of degenerating material too small to be observed with the light microscope.

The most characteristic feature in the regeneration of the mucosa is the appearance of basal cells of unknown origin. Their most distinguishing characteristic is their basophilic cytoplasm. This characteristic is generally associated with cells engaged in a rapid synthesis of protein which in turn is characteristic of cells undergoing a high rate of cell division.

Once the basal cells have formed, they begin to undergo mitosis. In the first division the nucleus divides but cytokinesis does not take place. Each of the resulting daughter nuclei seems to be surrounded by a small amount of cytoplasm and a plasma membrane but this observation cannot be positively verified and will probably require analysis with an electorn microscope. Division continues among the "daughter cells" within the limiting membrane and an apparently unorganized group of cells develops. Cytoplasm and plasma membranes become apparent around each nucleus and the cells become radially arranged forming a hollow nest of generative cells (Fig. 15).

During the period of cell nest formation, but preceding the extensive shortening of the small intestine, the muscle fibers of both muscle layers undergo a pronounced change with respect to their association with each other. They become loosely arranged

and remain in this state while the small-intestine contracts to approximately 1/8 of its original length. When contraction is complete, by Stage 7, the muscle fibers again assume their compact arrangement.

It is not possible at this time to account for the displacement of the muscle tissue during the contraction of the intestine. However, preliminary observations made during this study, of the changes undergone by the stomach during metamorphosis, indicate that there is a pronounced increase in the thickness of the gastric muscularis. This increase in thickness closely parallels the shortening of the intestine. In addition, there does not appear to be sufficient increase in mitotic activity to account for the increased amount of tissue in the stomach. It is therefore suggested that the loosening of the intestinal muscularis, the shortening of the small intestine without appreciable increase in thickness, and the thickening of the stomach are probably inter-related phenomena. It is possible that the smooth muscle cells of the intestine may actually migrate into the region of the stomach, thus redistributing the muscular tissue.

As the small intestine contracts in length, the tadpole mucosa is first pushed into folds but the cells retain their characteristic shape (Fig. 11). With continued contraction the cells of the mucosa are compressed and distorted into a mass and finally are sloughed off into the lumen (Fig. 19). The increase in thickness of the submucosa and the increase in lymphocytes in the submucosa is probably due to the compression of the submucosa by the contraction of the small intestine. The contraction also probably functions in bringing the cell nests closer together thereby facilitating their merging into a layer of generative cells.

In the ileum the mechanism involved in sloughing off the tadpole mucosa is different due to fewer cups of generative cells. The hollow spheres of generative cells are not adjacent to each other as they are in the duodenum but are widely spaced (Fig. 27). With continued cell division, the layer of generative cells expands around the periphery pinching and squeezing but not lifting the tadpole mucosa off into the lumen (Fig. 27).

Several previous investigators have described the frog mucosa as developing directly from basal cells (Kuntz, 1922 and Janes, 1932) without the basal cells forming nests and developing in the definite pattern observed in this study. If the previous investigators observed the regeneration of the mucosa as described above, it is conceivable that they could have interpreted the regeneration of the mucosa as forming directly from basal cells. Therefore it is possible that the different interpretations of regeneration of the new mucosa may be due to regional differences in the small intestine and not to variations among different species.

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PLATES

Abbreviations

BC, basal cell
BM, basement membrane
CC, cell cup
CE, columnar epithelium
CN, cell nest
GC, goblet cell
Ge, generative cell
Ly, lymphocyte
M, mucosa
MF, mitotic figure
Ml, muscularis
NM, new mucosa
PM, plasma membrane
SB, striated border
Sm, submucosa
TB, terminal bar
TM, tadpole mucosa
YG, yellow granule

Plate 1

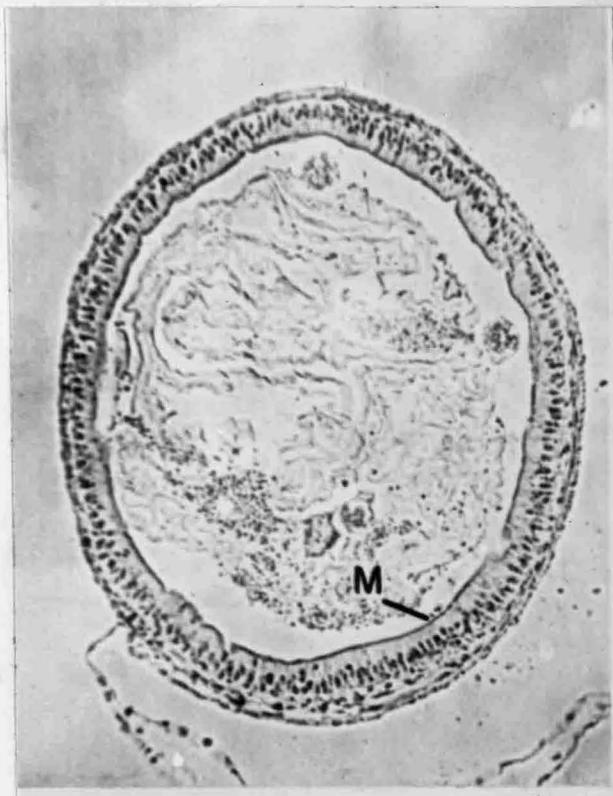
Explanation of figures

Transverse sections of the duodenum of a Stage 1 animal.

Figure 1 Showing the unfolded arrangement of the mucosa. x 840.

Figure 2 Showing the general arrangement of the mucosa, submucosa, and muscularis. The mucosa is composed of columnar epithelial cells and goblet cells. x 1500.

Figure 3 Showing the striated border, terminal bars, mitosis in a columnar epithelial cell, the general condition of the cytoplasm in the columnar epithelial cell, and a lymphocyte in the mucosa. x 3,250.



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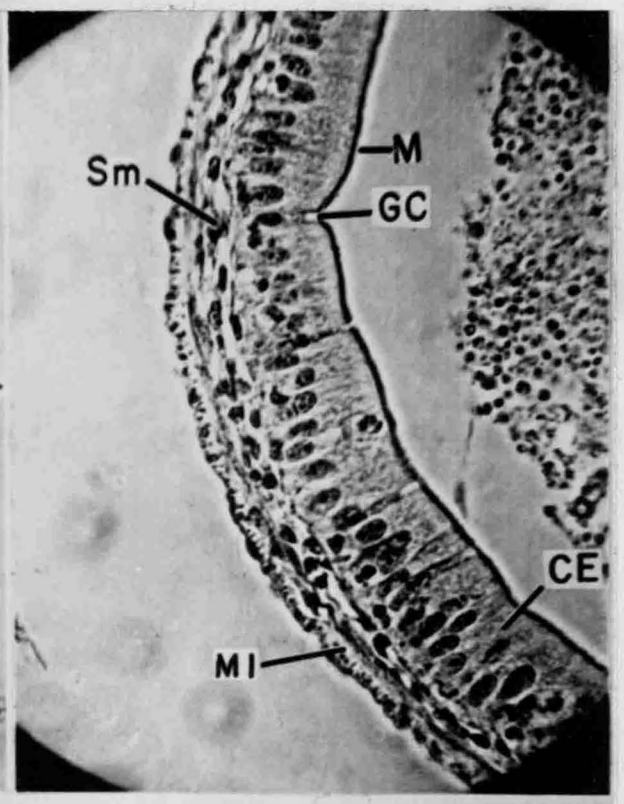

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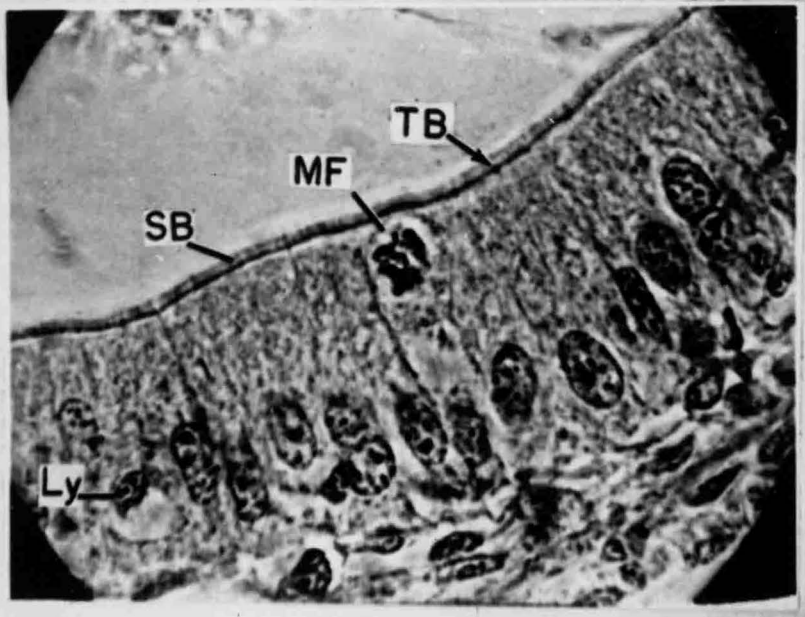

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Plate 2

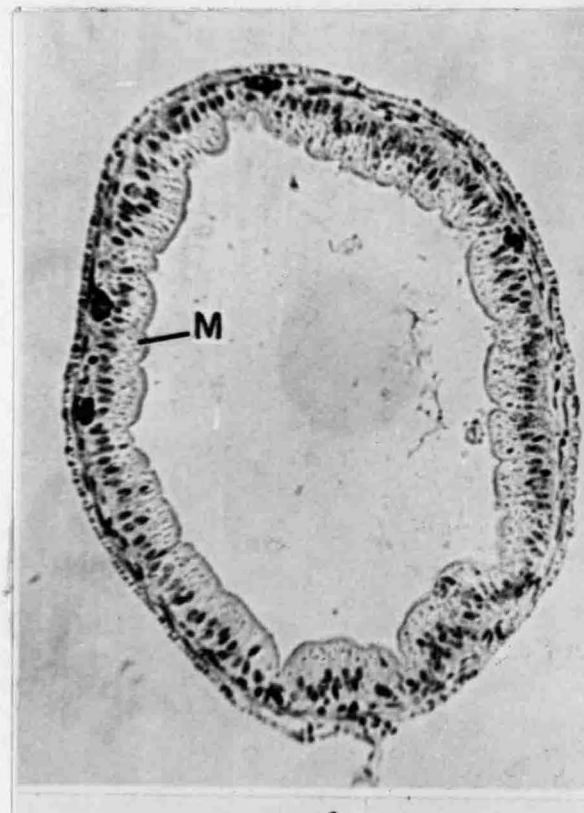
Explanation of figures

Transverse sections of the duodenum of a Stage 3 animal.

Figure 4 Showing the folded arrangement of the mucosa. x 840.

Figure 5 Showing the general arrangement of the mucosa, cell nests, and muscularis. x 1500.

Figure 6 Showing the degeneration of the cytoplasm. Clear areas are present in the cytoplasm and yellow granules are present in the clear areas. x 3,250.



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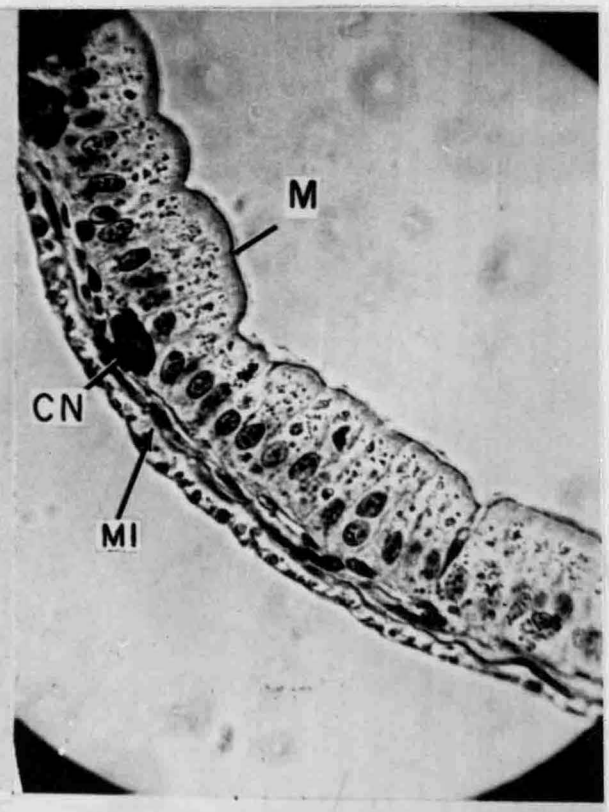

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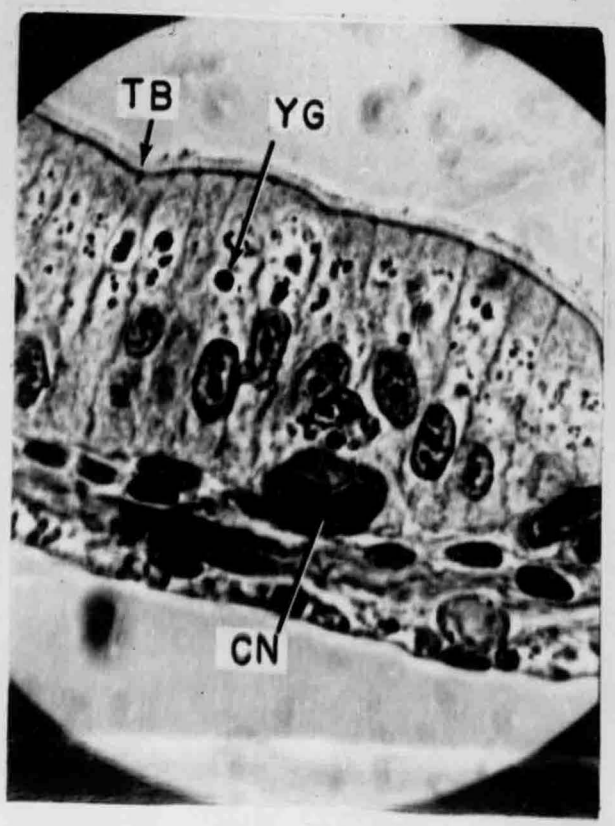

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Plate 3

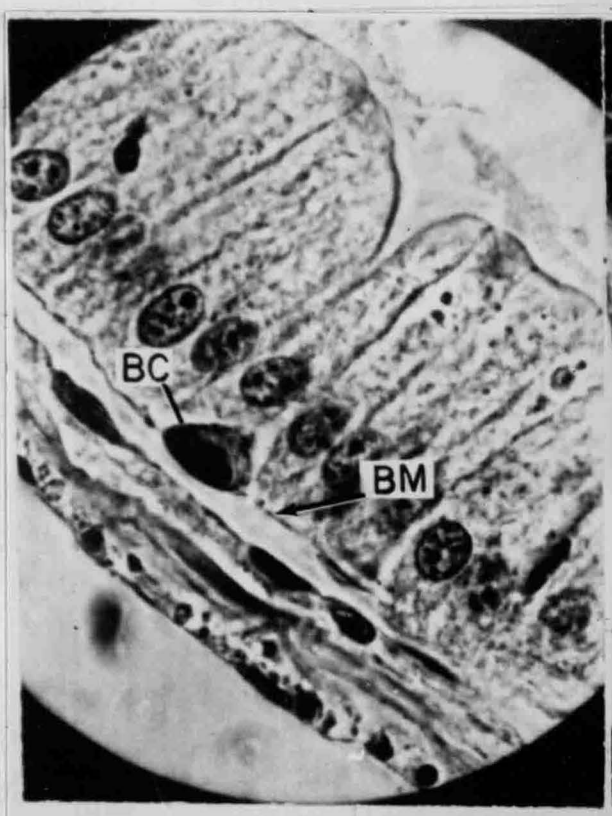
Explanation of figures

Transverse sections of the duodenum of a Stage 3 animal.

Figure 7 Showing a single basal cell above and adjacent to the basement membrane. x 840.

Figure 8 Showing a cell nest containing two basal cells and surroumded by the original plasma membrane. x 1500.

Figure 9 Showing mitosis within a cell nest. x 3,250.



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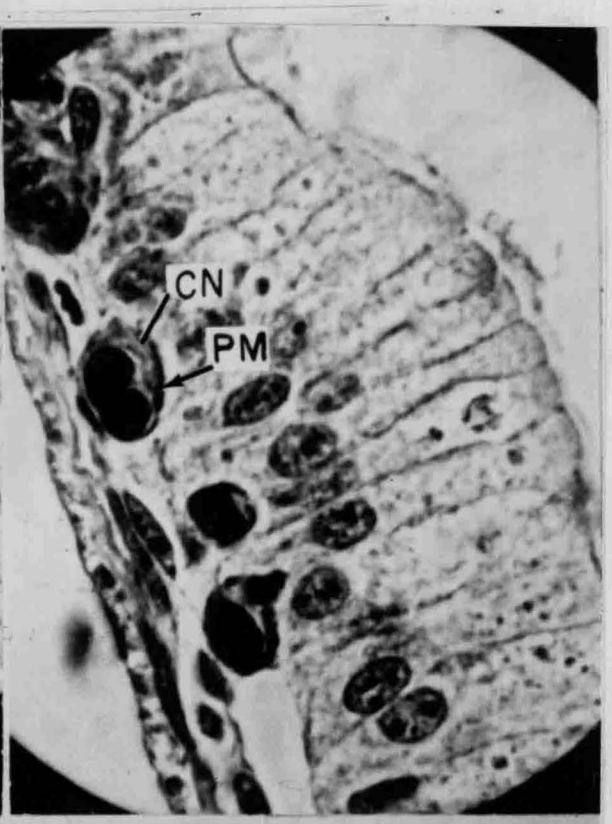

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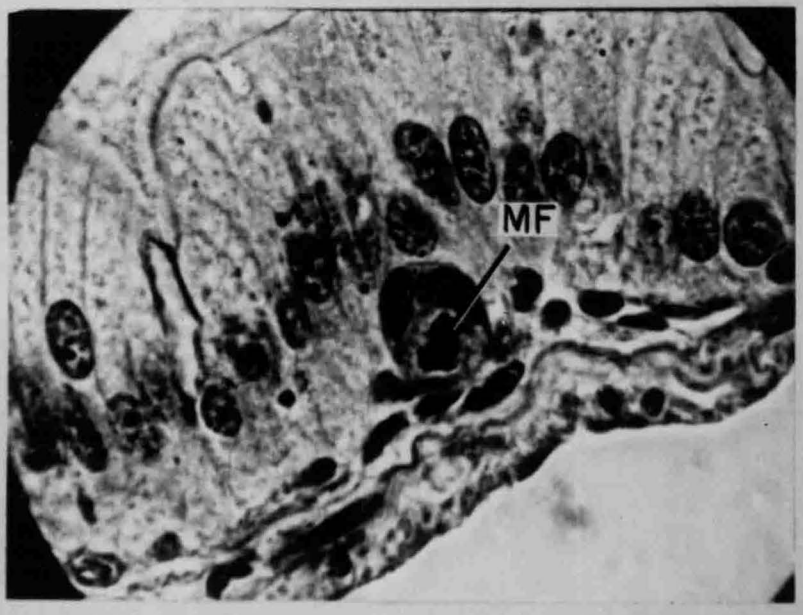

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Plate 4

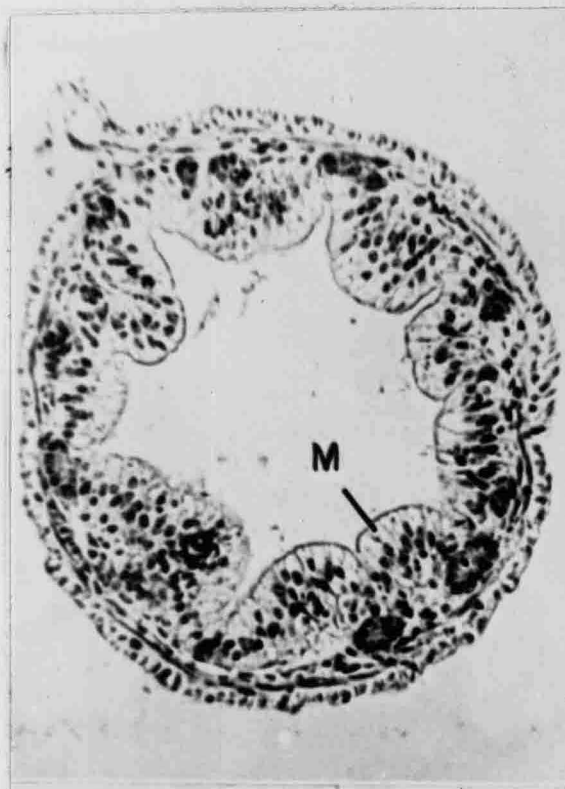
Explanation of figures

Transverse sections of the duodenum of a Stage 4 animal.

Figure 10 Showing the arrangement of the mucosa. x 840.

Figure 11 Showing the general arrangement of the mucosa, cell nests, submucosa, and muscularis. Note the cytoplasm of the epithelial cells is clear or absent but the cells still maintain their characteristic shape. x 1500.

Figure 12 Showing a cell nest. x 3,250.



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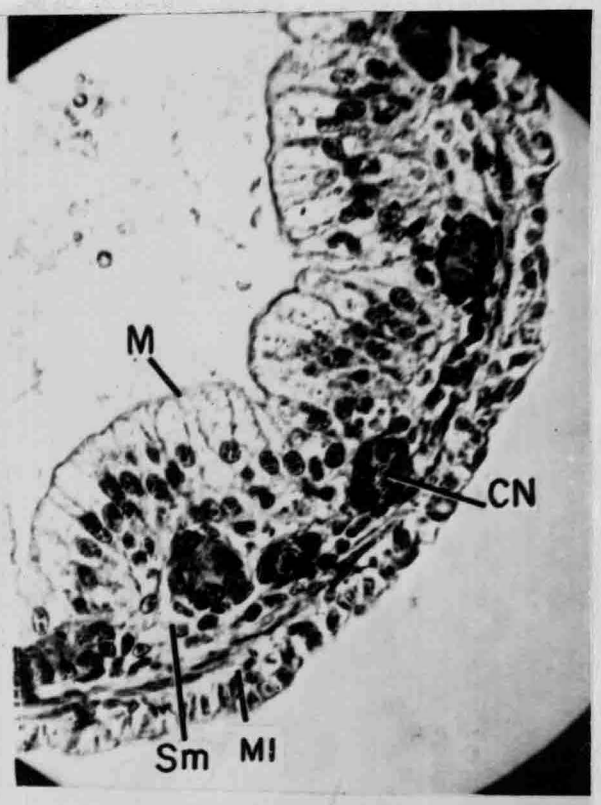

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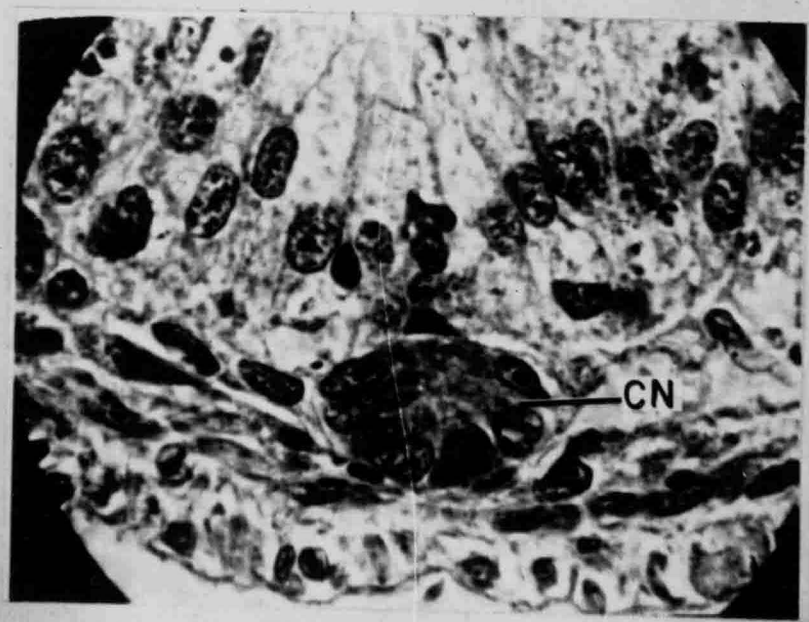

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Plate 5

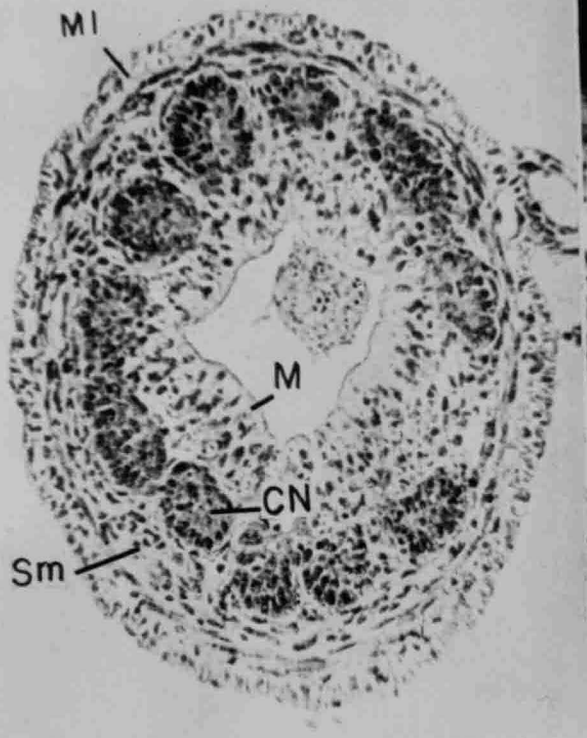
Explanation of figures

Transverse sections of the duodenum of Stage 5 and 6 animals

Figure 13 Showing the general arrangement of the mucosa, cell nests, submucosa, and muscularis. Note the increase in thickness of the submucosa. x 840.

Figure 14 Showing a hollow cell nest in which the generative cells are radially arranged with their long axes toward the center. x 1500.

Figure 15 Same as 14 except x 3,250.



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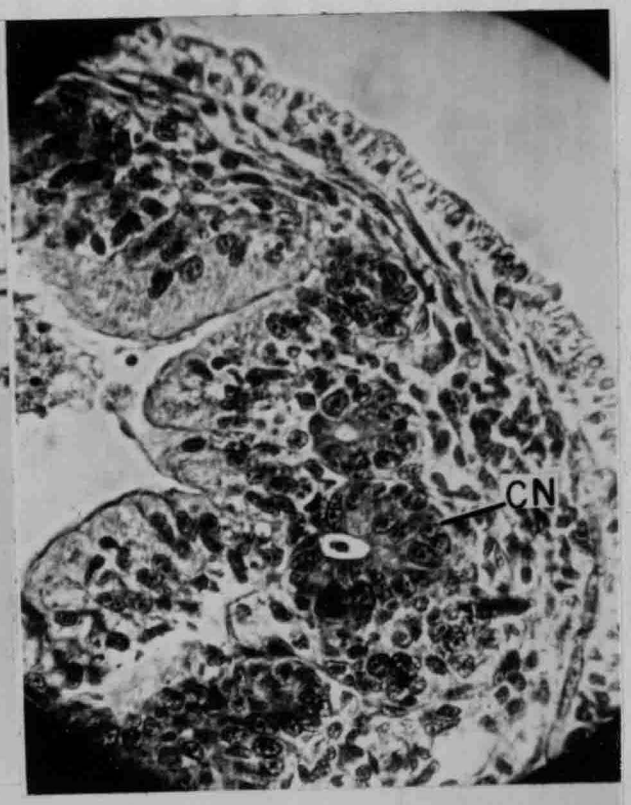

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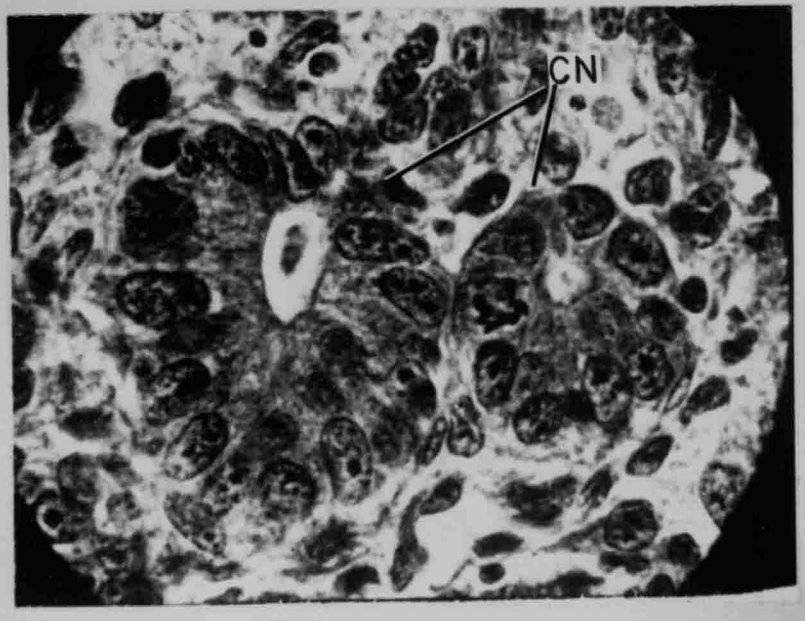

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Plate 6

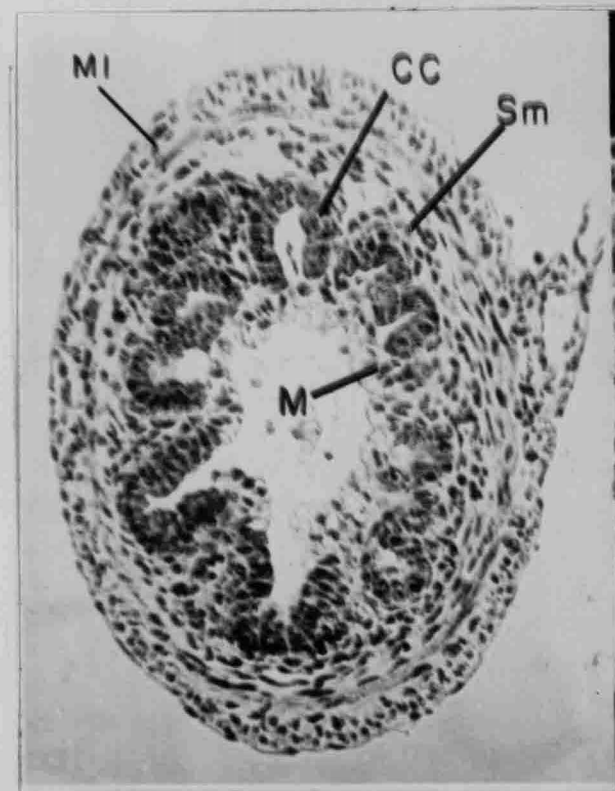
Explanation of figures

Transverse sections of the duodenum of Stage 5 and 6 animals.

Figure 16 Showing the general arrangement of the mucosa, cell cups, submucosa, and muscularis. x 840.

Figure 17 Same as 16 except x 1500.

Figure 18 Showing cell cups and mucosa. Note that cytoplasm is absent in the epithelial cells of the mucosa but the plasma membrane is still intact. x 3,250.



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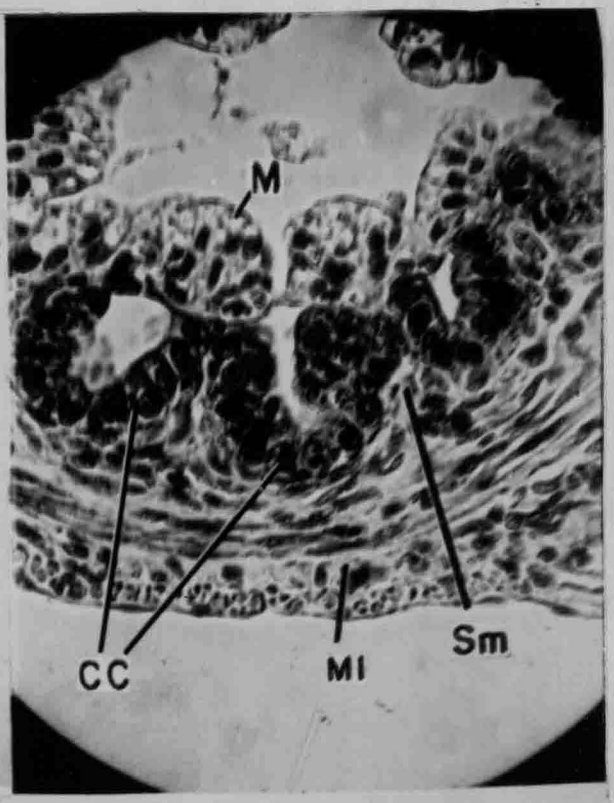

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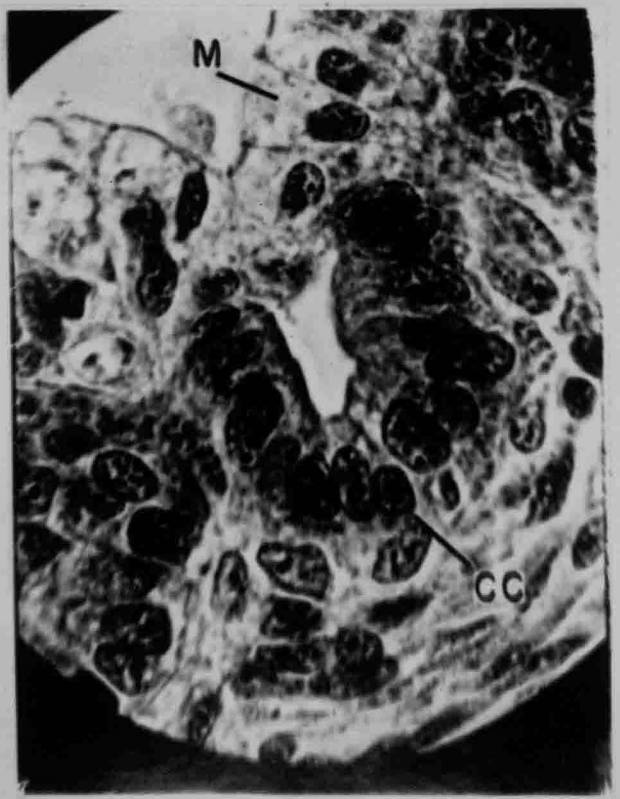


18

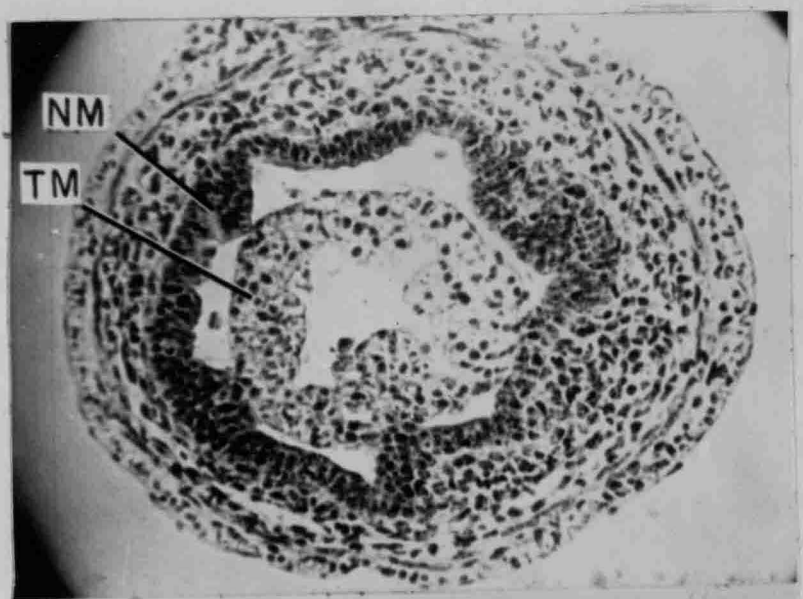
Plate 7

Explanation of figures

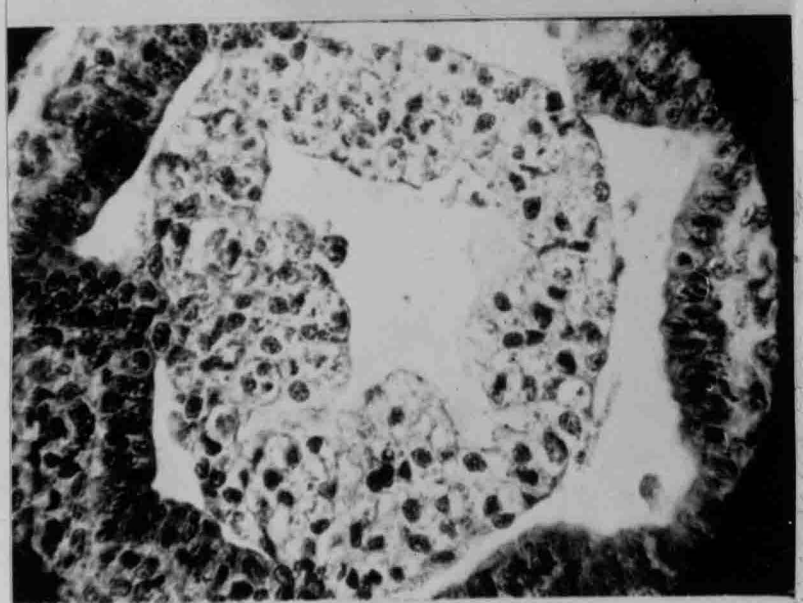
Transverse sections of the ileum of a Stage 6 animal.

Figure 19 Showing the tadpole mucosa in the lumen and the new mucosa in place over the submucosa. x 840.

Figure 20 Same as 19 except x 1500.



19



20

Plate 8

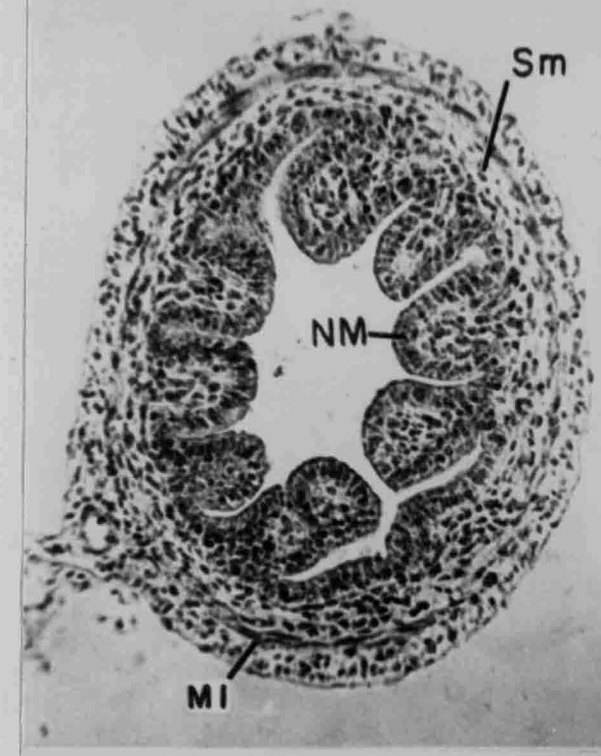
Explanation of figures

Transverse sections of the duodenum of Stage 6 and 7 animals.

Figure 21 Showing the arrangement of the new mucosa, submucosa, and muscularis. x 840.

Figure 22 Same as 21 except x 1500.

Figure 23 Showing the generative cells composing the mucosa. x 3,250.



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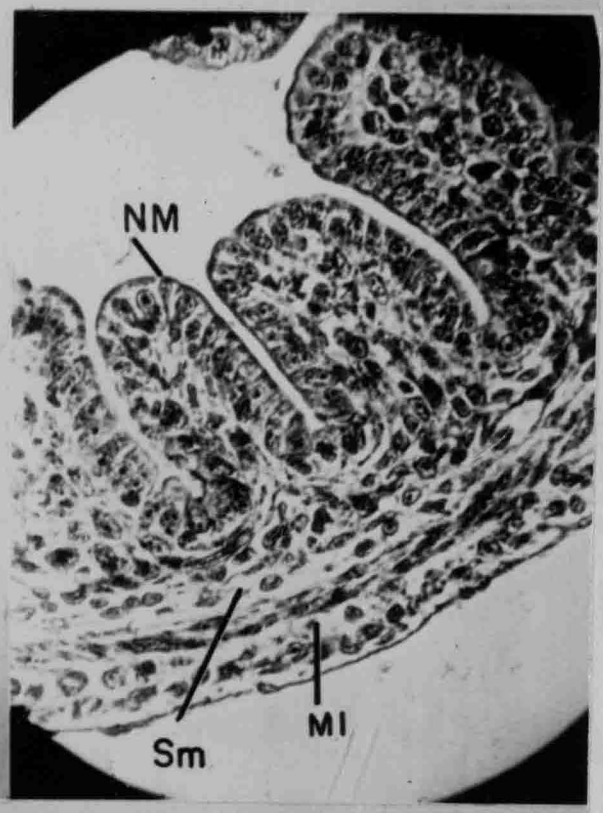

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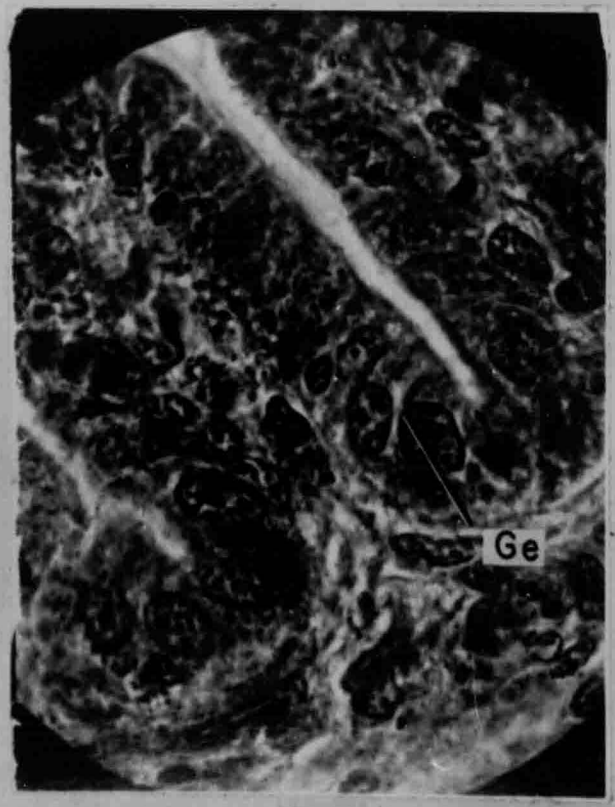


23

Plate 9

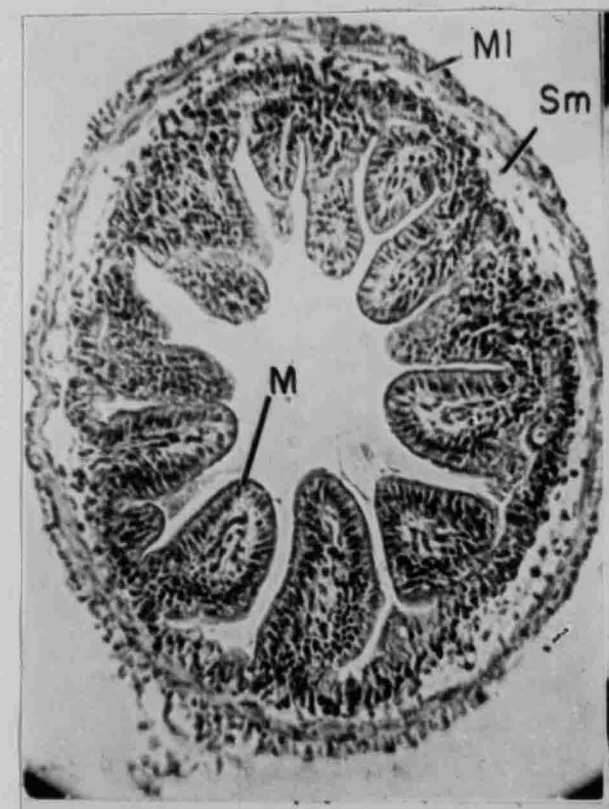
Explanation of figures

Transverse sections of the duodenum of a Stage 8 animal.

Figure 24 Showing the general arrangement of the frog mucosa, submucosa, and muscularis. x 840.

Figure 25 Showing the columnar epithelial cells and the goblet cells composing the mucosa. x 1500.

Figure 26 Showing a fold in the mucosa. x 3,250.



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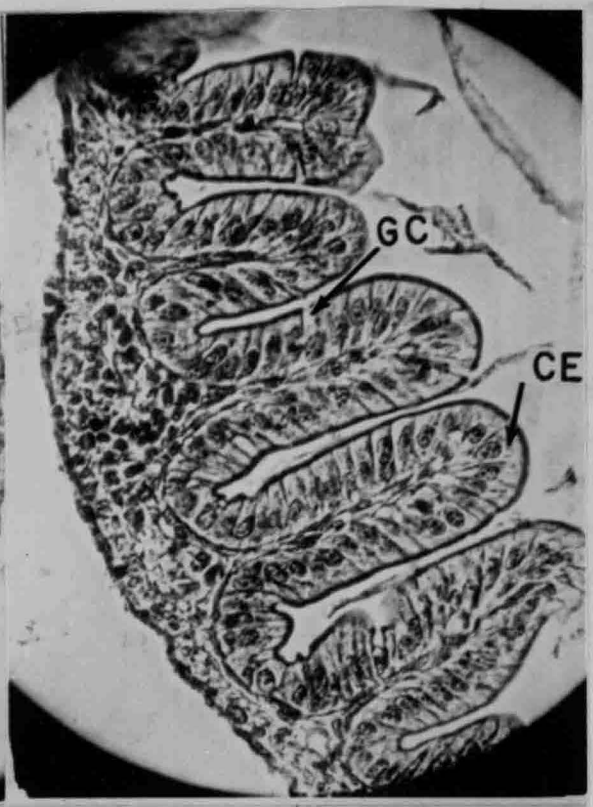

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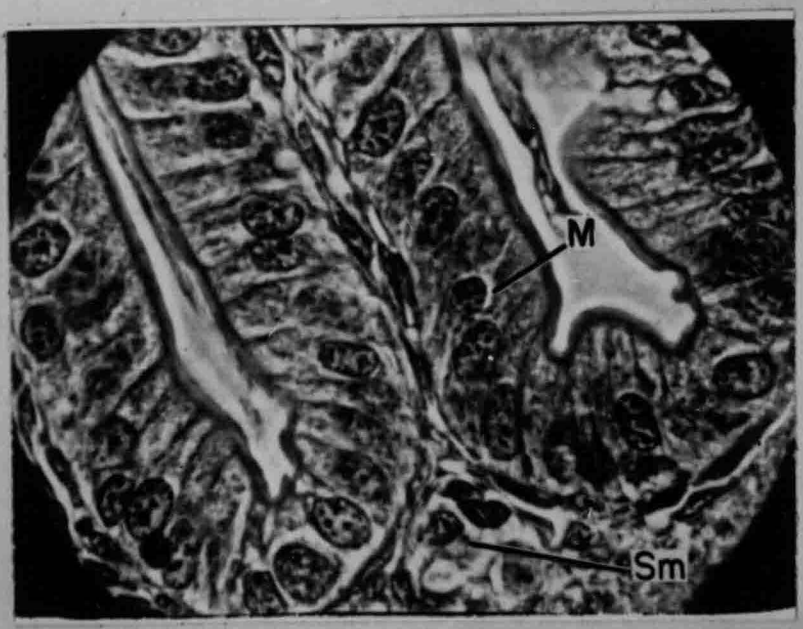

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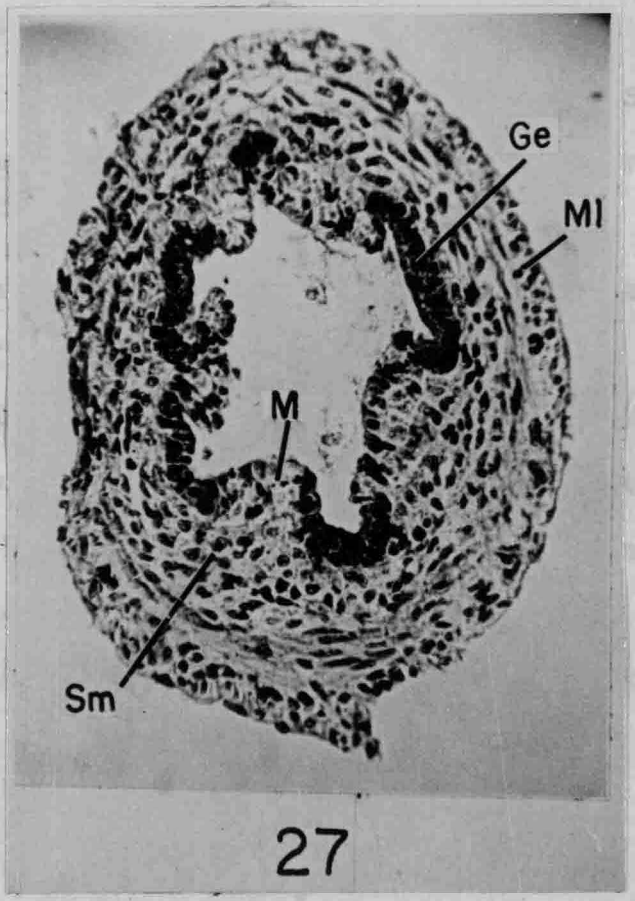
Plate 10

Explanation of figures

Figure 27 Transverse section of the ileum of a Stage 6 animal. Note that the generative cells have spread over the submucosa instead of forming and maintaining cups of generative cells. x 840.



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Vita

Henry B. Robinson was born in Welch, West Virginia on December 17, 1940. He attended elementary school in Roanoke, Virginia and secondary school in Portsmouth, Ohio where he was graduated from Portsmouth West High School in May 1958.

He matriculated at The Virginia Military Institute in September of 1958 and was graduated in June 1962 with a Bachelor of Arts Degree in biology.

During the summer of 1962 he studied at the Mountain Lake Biological Station of the University of Virginia and in the Fall of 1962 he began graduate work at the University of Richmond. While studying at the University of Richmond, he was a member of the Tri Beta National Honary Biological Society and also assisted in general biology and bacteriology labs.

In August of 1964 he graduated from the University of Richmond with a Master of Arts Degree in biology.