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OBSERVATIONS ON THE MECHANISM OF TAIL RESORPTION IN ASCIDIANS

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The resorption of the larval tail in simple ascidians is completed within 15 to 20 minutes after the beginning of metamorphosis. The remarkable speed of the process makes it an especially interesting morphogenetic problem.

According to Kowalevsky (1866), immediately following attachment in *Ciona intestinalis* the withdrawal of the tail is accompanied by the collapse of the notochord and the rounding up of the notochordal cells. The muscle cells which serve for the propulsion of the larval tail separate into simple rounded cells in the body cavity. Experiments by Weiss (1928) suggested that the involution of the tail in *Ciona* is caused by the contraction of the caudal epidermis and the tonic contraction of the musculature. Since excised tails from larvae failed to shorten by themselves, he postulated that some factor in the trunk must be necessary to trigger the involution mechanism.

Berrill (1929) reported that in *Boltenia hirsuta* and other species the destruction of the tail during metamorphosis is effected primarily by phagocytes. Conklin (1931) in a brief description of metamorphosis in *Styela partita* found no evidence for phagocytosis. He reported that the muscle cells, notochordal cells, and nerve tube cells become rounded and are then withdrawn into the trunk region where they remain identifiable. Only the ectodermal epithelium remains normal in staining reaction and general appearance during the process of metamorphosis. He expressed the opinion that proteolytic enzymes are involved in the process of separation of the tail tissues. Grave (1935) also disagreed with Berrill's opinion concerning phagocytosis. He concluded that in *Ascidia nigra* (Savigny) contraction of the muscle cells and the elasticity of the mantle (epidermis) are responsible for the initial resorption of the tail. After further studies Berrill (1947) developed another hypothesis:

> In the resorption of the tail, no phagocytosis is evident, the role of the notochord and muscle tissue appears to be passive, and the active agent in forcing the whole proximally is the shrinkage of the epidermis, which becomes nutritionally exhausted, progressing from the tip to the base. (pp. 263-264)

A detailed comparative histological study of tail resorption based on sectioned material has not been made previously. In the present study descriptions of the larva and metamorphosis of *Boltenia villosa* (Stimpson) based on studies of living specimens, and sections prepared for the light and electron microscopes are presented. The fate of excised fragments of the tail removed at different stages of metamorphosis and the effect of proteolytic enzymes on larvae are described. Tail resorption is compared in four different species.

MATERIALS AND METHODS

Adult specimens of *Boltenia villosa, Pyura haustor,* and *Styela gibbsii,* were dredged and collected intertidally in the San Juan Archipelago. *Ascidia callosa* was collected from floating docks at Friday Harbor. Gametes were obtained directly from the gonoducts of adult animals. Cultures were maintained between 11 and 15° C.

A stock solution of Janus Green B (National Aniline Dye Division, Allied Chemical and Dye Corp.; certification No. NJ11) was prepared with distilled water at a dilution of 1:10,000 and added to cultures of larvae to induce metamorphosis. This dye was first used for this purpose by Bertholf and Mast (1944).

Lyophilized crystalline trypsin and α-chymotrypsin, used in experimental studies, were dissolved in sea water at concentrations of 0.1

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and 0.25 mg/ml. The enzymes were obtained from Worthington Biochemical Sales Co., Freehold, New Jersey.

For light microscopy, specimens were fixed in a solution of 3 parts Bouin's fluid and one part sea water for 8 to 24 hours; they were then dehydrated in ethanol, cleared in toluene, and infiltrated with the following mixture; Fisher's tissuemat (M.P. 60-62°C) 300 gms, bleached beeswax 35 gms, and dry piccolyte 45 gms. Sections were cut at 5μ in a cold room at 5 to 10° C. The hardness of this mixture, in combination with a low sectioning temperature, made it possible to obtain thin sections without compression.

Sections were stained with Harris' hematoxylin and ethyl eosin or with Mallory's triple stain according to the recommendations of Pantin (1948). The PAS histochemical technique was used according to the method described by Gomori (1952).

Light photomicrographs were made on Kodak Microfile film and developed in Kodak D-23 developer at 28° C with constant agitation for about 2½ minutes. Contrast was controlled in special cases by varying the time of development. This method produces negatives with good gradation and extremely fine grain.

Specimens prepared for examination with the electron microscope were fixed in 2.7% OsO_4 and buffered at pH 7.5 with S-collidine buffer (Bennett and Luft, 1959) for one hour in an ice bath. Specimens were dehydrated for one hour in increasing concentrations of ethanol with a maximum of 5 minutes in absolute ethanol. They were transferred through two changes of propylene oxide and embedded in epoxy resin using 1% accelerator according to the methods of J. H. Luft (in press). Sections were stained for 3 hours in 1½% uranyl acetate.

Electron micrographs were taken on an RCA Model EMU 2C electron microscope fitted with a 40μ objective aperture, a Canalco compensator and a specially stabilized power supply. Exposures were made on Kodak fine grain positive film and developed in D-19 developer (Wood and Howard, 1959).

OBSERVATIONS

Larval morphology of Boltenia villosa

The following description of the larva will provide a foundation for a description and discussion of metamorphosis. Emphasis is given to the caudal tissues.

The external morphology of the fully developed larva of *Boltenia villosa* is illustrated in Figs. 1 and 9. One hundred fixed larvae of *B. villosa* measured from the tips of the adhesive papillae to the end of the cellular portion of the tail had an average length of .87 mm (range .78 to .93 mm). The caudal fin alone averaged .30 mm (range .25 to .33 mm) in length.

Tunic. The entire epidermis of the larva, including the atrial and branchial siphonal rudiments, is covered by a thin transparent tunic. The tunic contains several dozen free amoeboid cells. During the first few hours after hatching, the tunic appears to be composed of a single layer; in older larvae it is composed of two distinct layers (Fig. 1). The outer layer forms dorsal, ventral and caudal fins. Janus Green B stains both layers intensely.

Epidermis. The epidermis of the tail (Figs. 5, 7) is composed of a single layer of irregular epithelial cells. The basal surface of the epidermis extends into grooves between the underlying muscle, nervous, and endodermal strand tissues. The outer surface beneath the larval tunic is relatively smooth. At the base of the tail the epidermal cells are columnar in shape. At the level of the preoral pharynx, in many specimens, the epidermis forms a series of eight radially arranged thickenings. These are the rudiments of the ampullae and become prominent epidermal elaborations in the postlarval stages of development. At the anterior end of the larva, one ventral and two dorsal cone-shaped projections of epidermal cells form three adhesive papillae. The basal surface of the entire epidermis is bordered by a PAS-positive basement membrane. The cytoplasm of the epidermal cells is slightly basophilic and contains numerous eosinophilic PAS-positive yolk granules. Similar granules are found in all cells within the larva and early postlarval stages with the

exception of one type of amoeboid cell in the body cavity (macroblasts). These yolk granules have been previously described in the oocytes of *B. villosa* (Hsu and Cloney, 1958) and will be referred to as proteid yolk granules.

Rudiments of siphons, atrium, and digestive tract. The epithelium of the rudiment of the branchial siphon is continuous with the epidermis in the dorsal region of the trunk immediately anterior to the cerebral vesicle and the neurohypophysis (Fig. 5). The rudiments of the pharynx and digestive tract occupy most of the larval trunk. They consist of a simple cuboidal epithelium which remains relatively undifferentiated until after metamorphosis (Fig. 5). The posterodorsal region of the pharyngeal rudiment is divided into two elongate lobes each of which fuses with lateral lobes of the atrial rudiment. The atrial siphon develops in a region of fusion between the atrium and the epidermis.

Nervous system and sensory organs. The visceral ganglion, the otocyst, and the ocellus constitute the nervous and special sensory organs of the larval trunk. The neurohypophysis differentiates into the cerebral ganglion and subneural gland following metamorphosis. Tissues of the visceral ganglion (Fig. 5) extend posteriorly through the canal formed by fused lobes of the atrial and the pharyngeal rudiments into the tail; they become continuous with the hollow dorsal nerve tube.

Body cavity and mesenchyme cells. The body cavity between the pharyngeal rudiment and the epidermis is not lined by a mesothelium. It may properly be called a haemocoel or a pseudocoel. It contains mesenchyme cells of three morphologically distinct types tentatively designated as (1) microblasts, (2) macroblasts, and (3) free endodermal cells. Only the microblasts will be considered here. The trunk region posterior to the endodermal mass and anterior to the caudal muscle cells contains a dense accumulation of microblasts. These cells are also found in fewer numbers in the anterior trunk region. They are nearly spherical in shape and range from 5.5 to 7.0μ in diameter. The cytoplasm appears slightly basophilic when stained with Harris' hematoxylin. A total of 338 microblasts were counted in a single larva. Of these, 26 (7.5%) were in mitosis.

Notochord. The notochord extends from the posterior region of the trunk to the distal tip of the tail (Fig. 1). It is bounded by a thin acellular acidophilic, PAS-positive membrane. This membrane is referred to as the notochordal sheath. In electron micrographs it can be seen to contain numerous filaments approximately 110 A° in diameter. These are oriented at right angles to the axis of the tail and extend around the periphery of the notochord. In cross sections of the tail the filaments are cut longitudinally; in longitudinal sections they are cut transversely (Fig. 8). Filaments of the sheath appear to be continuous with filaments which extend into the intercellular spaces between muscle, nerve cord, and endodermal strand cells.

In the fully developed larva about 40 notochordal cells form a second membranous lining within the sheath (Figs. 6, 7, 8). The central axis of the notochord is filled with a matrix which appears between adjacent cells along the length of the cord before hatching. The development of the notochord was first accurately described by Kowalevsky (1866) in the ascidiid *Phallusia mammillata.*

The notochordal cells possess a well developed endoplasmic reticulum (ER). Dense particles approximately 150 A° in diameter are associated with the membranes of the ER and are also free in the cytoplasm.

Groups of agranular cisternae and small vesicles in the notochordal cells are identified as Golgi bodies (Fig. 8). Relatively few mitochondria are distributed throughout the cytoplasm. The nucleus is irregular in outline and is bounded by a nuclear envelope composed of two membranes. Pores are often observed in the nuclear envelope and the outer membrane is frequently seen to be continuous with the ER (Fig. 8).

The cytoplasm of the notochordal cells next to the sheath contains fine filaments which are oriented at right angles to the filaments of the sheath (Fig. 8). The combination of intracellular and extracellular filaments may be correlated with the fact that

when the tail is excised and some of the matrix of the notochord is allowed to escape, the tail does not collapse but retains its rigidity.

The plasma membranes of the notochordal cells are tortuous. This is particularly evident in regions of contact between neighboring cells. Adjacent cells are separated by a nearly constant spacing of about 200 A°. In the early stages of metamorphosis the notochordal cells move out of the sheath into the trunk region of the larva. At this time they can be observed as discrete nucleated subspherical cells (Figs. 2, 14, 17). Further evidence of the discreteness of these cells was obtained by treating excised tails with a solution of crystalline trypsin (about 0.1 mg/ml) in sea water. This causes the cells to separate from the notochordal sheath and from each other. As they pass into the surrounding medium they round up and assume a spherical shape (Fig. 23). There is no evidence of fusion of these cells in *B. villosa*. (Berrill has claimed (1947) that in many species the notochordal cells form a syncytium in the fully developed larva).

Muscle cells. On each side of the notochord there are three rows of muscle cells. The centrolateral rows consist of only four cells each and do not extend into the distal quarter of the tail. The dorsolateral and ventrolateral rows, consisting of seven cells each, converge distally and terminate near the tip of the notochord. The tail contains a total of 36 muscle cells in *B. villosa*. Each muscle cell is cylindrical with slightly constricted ends. In the dorsolateral and ventrolateral rows the muscle cells average 85μ in length and range in diameter from approximately 6.5 to 7.5μ. The centrolateral cells average 113μ in length and are slightly smaller in diameter than the other muscle cells.

A single nucleus, located near the middle of each muscle cell, is surrounded by an area of yolk-free cytoplasm (Fig. 5). Except for this area the central region of each muscle cell contains many large lipoidal and a few proteid yolk granules. In the living larvae the lipoidal yolk granules appear brilliant orange in color. Treatment with toluene or other clearing agents removes the lipoidal yolk from the cells leaving a reticular framework of cytoplasm outlining clear vacuoles. Fixatives containing OsO_4 stain the lipid black. Mitochondria are extremely abundant beneath the cortical myofibrils along the entire length of the muscle cells. The fine structure of the mitochondria corresponds closely to that described in many other cells (Palade, 1953).

Next to the sarcolemma there are numerous myofibrils. These myofibrils run a spiral course from posterior to anterior, and from left to right in a clockwise direction at an angle of approximately 12°. Myofibrils in the centrolateral muscle cells are limited to the surfaces adjacent to the notochord and bordering the dorsolateral and ventrolateral muscle cells. These myofibrils do not spiral; they extend parallel to the axis of the notochord. In both cell types the sarcomere periodicity is about 1.8μ.

In electron micrographs it is clear that the muscle cells of *B. villosa* do not form a syncytium. Furthermore, the muscle cells can be separated into individual units by treatment with trypsin (Fig. 22). During metamorphosis the muscle cells normally separate into 36 separate units within the body cavity of the postlarval organism, corresponding exactly to counts of muscle cell nuclei in the larva.

Metamorphosis

Initiation of metamorphosis in Boltenia villosa. In laboratory cultures the duration of the larval period is variable. A few larvae often begin to metamorphose about 6 hours after hatching, but within the same cultures other larvae may not begin until 4 or 5 days after hatching. Since it was impossible to predict when an individual larva was going to metamorphose it was convenient to use a relatively nontoxic chemical substance to initiate metamorphosis at the desired time. Ninety to 100% of the larvae of *B. villosa* responded to a concentration of about one part Janus Green B in 700,000 parts of sea water, by metamorphosing within 5 to 15 minutes. Although Janus Green B is a very effective stimulant it induces metamorphosis only in larvae older than 6 hours (posthatch-

ing age). This age corresponds closely to the minimum free swimming period observed in untreated cultures. Metamorphosis without artificial stimulation in culture dishes is usually, though not invariably, preceded by the attachment of larvae to a solid substratum. Following treatment with Janus Green B metamorphosis is generally not preceded by attachment. Otherwise, artificially stimulated larvae appear to metamorphose in the same way as untreated larvae.

General features of metamorphosis in Boltenia villosa. Figures 9 through 12 illustrate the early stages of metamorphosis. Larvae metamorphosing without artificial chemical stimulation first adhere to the surface of a culture vessel by secretions of the adhesive papillae. Within one or two minutes after attachment extensive functional and morphological changes begin to take place in the larva. The tail stops twitching; the tail and the trunk begin to shorten; the adhesive papillae are resorbed; and the inner layer of tunic (postlarval tunic) at the anterior end of the trunk increases its areas of attachment to the substratum. Resorption of the papillae is often complete within two to three minutes after the beginning of metamorphosis, but it may require 10 to 12 minutes. The larval epidermis does not come in direct contact with the substratum at any time during the attachment process. As the papillae are withdrawn the inner layer of the tunic remains as the only adhering surface.

The first indication of tail resorption which can be seen in living specimens, is the rupture of the proximal end of the notochordal sheath. This occurs approximately one minute after attachment. At the instant of rupture, the matrix and cells of the notochord begin to flow out of the notochordal sheath and into the posterior region of the trunk. Simultaneously the proximal muscle cells begin to buckle and fold (Fig. 2). In the early phases of tail resorption the distal tail tissues appear to be unchanged except for changes in the tunic to be described later. As the tail shortens, epidermal cells accumulate near the base of the tail and for a brief period they form a thick "collar" (Figs. 3, 10, 14).

Within 6 to 8 minutes after the beginning of metamorphosis all of the muscle cells in the tail are involved in the advancing region of buckling and folding which progresses distally (Fig. 10). After only 10 to 12 minutes the tail tissues are transformed into a cone-shaped mass behind the trunk tissues (Figs. 3, 11, 14). While the tail is being resorbed the trunk simultaneously undergoes shortening along its anteroposterior axis and increases in diameter (Figs. 2, 3, 4). The cerebral vesicle gradually collapses.

During the first ten minutes of metamorphosis, the anterior body cavity between the pharyngeal rudiment and the epidermis enlarges temporarily (Figs. 2, 3, 14). Within 30 minutes the endodermal tissues change in shape and come in contact with the anterior epidermis over a wide area. The anterior body cavity is shifted posteriorly and to the sides of the pharyngeal and digestive tract rudiments (Figs. 4, 16).

Between 10 and 30 minutes after the beginning of metamorphosis, the caudal tissues, with the exception of the epidermis are completely withdrawn into the body cavity and tend to spread out, away from the axis of the former tail. The cells in the thickened epidermal "collar" region spread out and an invagination forms behind the resorbed tail tissues (Figs. 4, 16, 17). The change in shape of the trunk during the first 30 minutes of metamorphosis involves only a slight shift in the axes of the atrial and branchial siphonal rudiments, but these structures move closer together along the anteroposterior axis of the trunk (Fig. 16).

From 30 minutes to 1 hour after the beginning of metamorphosis the epidermis adjacent to the anterior pharyngeal rudiment begins to develop eight short radially arranged epidermal outpocketings, the rudiments of the ampullae. Two to three hours later the ampullae become prominent lobes. Cells within the body cavity, including the tail elements, continue to spread out and often extend into the ampullar lumina.

At the time of settling the siphonal axes of the larva are oriented approximately parallel to the plane of the substratum. The axes of the nervous and the endodermal

tissues are perpendicular to the surface of the substratum. Within 20 hours after metamorphosis begins, these axes are reoriented approximately 90°. Although the nervous and digestive systems undergo considerable rearrangement in shape and position during the first 20 hours of metamorphosis, there is little histological change.

Histological and cytological changes associated with tail resorption in B. villosa, P. haustor, and S. gibbsii. The epidermis increases in thickness and the inner surface of the epithelium tends to extend into folds of the muscle cells which develop as the tail shortens (Fig. 13). Near the base of the tail, in the region of the "collar," the epidermal cells become elongated at right angles to the tail axis.

As soon as tail resorption begins the inner layer of tunic immediately outside the epidermis begins to form a pleated annular ring around the distal tail region (Fig. 2). This ring enlarges as the tail shortens and 20 to 30 minutes after the beginning of metamorphosis it becomes incorporated into an invagination of the epidermis which develops behind the notochordal sheath in the original caudal axis (Figs. 4, 17). The outer layer of tunic with its dorsal, ventral, and caudal fins is shed as a transparent cuticular "molt" along with about 16 free amoeboid cells (Figs. 2, 14).

As the muscle cells shorten the myofibrils become folded and disorganized but remain in the cortical regions of the cells and retain their strong staining properties with acid fuchsin and eosin (Figs. 13, 14). The muscle cells become more extensively folded in the regions near the surface of the notochord than in the regions adjacent to the epidermis (Figs. 2, 3, 13, 14). It is apparent in electron micrographs that the myofibrils remain close to the sarcolemma during the initial buckling and folding of the muscle cells (Fig. 15).

From 10 to 30 minutes after the beginning of metamorphosis the muscle cells move away from the notochordal sheath into the enlarging body cavity around the pharyngeal and digestive tract rudiment. For a short time the muscle cells retain folded surfaces (Figs. 4, 17), but they gradually round up into spherical bodies. The lipoidal granules remain centrally located.

During the first 3 or 4 days after the beginning of metamorphosis in *B. villosa,* 36 degenerate muscle cells become randomly distributed throughout the body cavity. In later stages they become arranged in the form of a crescent around the developing branchial basket and gut.

The cells which pass into the body cavity from the notochordal sheath in *B. villosa, P. haustor,* and *S. gibbsii* show no signs of necrosis (Figs. 13, 14, 17). The notochordal sheath stains strongly with aniline blue in Mallory's triple stain and can be identified in all stages of tail resorption. As the sheath shortens it decreases in diameter, and develops annular folds along its length (Figs. 4, 14, 17). For many hours after tail resorption it remains as a collapsed and folded membrane in the body cavity.

In electron micrographs of *B. villosa* larvae the filaments of the sheath appear to become disoriented during the early stages of tail resorption. The layer of intracellular filaments in the notochordal cells increases in thickness (Fig. 15). The thickening of this layer of filaments is probably due to a general rounding up of these cells rather than to the formation of new filaments.

Disrupted cells of the dorsal nerve tube and the ventral endodermal strand retain their relative positions as they pass into the body cavity during the first 20-30 minutes of metamorphosis (Fig. 16).

Tail resorption in Ascidia callosa. The caudal muscle cells in the larva of *A. callosa* are of one type. The periphery of each cell is provided with myofibrils which spiral from posterior to anterior in a clockwise direction. The structure of the notochord is similar to that described in *B. villosa* (Figs. 18, 19).

At the beginning of metamorphosis the tail is withdrawn, within about 10-15 minutes, into the posterior region of the trunk and is formed into a coil as previously described in *Ascidia nigra* by Grave (1935). The tail tissues coil clockwise in the same direction as the myofibrils. For a short time after the formation of the coil, the tissues of the tail.

except for the epidermis, retain the same structural relationships as they had before tail resorption (Figs. 20, 21). The matrix in the lumen of the notochord passes into the body cavity. The notochordal cells remain within the sheath and the muscle cells buckle and shorten but continue to adhere to the notochordal sheath. The nerve tube retains its "dorsal" position above the notochord. The epidermis, however, separates from the underlying tissues and forms a mass of cells behind the other tissues. A small invagination formed by the epidermal mass becomes partially filled with the inner layer of the tunic. Numerous yolk granules are present in the epidermal cells, both before and after tail resorption (Figs. 18, 20).

Experimental studies of tail resorption in B. villosa

Histological studies of tail resorption in *B. villosa, P. haustor,* and *S. gibbsii* show that the shortening phenomenon begins at the base of the tail and gradually progresses to the tip. This suggests that some factor necessary for tail resorption emanates from the trunk or the anterior end of the tail. It would be of interest to know if the tail tissues will shorten if they are separated from the trunk.

Tail excision before the beginning of metamorphosis. Larvae were placed in a small wax-coated Stender dish with about 15 ml of sea water. The tails of about one dozen individuals were cut off at various points with a fragment of a razor blade or glass, mounted on the end of a small wooden stick. The larval tunic around the tail tended to stick in small fissures in the wax formed by the cutting edge but the tissues themselves separated cleanly. This made it possible to keep both parts of the larvae together and to keep records of the fate of isolated tails and of the trunks with their tail fragments.

About half of the specimens metamorphosed and the fragment of the tail proximal to the point of excision was resorbed into the posterior trunk region (Fig. 25). All isolated distal pieces of tail, both from individuals which had metamorphosed and from individuals which had not metamorphosed, failed to shorten (Fig. 24). They remained intact and exhibited spontaneous twitching movements for several days after isolation. After four or five days they began to disintegrate, but they never shortened. Weiss (1928) obtained similar results with excised tails of *Ciona* larvae.

A more extensive experiment of this type was carried out using Janus Green B to induce the onset of metamorphosis. In one dish (A) tails were excised from 108 larvae. Cuts were made within the proximal half of the tail. In another dish (B) 255 larvae were left undisturbed. A few drops of Janus Green B stock solution was added to each dish to give a final concentration of approximately 1:700,000. After 2½ hours the Janus Green B solution was removed and replaced with fresh sea water. After 6 hours the results of the experiment were tabulated (Table 1).

These experiments show that the anterior end of the tail, the trunk, or both of these structures are necessary, at least for the initiation of tail resorption. There is no indication, however, that either or both of them are necessary after resorption has started.

Tail excision through the region of shortening. A group of larvae were first stimulated to metamorphose with Janus Green B. After

TABLE 1

	Number of Cases
Dish A—Experimental	
Metamorphosis in progress; proximal half of tail resorbed. Isolated distal half of tail unshortened; displays sporadic twitching.	66
Same as above but tail not observed to twitch.	41
No signs of metamorphosis in trunk. Isolated distal half of tail unshortened; displays sporadic twitching.	1
Total Cases	108
Dish B—Control	
Metamorphosis in progress	254
No metamorphosis	1
Total Cases	255

the beginning of resorption the tails of 34 larvae were excised by a transverse cut through the proximal region of shortening. Some of the tissues which had undergone partial resorption were thus included with the isolated distal pieces of tails. Two and a half hours after isolation 30 of the tails had shortened, 4 had not. This suggests that resorption involves histological changes within the tail tissues themselves. It rules out the possibility that a mechanical force, such as reduced pressure in the trunk sucks or pulls in the tail tissues. It also shows that stimulation from the central nervous system of the trunk is not a factor, at least after the beginning of tail resorption.

Tail excision posterior to the region of shortening. If resorption of the tail involves changes within the tail tissues themselves and if some important factor (physical or chemical) passes gradually from the proximal to the distal end of the tail, it should be possible to interrupt its path by excising the tail distal to the region of shortening and thus inhibit the shortening of the isolated distal piece.

Forty-five such excisions resulted in the shortening of 25 distal pieces. In 20 cases they failed to shorten; twelve of these distal pieces continued to twitch for many hours after the operation.

The failure of these fragments to shorten strongly suggests that the path of some factor has been interrupted. Operational errors of cutting within the shortening area instead of posterior to it may explain why the majority of fragments shortened. It is possible that a portion of the tail may not appear to be shortening at a time when the factor initiating shortening has already passed into it.

Experiments and histological observations together suggest that the shortening of the tail during metamorphosis is brought about by some factor or factors, perhaps chemical, which originate in the trunk or anterior tail region and advance posteriorly. This is further supported by observations presented in the following section.

Experiments with trypsin. While trying to isolate cells of the tail, it was discovered that in larvae older than 6 hours the enzyme trypsin both initiated general metamorphosis and also initiated shortening in tails which had been excised before the beginning of metamorphosis.

In a solution of trypsin (0.1 mg/ml lyophilized crystalline trypsin in sea water) excised tails do not dissociate into an unorganized mass of cells. The tail fragments shorten gradually. The process begins at the anterior cut surface and advances towards the posterior end of the tail fragment (Figs. 26-28). While the anterior muscle cells shorten and become dissociated the posterior ones continue to twitch. As a tail fragment shortens a few notochordal cells are slowly pushed out of the cut end of the notochordal sheath and assume spherical shapes in the medium. Unlike the muscle and notochordal cells, the epidermal cells are not dissociated by the enzyme. They gradually seal over the cut ends of the tail fragment and the stump attached to the trunk.

Chymotrypsin (2.5 mg/ml in sea water) produces similar results, but preliminary experiments with solutions of papain and pancreatin at concentrations as high as 25 mg/ml were not effective in causing the shortening of isolated tails.

Treatment of excised tails of newly hatched larvae (30 minutes to 2 hours old) with trypsin fails to induce the degree of shortening which occurs in tail fragments of older larvae. Although the muscle cells tend to round up and separate from other cells near the cut surface in the excised tails, the notochordal cells are not pushed out of the cut end of the notochordal sheath as they are in older larvae. The epidermis fails to seal over the cut surfaces of the tail. Even after prolonged treatment (1 hour) the adhesive papillae and the tails in whole larvae are only partially resorbed (Figs. 29, 30).

DISCUSSION AND CONCLUSIONS

In *B. villosa, P. haustor* and *S. gibbsii* the resorption of the tail, contrary to Berrill's conclusions based on other species, has been shown by histological techniques to begin at the base of the tail rather than at the tip. The experiment of removing a section of the tail of *B. villosa* larvae posterior to the shortening region, after the beginning of

metamorphosis confirms this. The distal half fails to shorten and even continues to twitch sporadically for many hours.

Resorption of the tail in ascidians is typically synchronized with changes in the trunk of the larva. Berrill's theory (1947) that resorption is caused by nutritional exhaustion and shrinkage of the epidermal cells of the tail would seem to require that larvae metamorphose at the moment when these cells become depleted of nutrients, whether the larva has settled or not. This theory does not appear to be acceptable for the following reasons. (1) In normal as well as artifically induced metamorphosis, the epidermal cells in larvae of 3 different families have been shown to contain abundant yolk granules both before and after metamorphosis. (2) Tail resorption and metamorphosis can be artifically induced at any time (after a short aging period) with a variety of chemical agents. It is not necessary to wait until the epidermal cells become "nutritionally exhausted." (3) Isolated excised tails do not shorten but retain their normal structure and continue to twitch sporadically for several days while the anterior half of the tail is resorbed as the trunk metamorphoses. This strongly indicates that it is not "nutritional exhaustion" which causes the epidermal cells to shrink.

The muscle cells might be considered structurally adapted to withdraw the tail by contraction as suggested by Grave (1935) but microscopic and submicroscopic examinations show that as the muscle cells shorten they buckle and the contractile myofibrils gradually become folded and displaced. They bear no consistent relationship to the axis of the shortening tail. In fact, many segments of the fibrils become oriented at nearly right angles to the axis of shortening. Furthermore, Weiss (1928) has shown that narcotized *Ciona* larvae which are incapable of swimming movements can nevertheless undergo normal metamorphosis. If the muscle cells exert a force which causes the tail to shorten, it is unlikely that the displaced myofibrils are involved in the mechanism. The change in shape of the muscle cells due to their fluid and surface properties may, however, contribute to the overall shortening of the tail.

The experiments with trypsin and chymotrypsin demonstrate that isolated tails shorten when the muscle cells and notochordal cells are caused to dissociate. This, together with the histological observations that intercellular connections are actually broken in the normal process of resorption, and the fact that excised tails cut through the proximal resorbing region will shorten in isolation, suggests that normal tail resorption may involve, at least in part, the production of a proteolytic or another hydrolytic enzyme which gradually diffuses or is activated along the length of the tail.

If a hydrolytic enzyme is secreted near the base of the tail, it would be of interest to know its source. No direct evidence of secretion by any special group of cells has been obtained, but it will be recalled that the microblasts are concentrated in an almost solid mass around the anterior ends of the notochord and the muscle cells. This is precisely the region where histological changes have been demonstrated to begin. The secretion and subsequent diffusion of a hydrolytic enzyme from this point would seem to offer an explanation for the abrupt rupture of the notochordal sheath, the initial release of its matrix and cells, and the observed dissociation of the caudal tissues. These microblasts should be investigated with some of the new histochemical methods for enzymes such as leucine amino peptidase (Nachlas *et al.*, 1959) and chymotrypsin (Benditt and Arase, 1959).

The dissociation of the caudal tissues alone may not be sufficient to cause complete tail resorption. In *A. callosa* the tail is initially withdrawn without the separation of the muscle and notochordal cells from the notochordal sheath. If tail resorption in this species involves the same mechanisms as tail resorption in *B. villosa, P. haustor* and *S. gibbsii,* and if notochordal and muscle cells play only a passive role in this resorption, then an explanation is needed to show how a similar basic mechanism can cause different patterns of resorption. The rapid resorption of the adhesive papillae, the formation

of the epidermal ampullae and the characteristic invagination of the epidermis behind the resorbed tail, which occurs in all species, are evidences of active movements and changes in the shape of the epidermal cells. Although the caudal epidermal cells change shape during tail resorption, it has not been possible to demonstrate conclusively that the epidermis can actively contract.

In the experiments with trypsin, the epidermal cells did not dissociate like the muscle and notochordal cells during the shortening of isolated tails. The intercellular attachments in the epidermis are evidently more resistant to the enzyme.

The failure of trypsin to evoke complete tail resorption in larvae and isolated excised tails treated immediately after hatching suggests that the dissociation of cells beneath the epidermis alone, may not be enough to cause complete tail resorption or shortening in *B. villosa*. It is possible that immediately after hatching some mechanism is not yet competent to exert a force on the separated tail tissues and propel them into the trunk. This competence may develop only some hours later. The mechanism may reside in the epidermis of the tail.

The following tentative hypothesis is proposed as an explanation of tail resorption in pyurids and styelids: After larvae are stimulated to metamorphose a proteolytic enzyme is secreted at the base of the tail which causes directly or indirectly the local breakdown of the notochordal sheath and intercellular cementing substances. The enzyme gradually moves toward the tip of the tail and alters intercellular cohesive substances at the surfaces of all the cells in the tail except the epidermis. Simultaneously the epidermal cells are stimulated to change shape if they have reached a state of competence. The epidermis exerts a mechanical force beginning at the base of the tail which causes the tail tissues to move into the posterior end of the trunk.

It is suggested that in *A. callosa* the proximal end of the notochordal sheath is ruptured by a hydrolytic enzyme with the consequent release of the matrix in its lumen, but without the release of the notochordal cells. A contraction of the epidermis forces the other caudal tissues into a coiled unit, as they are moved into the posterior end of the trunk.

Further work must be done to analyze the process of tail resorption in different species of ascidians. Assuming the basic mechanisms are similar, the differences in the pattern of resorption should draw attention to the fundamental or essential features of the processes. An electron microscopic study of this subject is in progress.

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SUMMARY

1. Histological and experimental evidence indicates that the resorption of the tail of *B. villosa* begins proximally and progresses distally.

2. The sheath at the anterior end of the notochord ruptures at the beginning of tail resorption. The matrix and cells of the notochord flow into the body cavity of the trunk.

3. The muscle cells shorten and buckle as the tail shortens; the myofibrils of these cells become disarranged. Therefore, they probably do not cause the shortening of the muscle cells.

4. Experimental removal of the distal portion of the larval tail of *B. villosa* before metamorphosis showed that excised distal halves of tails normally cannot shorten although the tissues continue to live for several days. The proximal halves of the same tails continuous with the trunk are resorbed when the trunk tissues undergo metamorphosis.

5. Fragments of the tails excised within the proximal region of shortening after the

beginning of metamorphosis will shorten in isolation. These findings indicate that the mechanism of shortening resides within the tail tissues themselves.

6. Fragments of tails excised posterior to the region of shortening after the beginning of metamorphosis normally do not shorten in isolation. This experiment indicates that the shortening process can be interrupted after it starts.

7. Excised tails of larvae of *B. villosa* older than 6 hours will shorten if treated with solutions of trypsin and chymotrypsin. The shortening proceeds from the cut surface of the tail toward the tip. The enzymes also induce metamorphosis in whole larvae older than 6 hours.

8. It is suggested that a hydrolytic enzyme is normally involved in the phenomenon of tail resorption—that it weakens the intercellular binding forces between the muscle and notochordal cells and causes their separation. Histological observations point to the microblasts located at the base of the tail as the possible origin of this enzyme.

9. The epidermis exhibits morphogenetic activity in such phenomena as the resorption of the adhesive papillae, the formation of an invagination behind the tail tissues near the end of tail resorption and in the formation of ampullae.

10. A hypothesis is proposed to explain tail resorption through the interaction of a hydrolytic enzyme and the contraction of the epidermis.

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EXPLANATION OF FIGURES

KEY TO ABBREVIATIONS

AP, adhesive papilla
AS, rudiment of atrial siphon
BC, body cavity
BS, rudiment of branchial siphon
CV, cerebral vesicle
E, epidermis
EC, epidermal collar
EI, epidermal invagination
EN, endodermal strand
ER, endoplasmic reticulum
FS, filaments of notochordal sheath
G, Golgi apparatus
IF, intracellular filaments
L, lumen of notochord
LY, lipoidal yolk of muscle cell
M, mitochondria
MC, muscle cell
MY, myofibril of muscle cell
N, notochord
NC, notochordal cell
NH, neurohypophysis
NU, nucleus
NP, nuclear pore
NS, notochordal sheath
NV, nerve tube
OP, pigment granule of ocellus
OT, otolith
PM, plasma membrane
PR, pharyngeal rudiment
PY, proteid yolk
S, sarcolemma
TI, inner layer of tunic
TO, outer layer of tunic
VG, visceral ganglion

PLATE 1

The diagrams illustrate many of the major changes which take place in the initial stages of metamorphosis of *Boltenia villosa*. All diagrams are seen in dorsal view. Further explanation in text.

1. Larva. X 180.

2. 2-3 minutes after beginning of metamorphosis. X 180.

3. 10-12 minutes after beginning of metamorphosis. X 180.

4. 20-30 minutes after beginning of metamorphosis. X 180.

PLATE 2

Photomicrographs of *Boltenia villosa* larva

5. Sagittal section of larva cut slightly to the right of the median sagittal plane. Double arrows indicate the approximate level of the branchial siphonal rudiment. Single arrow indicates approximate level of the atrial siphonal rudiment. X 620.

6. Frontal section of tail of fully developed larva showing matrix filled lumen of notochord (L), notochordal cell (NC), muscle cells (MC), and lipid yolk (LY). X 620.

7. Cross section of larval tail. The notochord forms the central axis of the tail. The nerve tube (NV) is dorsal to the notochord. The endodermal strand (ES) is ventral. Six rows of muscle cells (MC) extend along the lateral surfaces of the notochord. The epidermis (E) forms a simple epithelium of varying thickness. X 840.

PLATE 3

8. Electron micrograph of the larva of *B. villosa* showing longitudinal section of portion of notochord and muscle cell. Filaments of the notochordal sheath (FS) lie near the plasma membrane of a notochordal cell. Beneath the plasma membrane are intracellular filaments (IF). The endoplasmic reticulum (ER) and Golgi apparatus (G) are well developed in notochordal cells. Membrane bound proteid yolk (PY) granules of various sizes are distributed throughout the cytoplasm. The nuclear envelope is perforated by nuclear pores (NP). The lumen of the notochord (L) contains a matrix of low electron density. X 24,000.

PLATE 4

9. *B. villosa* larva, living specimen, before the beginning of metamorphosis. X 130.

10. *B. villosa,* living specimen, 7-8 minutes after beginning of metamorphosis. X 130.

11. *B. villosa,* living specimen, about 10 minutes after beginning of metamorphosis. The outer layer of tunic has been cast off. The inner layer of tunic covers the entire organism. X 130.

12. *B. villosa,* living specimen, about 15 minutes after the beginning of metamorphosis. X 130.

13. Longitudinal section through anterior tail region of *Pyura haustor* shortly after beginning of metamorphosis. The proximal portion of the tail exhibits marked histological changes. The muscle cells (MC) are buckled anteriorly, but relatively unchanged distally. Myofibrils (MY) are folded within the muscle cells. Notochordal cells (NC) are beginning to pass into the body cavity from the sheath (NS). X 470.

14. *B. villosa,* sagittal section, 10-12 minutes after beginning of metamorphosis. All of the muscle cells (MC) have undergone shortening and buckling. Folds of the muscle cells are more numerous next to the notochord than next to the epidermis. Most of the notochordal cells (NC) have passed from the sheath (NS) into the body cavity of the trunk. Epidermal cells form a thickened ring or collar (EC) around the base of the tail. The pharyngeal rudiment (PR) shortens along its anterioposterior axis and increases in diameter. The body cavity (BC) has temporarily enlarged in the anterior trunk region. X 470.

PLATE 5

15. Electron micrograph of *B. villosa* showing early changes in the tail during resorption. The muscle cell on the right is beginning to develop folds. Myofibrils (MY) lie near the sarcolemma (S) as in the larva. Filaments of the sheath (FS) have become disarranged and are cut at different angles in this section. As the notochordal cells round up due to the shortening of the tail the layer of intracellular filaments beneath the plasma membrane of the notochordal cells increases in thickness (IF). X 41,000.

PLATE 6

16. *B. villosa,* median sagittal section, 20 to 30 minutes after beginning of metamorphosis. The anterior body cavity has been reduced in size by movements of the pharyngeal rudiment. The rudimentary branchial (BS) and atrial siphons (AS) have moved closer together with the shortening of the trunk. The epidermal collar seen at earlier stages is no longer prominent. An invagination of the epidermis (EI) behind the resorbed tail tissue has formed and become partially filled with the inner layer of tunic from the tail. Resorbed tissues of the nerve tube (NV) and endodermal strand (EN) retain their dorsal and ventral positions at this stage. The cerebral vesicle (CV) has become reduced in size. The collapsed notochordal sheath (NS) no longer contains cells. X 620.

17. *B. villosa,* frontal section, approximately the same stage as Fig. 16. A few resorbed muscle cells (MC) are clearly visible. Notochordal cells (NC) lie close to the pharyngeal rudiment (PR). The sheath (NS) is collapsed and folded. The body cavity (BC) has shifted to the posterior region of the trunk. X 620.

PLATE 7

Photomicrographs of *Ascidia callosa*

18. Longitudinal section of larval tail. The cells of the notochord (NC) form a membrane within the notochordal sheath. A matrix filled lumen (L) extends through the center of the notochord. Muscle cells (MC) lie next to the notochord. A few spherical proteid yolk granules are visible in the epidermis (E). X 470.

19. Cross section of the larval tail. Six rows of muscle cells (MC) surround the notochord. The hollow nerve tube (NV) lies dorsal to the notochord. X 470.

20. Sagittal section through the posterior region of the trunk in advanced stage of tail resorption. The muscle cells (MC), notochordal cells (NC), and nerve tube (NV) are formed into a coil during tail resorption. The epidermis forms an invagination behind the caudal tissue. X 470.

21. Cross section through the resorbed tail tissues at approximately the same stage of tail resorption as Fig. 20. Individual notochordal cells (NC) are located within the notochordal sheath but the matrix of the notochord has escaped into the body cavity. Muscle cells (MC) remain attached to the sheath during the initial shortening of the tail. X 470.

PLATE 8

Experimental studies with *B. villosa*

22. Muscle cells isolated from excised larval tail by treatment with a solution of trypsin and application of slight pressure under a cover slip. Lipid yolk granules (LY) are visible in each cell. X 470.

23. Isolated notochordal cells (NC) following treatment of excised tails with a solution of trypsin. X 230.

24. Tail fragment from larva. Excised tails fail to shorten with or without treatment with Janus Green B. They continue to twitch sporadically for several days, finally disintegrating without shortening. X 95.

25. Metamorphosis progresses without special treatment following the excision of the distal 1/3 of the tail. Postlarval development is not adversely affected by removal of the tail. X 95.

Figs. 26-28 demonstrate the result of treatment of fully developed living larvae (posthatching age about 15 hours) with a solution of crystalline trypsin.

26. Larva with excised tail immediately after being placed in solution of enzyme. X 95.

27. After 5 minutes. Changes in the excised tail appear to begin at the cut surface and progress distally. X 95.

28. After 30 minutes. As the excised tail shortens notochordal cells escape from the notochordal sheath. X 95.

Figs. 29-30 demonstrate the results of treatment of newly hatched living larvae (posthatching age about one hour) with a solution of crystalline trypsin.

29. Larvae older than about 6 hours are induced to metamorphose by trypsin, but newly hatched larvae respond differently. The tail and adhesive papillae are incompletely resorbed even after treatment for 1 hour. X 95.

30. The response of the excised tail to trypsin is different from that exhibited in older larvae (Figs. 26-28). The muscle cells appear to dissociate but the notochordal cells are not extruded from the cut end of the sheath. The epidermis does not seal over the cut surfaces. X 225.

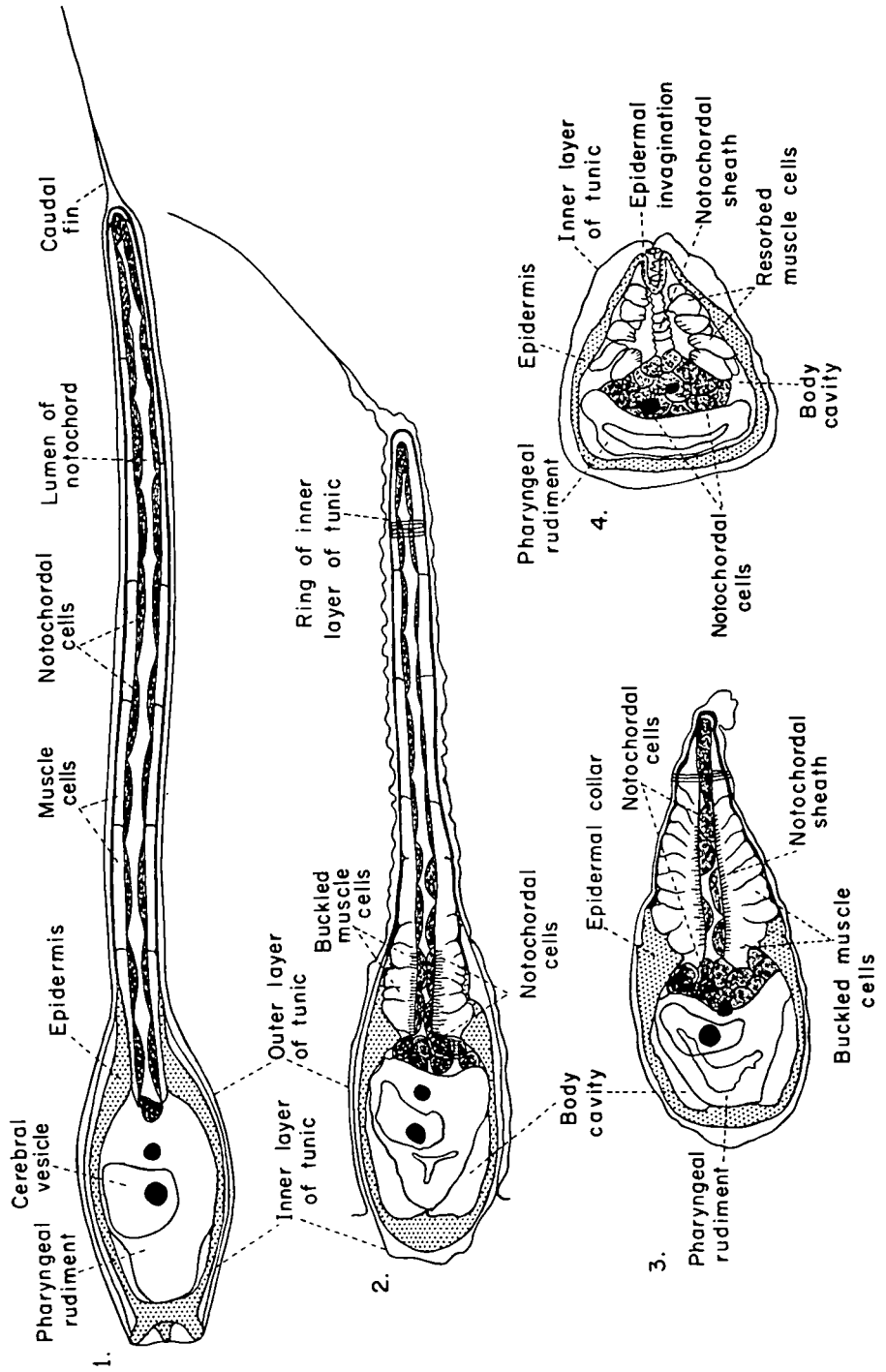
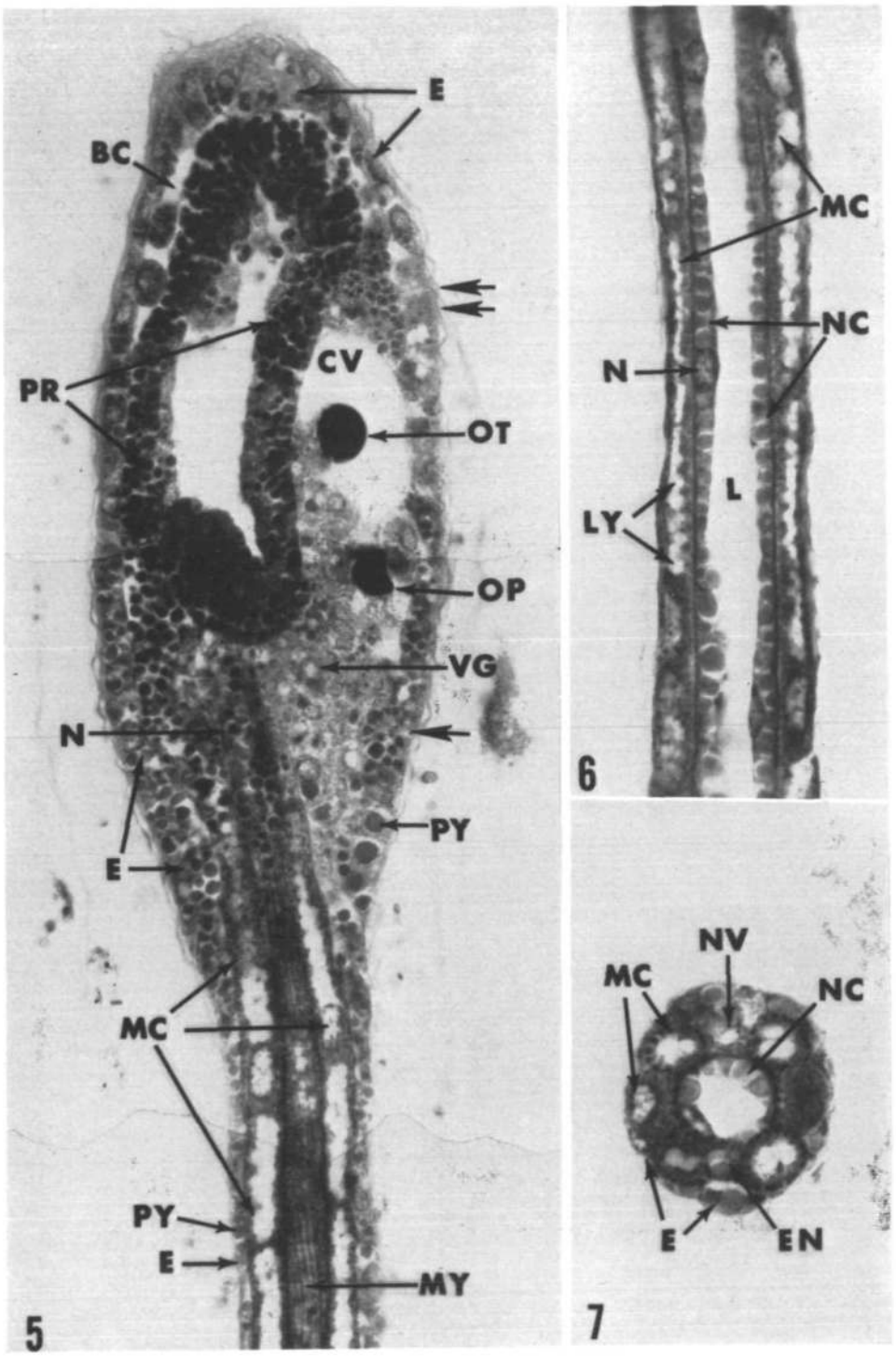


PLATE 1



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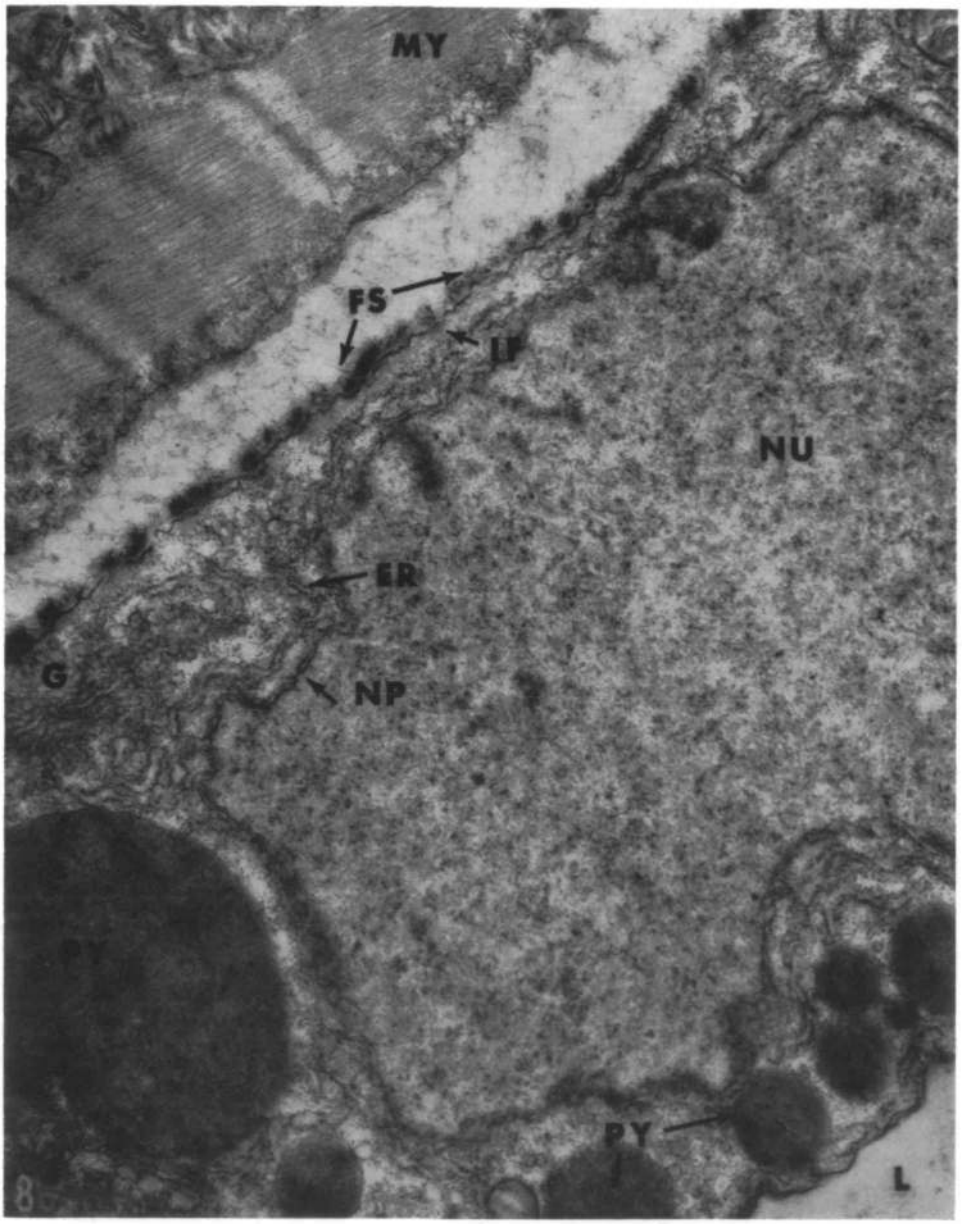

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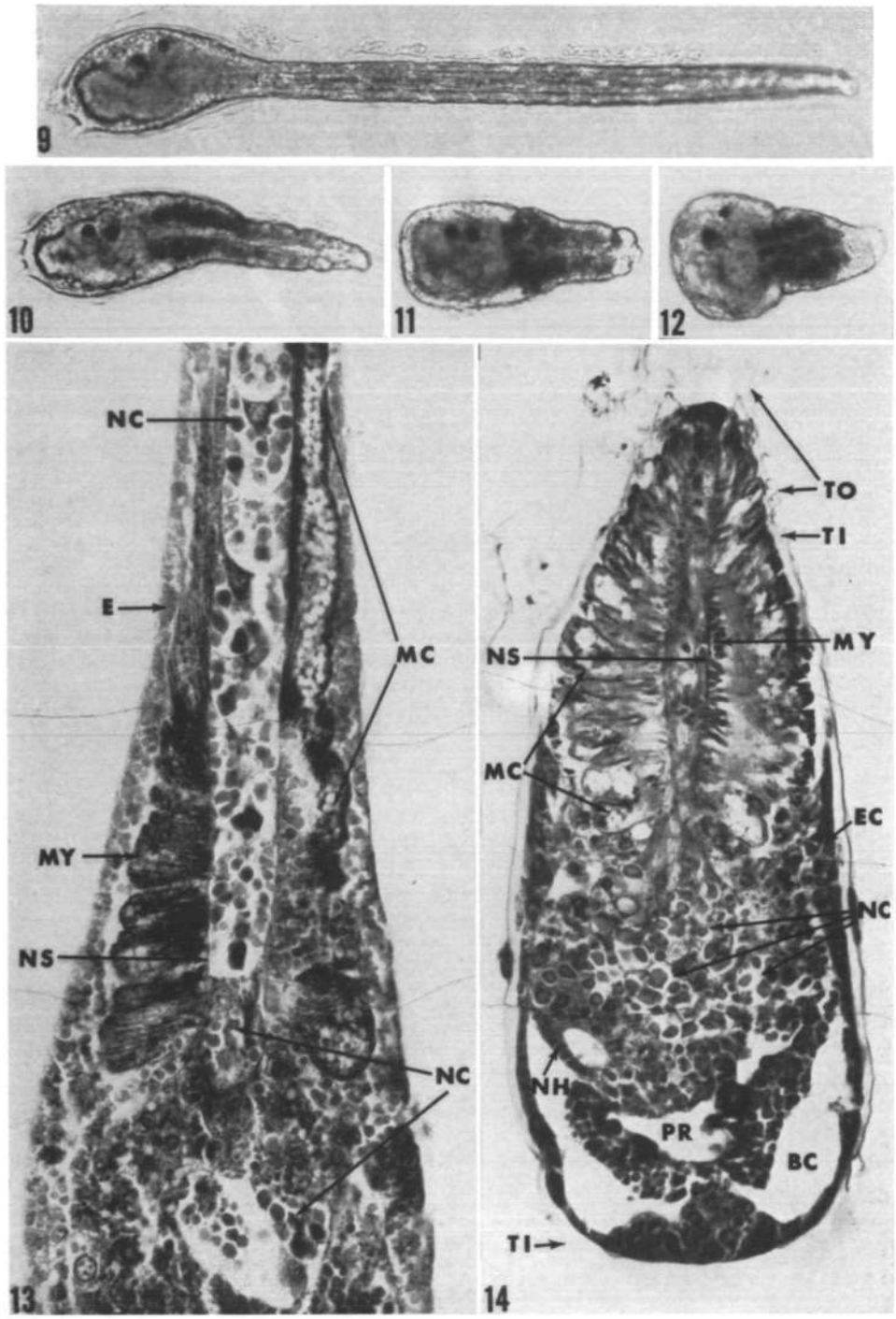

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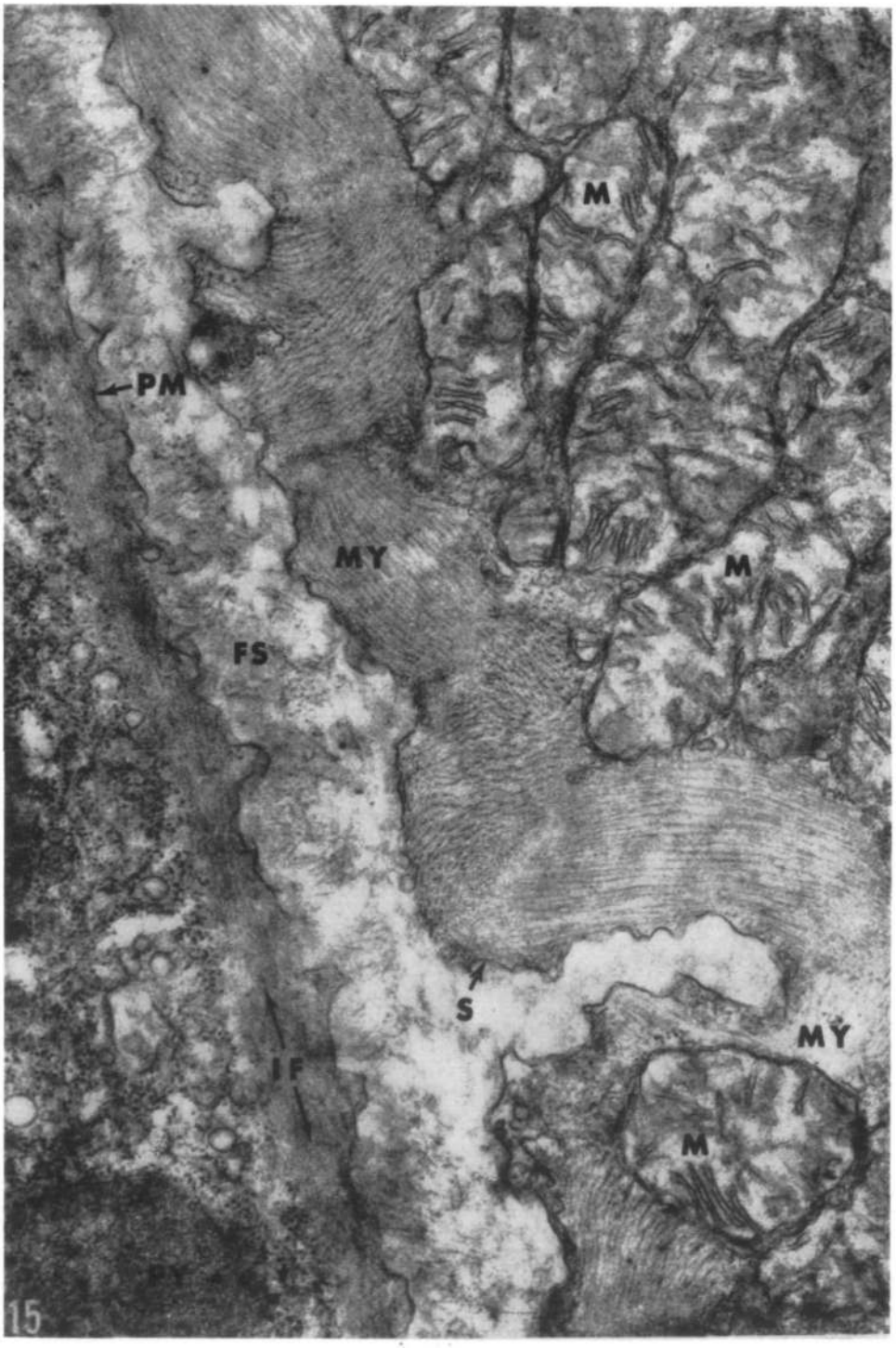

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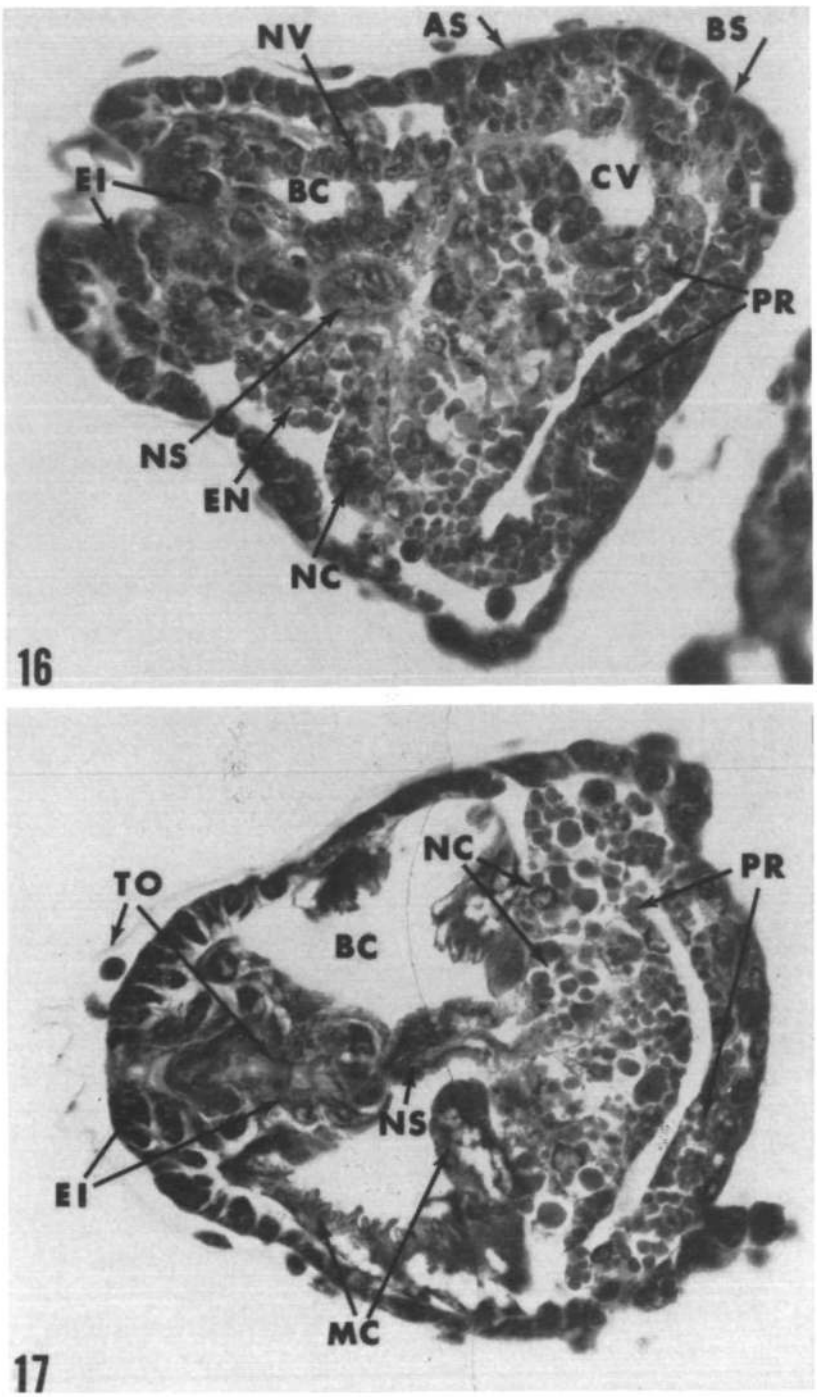

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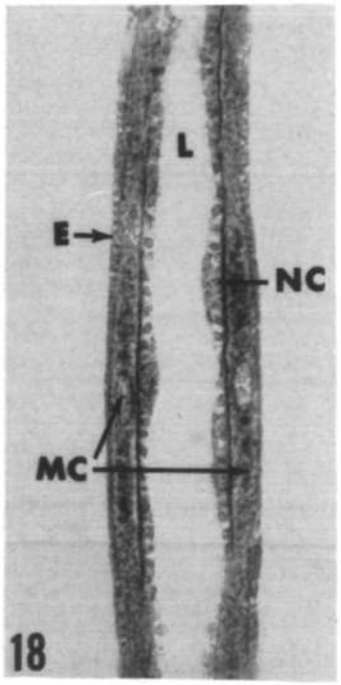

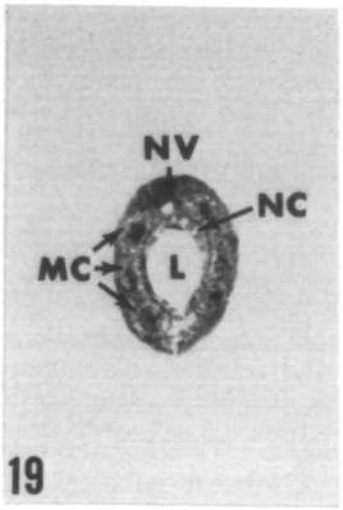

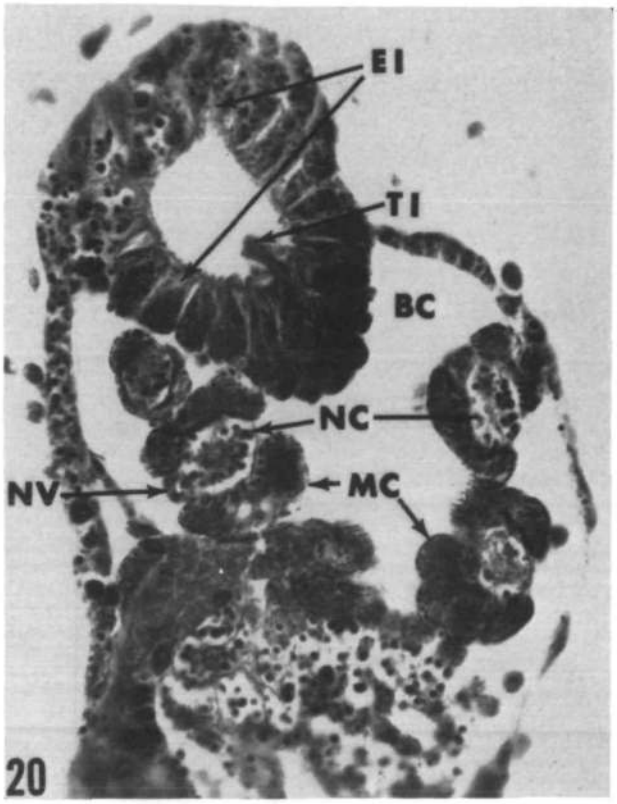

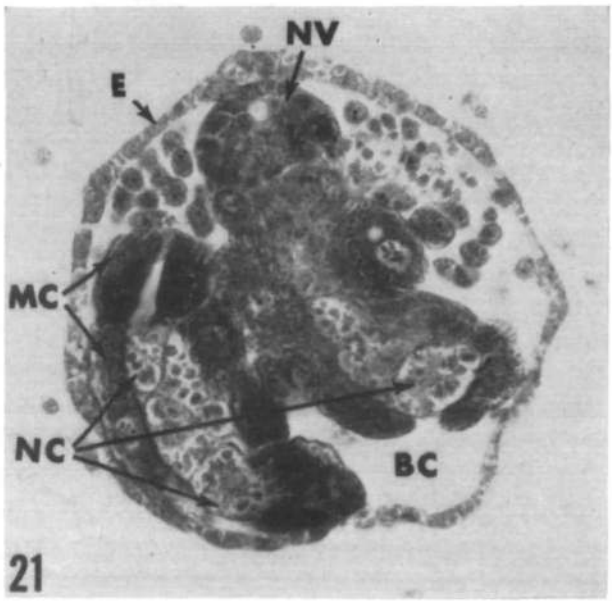

PLATE 7

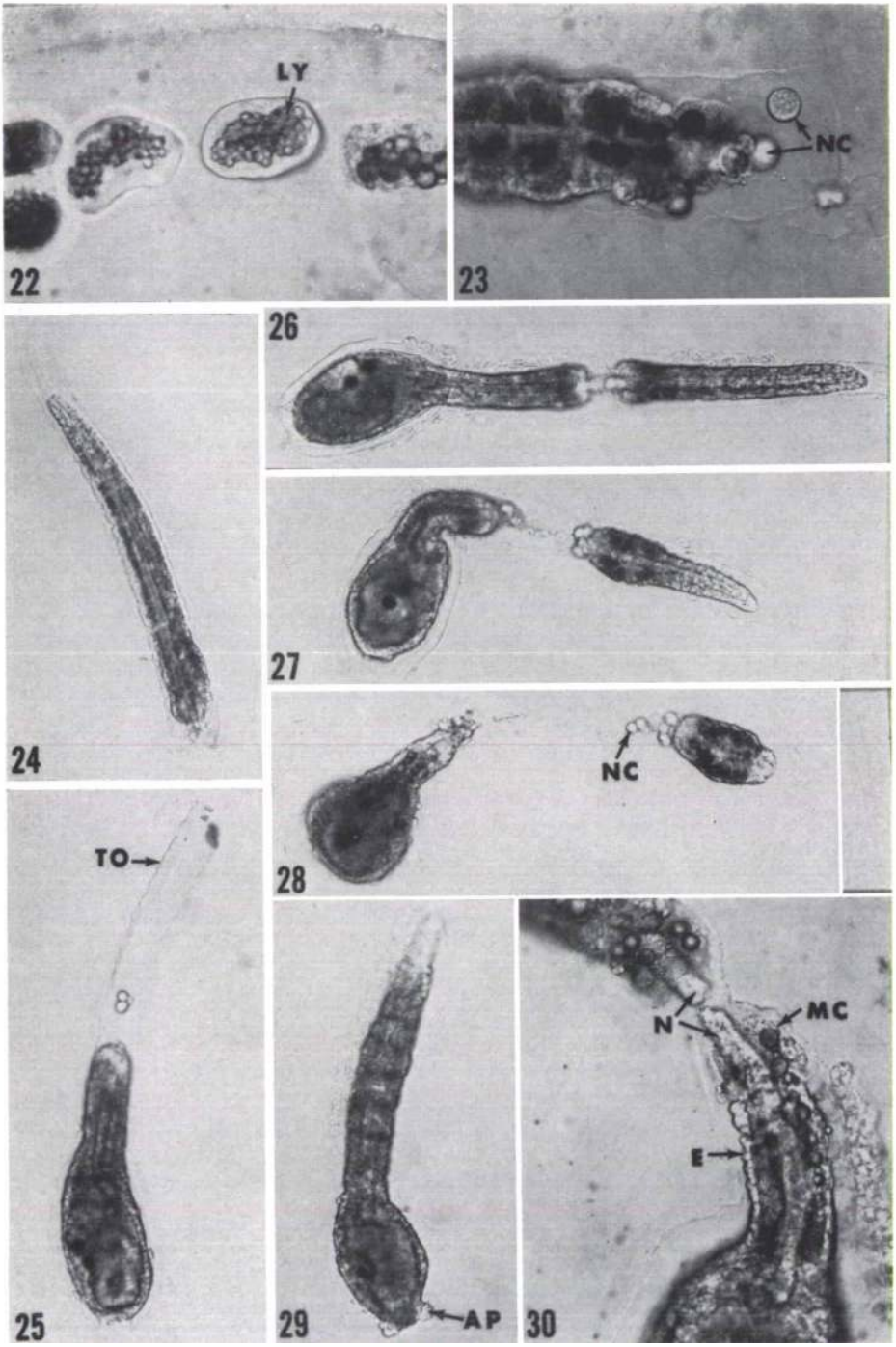

PLATE 8