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STUDIES ON REGENERATION IN THE HOLOTHURIAN *HOLOTHURIA (METRIATYLA) SCABRA* JAEGER

M. MARY BAI

Southern Regional Station, Zoological Survey of India, Madras - 600 028

ABSTRACT

The histological studies made on regeneration of *Holothuria (Metriatyla) scabra* revealed that the alimentary canal regenerates as a thickening from the original mesentery through mitotic proliferation. The presence of groups of morula cells at the anterior and posterior remnants, during regeneration suggests their possible role in the process of wound healing. The circular muscle is formed by the de-differentiation and reorganization of the circular muscle fibres that are already present. The haemal rudiment is formed within three days as a projection of the mesenterial thickening. The respiratory tree originates as a solid rudimentary protrubarance from the ruptured end of the respiratory tree. The rate of regeneration, in all the tissues studied, seems to be more rapid when compared to that of temperate forms.

INTRODUCTION

Echinoderms have long been known to be interesting forms for the study of regeneration. Experimental studies on regeneration of different organs are on record (Hyman, 1955; Nichols, 1964; Swan, 1966; Bakus, 1973). In holothurians, evisceration has been observed as a response to external stimuli (Kille, 1931, 1935). However, there are records of definite occurrence of this process seasonally also (Bertolini, 1932; Kille, 1936; Mosher, 1965; Swan, 1961). Even though detailed investigations on regeneration in holothurians have been carried out in temperate forms, no information is available for tropical forms except for the preliminary report in *Holothuria (Metriatyla) scabra* (Semper, 1868). Further the earlier works have failed to describe in detail the histology of the early stages in regenerative process. Anderson (1965) pointed out that a study of events in visceral regeneration in holothuroids with particular attention to histological details, localization of mitotic activities and cellular differentiation will provide a valuable contribution for comparative purpose. In the present report, therefore, attempts were made to study the evisceration and regeneration of different systems in the holothurian *Holothuria (Metriatyla) scabra* using histological techniques.

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MATERIAL AND METHODS

Method of collection and maintenance : The holothurians *Holothuria (Metriatyla) scabra* used in the present studies were collected from an area of 14 sq. km near Pamban (09° 16' N, 79° 13' E), around Krusadai Islands, 97 miles away from Madurai. Forth to fifty animals were collected at weekly intervals for a period of two years and dissected for the presence of complete viscera. Sampling was made at random by hand picking from the same location. They were transported to the laboratory in well oxygenated polythene bags.

For the experimental studies in the field, the holothurians were maintained inside a pen covering an area of 100 sq. m at Pamban, where the sea water never recedes below the depth of one metre.

In the laboratory, they were maintained at room temperature (28° - 30° C) in cement concrete tanks of 120 - 150 litres capacity. Arrangements were made to aerate and circulate the sea water continuously. This system mainly consisted of an air-lift siphon operated with compressed air and an alkathene, or glass cylinder of 60 cm long with a diameter of 7 cm filled with shore jelly plugged with glass wool. Water pumped by the air-lift recirculates through the glass wool and pebbles and becomes cleared of the debris and excreta of the animals (Fig. 1).

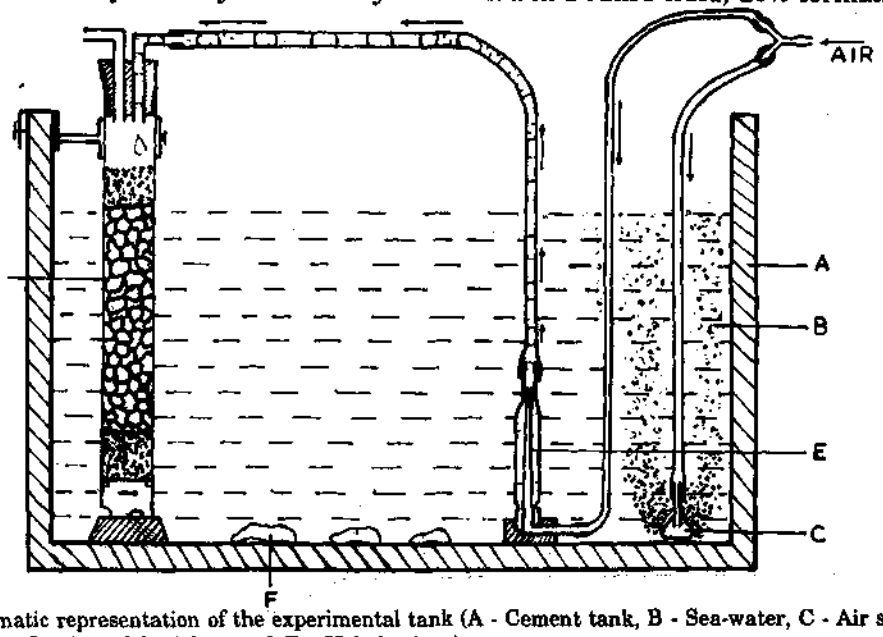

FIG 1. Diagrammatic representation of the experimental tank (A - Cement tank, B - Sea-water, C - Air stone, D - Filtering apparatus, E - Air - lift siphon and F - Holothurians).

Evisceration

Evisceration of the internal organs in holothurians weighing 80 - 200 gms, was induced by injecting 10 - 100 ml of distilled water into the coelom following the method of Dawbin (1948 a). After evisceration the animals were returned to the experimental pen. In the laboratory they were maintained in cement cisterns as described earlier.

Histology

For histological studies, the regenerated tissue samples of 2 cm long were taken to study from five different regions; the junction of the remaining original constriction and the anterior portion of regenerating alimentary canal, the posterior end of the dorsal mesentery, the middle portion of the lateral mesentery, the end of the ventral mesentery at the junction with the cloaca and respiratory tree. The tissues were fixed in Bouin's fluid, 10% formalin, Susa's fluid (all made with sea water), Zenker-formal and Helly's fluid, cleared in oil of winter green and embedded in paraffin wax. Sections of 5 μ thickness were cut and stained with Heidenhein's iron hematoxylin, Mallory's triple strain (MTS), Heidenhein's azan (HA) and Masson's trichrome strain (MT).

RESULTS

The observations on the morphological details of the evisceration and regeneration of *Holothuria (Metriatyla) scabra* have been reported by Mary Bai (1971).

The closure of the anterior and posterior ruptured ends

As a result of evisceration, the coelomic cavity was left in open communication with the exterior through the cloaca. However, the ruptured ends at the anterior and posterior regions of the gut were closed within 24 hours after evisceration. The closed ends consisted of an outer layer of peritoneal epithelium and an inner layer of connective tissue with morula

cells and muscle fragments (Pl. I A). As a result of contraction of muscle fibres, the villi of the lining epithelium were drawn together, thereby obliterating the lumen (Pl. I B). Groups of morula cells were observed in the connective tissue and in the lining epithelium of the 'constriction' (anterior to the cut end) and the cloaca (Pl. I C).

On the second day, the important change noticed at the anterior cut end, was the histolysis of the epithelium. The villi of the 'constriction' were disorganised and their size was reduced (Pl. I D). Inside these villi, pynotic phagocytic cells and morula cells were seen (Pl. I E). Changes like histolysis and disorganisation of the lining epithelium noticed at the anterior ruptured end were not seen at the posterior ruptured end.

In the 'constriction' histolysis of the lining epithelium continued on the third day also. The villi started diminishing in numbers on the third day and by the end of the fourth day they disappeared completely (Pl. I F). As a result, a reduction in the thickness of the wall of 'constriction' was noticed. The number of fragmented muscle fibres found on the first and second day at the closed end were reduced on the third day (Pl. II A) and by the fourth day these were completely missing.

Regeneration of the alimentary canal

After 24 hours of evisceration : There was no change in the course taken by the mesentery. The torn edge of the mesentery was, however, healed and slightly thickened. This thickening which was uniform throughout its length consisted of an outer peritoneal epithelium and an inner mass of mesenchyme with spindle - or oval - shaped de-differentiated muscle fragments and morula cells (Pl. II B).

After 48 - 72 hours of evisceration : There was a distinct increase in the mesenterial thickening. Histological examination revealed that the mesenterial thickening consisted of an inner mass of mesenchyme with de-differentiated muscle fibres and morula cells (Pl. II C, D). Further the cells of the epithelium of the peritoneum were mitotically active.

Four and five days after evisceration : The primordium of the alimentary canal was a rod-like straight thickening along the entire length of the mesentery. The circular muscle fibres have become differentiated on the fourth day (Pl. II E). The cells of mesenchyme and peritoneal epithelium showed evidence of proliferation. On the fifth day the closed end of the alimentary canal became fused with the mesentary.

Six days after evisceration : A complete tubular alimentary canal was formed by the formation of a lumen in the mesenterial thickening, connecting antero-posteriorly the old and regenerating alimentary canal. The newly formed alimentary canal consisted of an outer peritoneal epithelium, a thin layer of circular muscle fibres, a connective tissue layer with amoebocytes and morula cells and a lining epithelium (Pl. II F).

An antero-posterior differentiation in the structure of the alimentary canal was noticed on the seventh day of regeneration. Three distinct changes *viz.*, 1. the peritoneal and lining epithelium had acquired columnar structure, 2. the muscle layer had increased in thickness and 3. the connective tissue was differentiated into an outer thick and inner fluid-like mass so characteristic of the normal intestine, were observed (Pl. III A).

Eight days after regeneration : By the eighth day, the alimentary canal showed evidence of normal functioning, like pulsation and movements. Further, mud and bottom material were seen in the alimentary canal. Faecal pellets were also found indicating that the normal feeding activities had started. Even though the lining epithelium of the anterior part was well organised with secretory glands and goblet cells in the villi (Pl. III B), the differentiation of the mid and posterior region was incomplete. From the seventh to ninth day there was an extensive growth in the regenerating alimentary canal, leading to the formation of intestinal loop (with ascending and descending limbs), simulating the condition in the normal animal.

Nine days after regeneration : On the ninth day, the cells of the lining epithelium of the middle region were columnar and simple (Pl. III C).

Mucus secretion was noticed in both the anterior and middle parts (Pl. III D). From tenth day onwards, there was a gradual thickening of all the layers of the alimentary canal and by 23rd day the structure of the anterior part resembled that of the normal one (Pl. III E).

Changes in the descending small intestine during regeneration : Even though a functional alimentary canal was formed by the ninth day, the differentiation of the posterior region had not completed as mentioned earlier. From fourteenth to thirty-second day, great changes were seen in the descending small intestine. A gradual development of the villi from its simple form to normal complicated and elongated structure was noticed. On the thirty-second day the descending portion of the small intestine had assumed its normal form.

Changes seen in the ascending small intestine : From fourteenth to thirty-second day, increase in the thickness of the different tissue layers were seen. Further, the development of the villi was accompanied by the formation of secretory glands in the lining epithelium. On the thirty second day, the ascending small intestine showed histological details resembling normal intestine (Pl. IV A).

Changes seen in the large intestine : The large intestine which started as a straight tube on the ninth day showed progressive lengthening and changes in the structure. The normal form was attained on the thirty-second day.

Regeneration of haemal system

During evisceration the entire haemal system except the haemal ring and the anterior part of the haemal system were expelled. The ruptured end of the ventral haemal vessel was closed at the end of 24 hours after evisceration. This closure was brought about by the fusion of the epithelial cells of the peritoneum of the adjacent region. A mass of connective tissue with morula cells and muscle fibres were also seen next to the layer of epithelial cells (Pl. IV B). On the third day the rudiment of the ventral vessel appeared as a projection consisting of an outer peritonial epithelium covering a mass of mesenchymal tissue with de-differentiated muscle fibres and a few morula cells at the free edge of the mesenterial thickening (Pl. IV C). The circular muscle fibres were noticed on the fourth day. Mitotic activity was seen in the mesenchyme and peritoneal epithelium of the haemal rudiment of five day regeneration (Pl. IV D). The dorsal haemal vessel developed as a mesenterial thickening at the junction of the alimentary canal and mesentary. The wall of the vessel is composed of a layer of peritoneal epithelium over a layer of connective tissue with morula cells. The process involved in the formation of lumen of both the dorsal and ventral vessel, is similar to that of the alimentary canal. On the eighth day the dorsal haemal vessel started branching. Both the dorsal vessel and the branches consisted of an outer columnar epithelium, a layer of thin circular muscle fibres and a layer of inner connective tissue. Morula cells and amoebocytes were also present (Pl. IV E). With the development of the intestine, the haemal vessels adjacent to the descending small intestine became differentiated into the main dorsal vessel. The haemal vessels close to the ascending small interesting became heavily branched and gave rise to *rete-mirable*. By the end of twelveth day both the dorsal and ventral haemal vessels assumed the normal structure (Pl. IV F). The haemal system attained its full development by the thirty second day.

Regeneration of respiratory tree

The ruptured end of the respiratory tree was covered by the fusion of peritoneal epithelium by the end of 24 hours after evisceration (Pl. IV G). On the second day a layer of connective tissue with morula cells covered by a layer of peritoneal epithelium as a protuberance from the healed end was noticed (Pl. IV H). On the fourth day the regenerated part assumed a tubular form as the result of the formation of lumen connecting it with the main vessel. The respiratory tree was made up of a layer of thin peritoneal epithelium. From sixth day onwards there was a general thickening of the different tissue layers. There was an elongation of the respiratory tree from the seventh to the eighteenth day after the evisceration. Three main branches, measuring 3.5 mm in length, were formed. The respiratory tree entered into the coelom making contact

with *rate-mirable* by the nineteenth day. Further development continued until the thirty second day by which time the normal structure and shape had been attained.

Discussion

In *Holothuria* (*Metriatyla*) *scabra* it was reported that evisceration does not occur in nature either spontaneously or seasonally (Mary Bai, 1971).

Regeneration of alimentary canal : While looking into the origin of the alimentary canal during regeneration two different views have been expressed. Torelle (1909), Bertolini (1932) and Kille (1936) are of the opinion that the alimentary canal is formed by the growth of blind tubular elements, one growing from the anterior, and the other from the posterior end. Later they unite in the middle forming a continuous structure. Conversely, Scott (1914), Bertolini (1930), Kille (1935), Dawbin (1949) and Mosher (1956) have shown that the alimentary canal regenerates as a thickening from the original mesentary, and the anterior and posterior fragments apparently have no influence on the regeneration of the intestine. The histological studies made on consecutive days of regeneration in *H.* (*M.*) *scabra*, have revealed that the latter method of regeneration (namely from the mesenterial thickening) appears to be true in this species as in *Stichopus mollis* (Dawbin, 1949).

The closure of wound at the anterior and posterior ruptured ends is provisional and by muscular contraction of the gut remains, as described by Needham (1952) and Smith (1971).

It is interesting to note that in *H.* (*M.*) *scabra* mitotic activity is encountered in the peritoneal epithelium and mesenchyme cells of the mesenterial thickening, even as early as 72 hours after evisceration. This is indicative of the rapidity with which regeneration takes place. In the other holothurians, in *Holothuria* sp. (Bertolini, 1930 a), in *Thyone briareus* (Kille, 1935) and in *Actinopyga agassizi* (Mosher, 1956) the authors failed to make clear whether mitotic activity is involved or not. However, in *S. mollis* (Dawbin, 1949) noticed distinct mitotic figures in mesenchymal cells lining the lumen and the peritoneal epithelium, only after 4 days of regeneration.

While looking into the regeneration of muscle fibres in the gut, Dawbin (1949) has reported that these fibres in the regenerating gut of *S. mollis* originate from the mesenchyme cells 80 days after regeneration. However in *H.* (*M.*) *scara* it was found that the muscle fibres are formed by de-differentiation of already existing muscle fibres in the mesentary, followed by re-differentiation.

Even though the lumen of the regenerating gut is formed within the mesenterial thickening, there seems to be divergence in the process in different genera. For instance, in *S. mollis* the lumen is shown to have developed by extension and fusion of cleft-like structures in the mesenterial thickening and the lining epithelium is formed later by differentiation of mesenchymal cells (Bertolini, 1931; Dawbin, 1949). In *Thyone briareus* (Kille, 1935) and in *Holothuria* sp. (Bertolini, 1932), the lumen is shown to have formed as a result of the secondary invasion of tubular ingrowth from one or both ends of mesenterial thickening. Mosher (1956) has described in *A. agassizi* that the epithelium of the mesenterial thickening proliferate followed by folding of the surface of the rudiment resulting in the inclusion of blind lumina within the connective tissue core. The lumina later fuse into a single continuous lumen. The present observations on *H.* (*M.*) *scabra* reveals that a hollow space in the middle protion of the mesenterial thickening is formed by the reorganisation of mesenchyme cells. The lumen appears without any constant relation in the position along the regenerating alimentary canal. These finding are in agreement with that of Dawbin (1949) and Bertolini (1931 b) who report the similar features in *S. mollis* and *S. regalus* respectively.

As reported for *S. mollis* by Dawbin (1949), the formation of alimentary canal as a straight tube at an earlier period of regeneration and the antero-posterior differentiation of different layers in *H.* (*M.*) *scabra* may enable the animal to start its feeding earlier and to supply the nutritive material for its further regeneration.

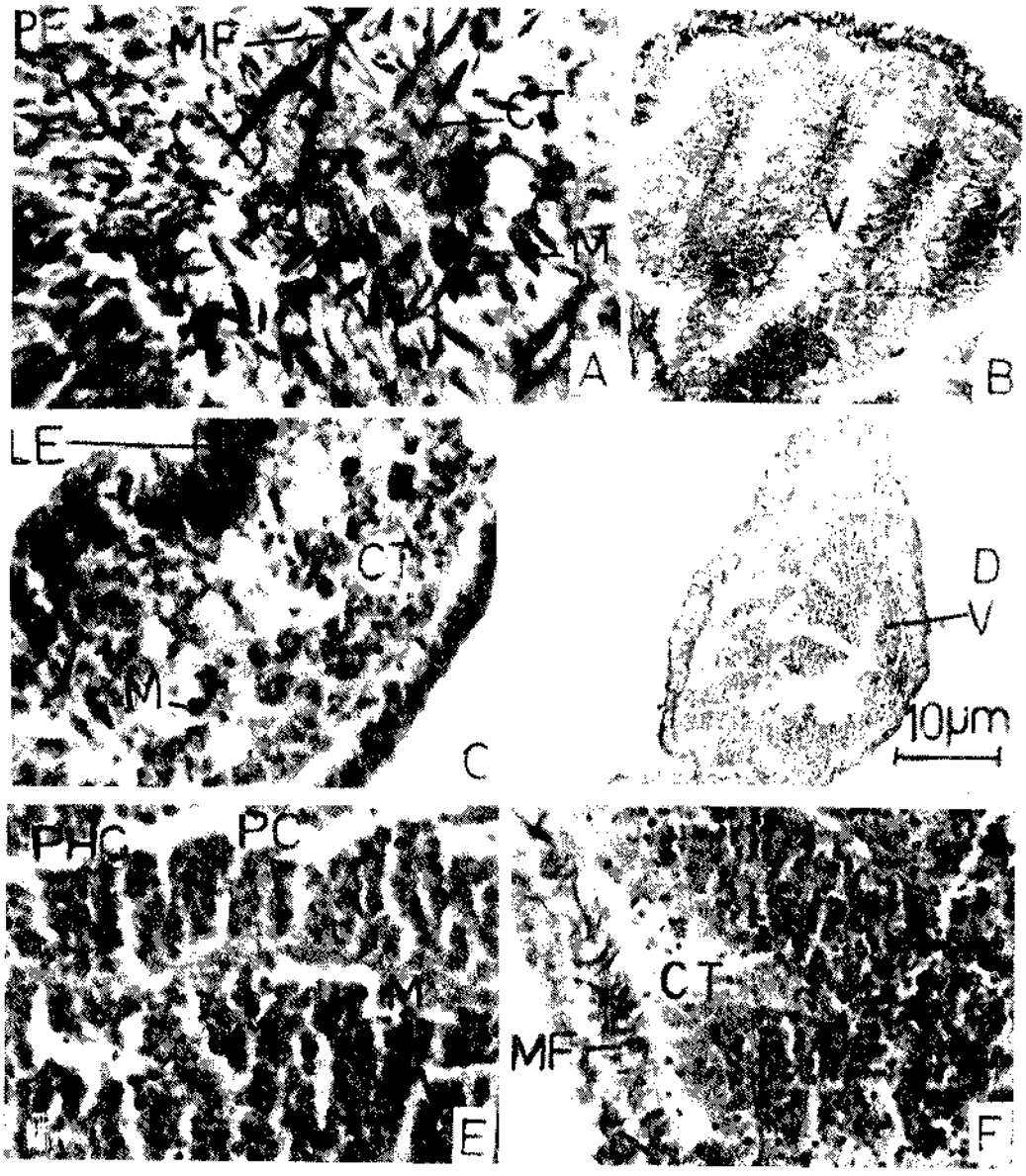

Plate I A. T.S. of the closed end at 'constriction' 24 hours after evisceration showing outer peritoneal epithelium (PE), inner connective tissue (CT) with morula cells (M) and muscle fibres (MF) X 320; B. T.S. of the 'constriction' 24 hours after evisceration. Note the villi (V) are drawn closer together obliterating the lumen X 80; C. T.S. of the cloaca 24 hours after evisceration showing the morula cells (M) in the connective tissue layer (CT) and lining epithelium (LE) X 320; D. T.S. of the 'constriction' of 48 hours after evisceration showing the disorganisation of the villi (V) X 80; E. Portion of T.S. of the 'constriction' showing the presence of Pycnotic cells (PC), Phagocytic cells (PHC) and morula cells (M) X 320 (A-E. Heidenhains iron haematoxylin) and F. T.S. of the 'constriction' 72 hours after evisceration showing the diminution of villi (V) X 320 (Masson's trichrome).

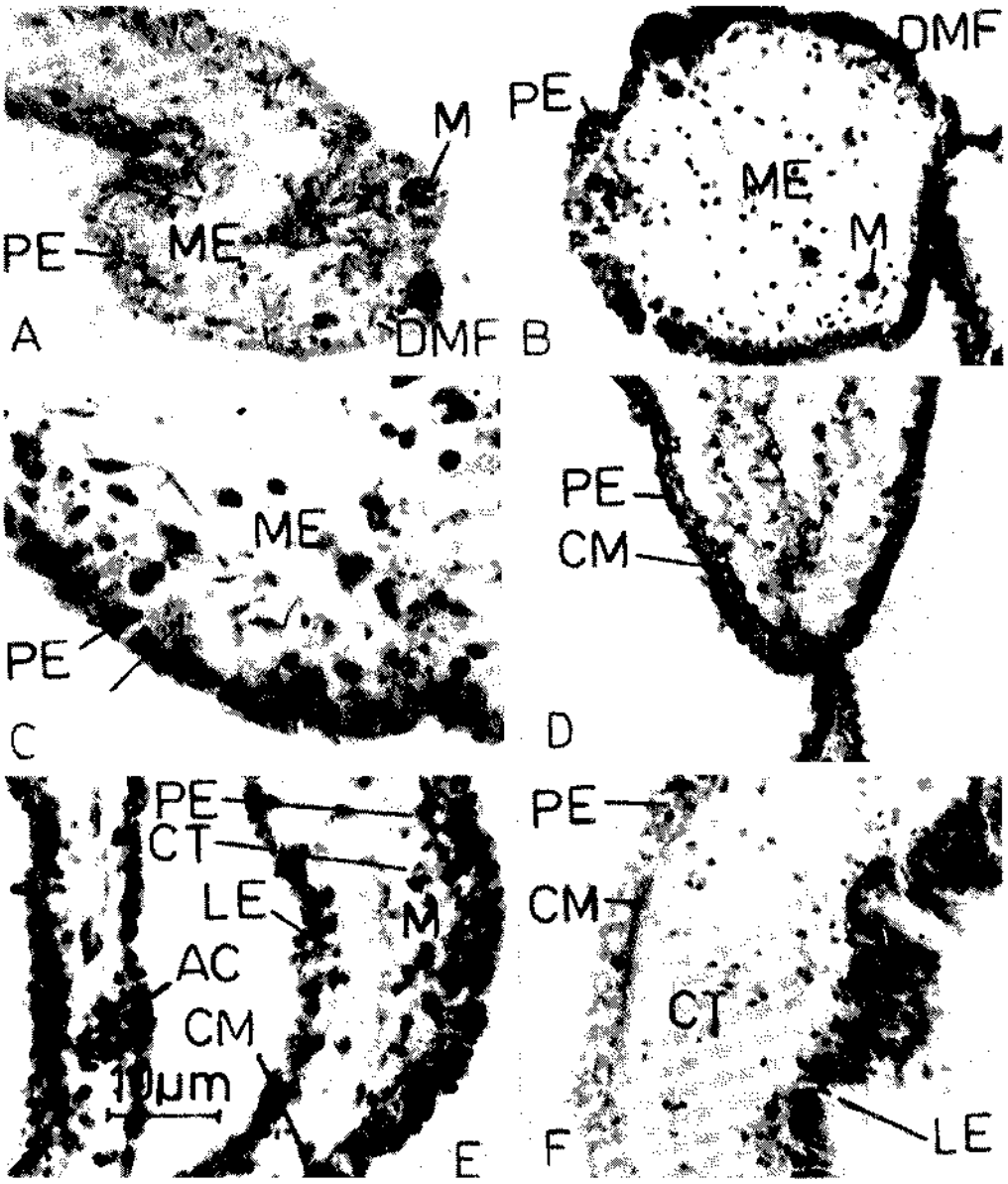

Plate II A. Plate II A. T.S. of the anterior closed end 72 hours after evisceration showing the reduction of muscle fibres (MF) in the connective tissue (CT) X 320; B. T.S. of the mesenterial thickening at the anterior region 24 hours after evisceration showing outer peritoneal epithelium (PE) and an inner mass of mesenchyme (ME) with de-differentiated muscle (DMF) and morula cells (M) X 320; C. T.S. of the mesenterial thickening 48 hours after evisceration showing an outer peritoneal epithelium (PE) inner mass of mesenchyme (ME) with de-differentiated muscle fibres (DMF) and morula cells (M) X 320; D. T.S. of the mesenterial thickening 72 hours after evisceration showing the mitotic activity in peritoneal epithelium (PE) and mesenchyme. The arrow indicates the dividing cells X 800; E. T.S. of the mesenterial thickening 3 days after regeneration. Note the circulatory muscle fibres (CM) are rearranged under peritoneal epithelium (PE) X 320 and F. T.S. of the intestine of 6 days regenerate showing the outer peritoneal epithelium (PE), thin circular muscle layer (CM), connective tissue layer (CT) and inner lining epithelium (LE) X 320 (All in Heidenhain's iron haematoxylin).

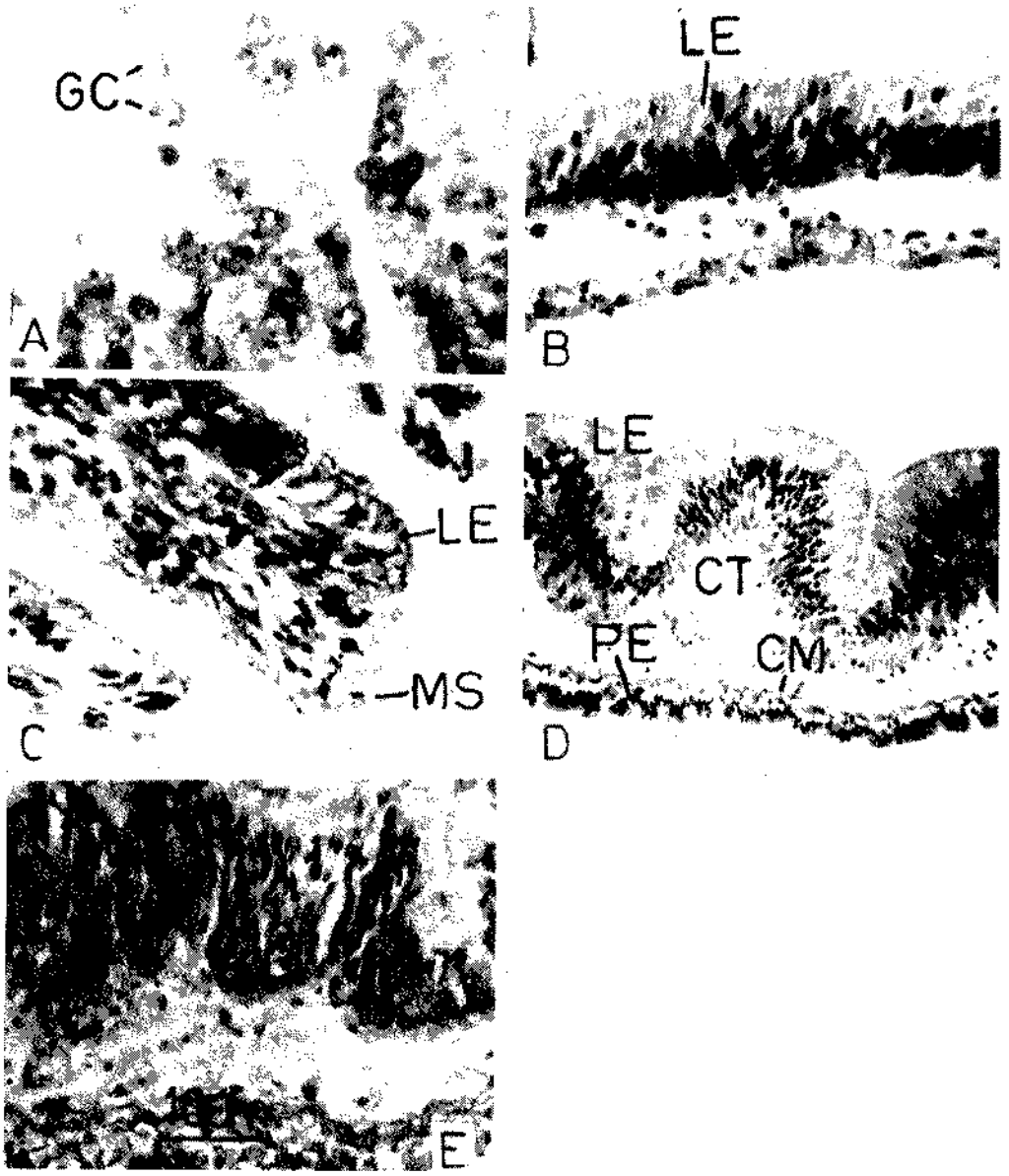

Plate III A. T.S. of the anterior part of intestine 7 days regenerate. Abbreviations as in Pl. II F. X 320 (Masson's trichrome); B. T.S. of the anterior part of the intestine 8 days regenerate. Note the presence of goblet cells (GC) and secretory glands in the lining epithelium X 800 (Heidenhain's iron haematoxylin); C. T.S. of the middle part of the intestine of 9 days regenerate. Note that the lining epithelial (LE) cells had become columnar X 320 (Heidenhain's iron haematoxylin); D. T.S. of the anterior part of intestine of a 9 days regenerate showing the presence of mucus secretion (MS) in the lining epithelium (LE) X 320 (Mallory triple stain) and E. T.S. of the anterior part of the intestine of 23 days regenerate, showing the outer peritoneal epithelium (PE) the muscle layer of circular fibres (CM), connective tissue layer (CT) and inner lining epithelium (LE) X 320 (Heidenhain's iron haematoxylin).

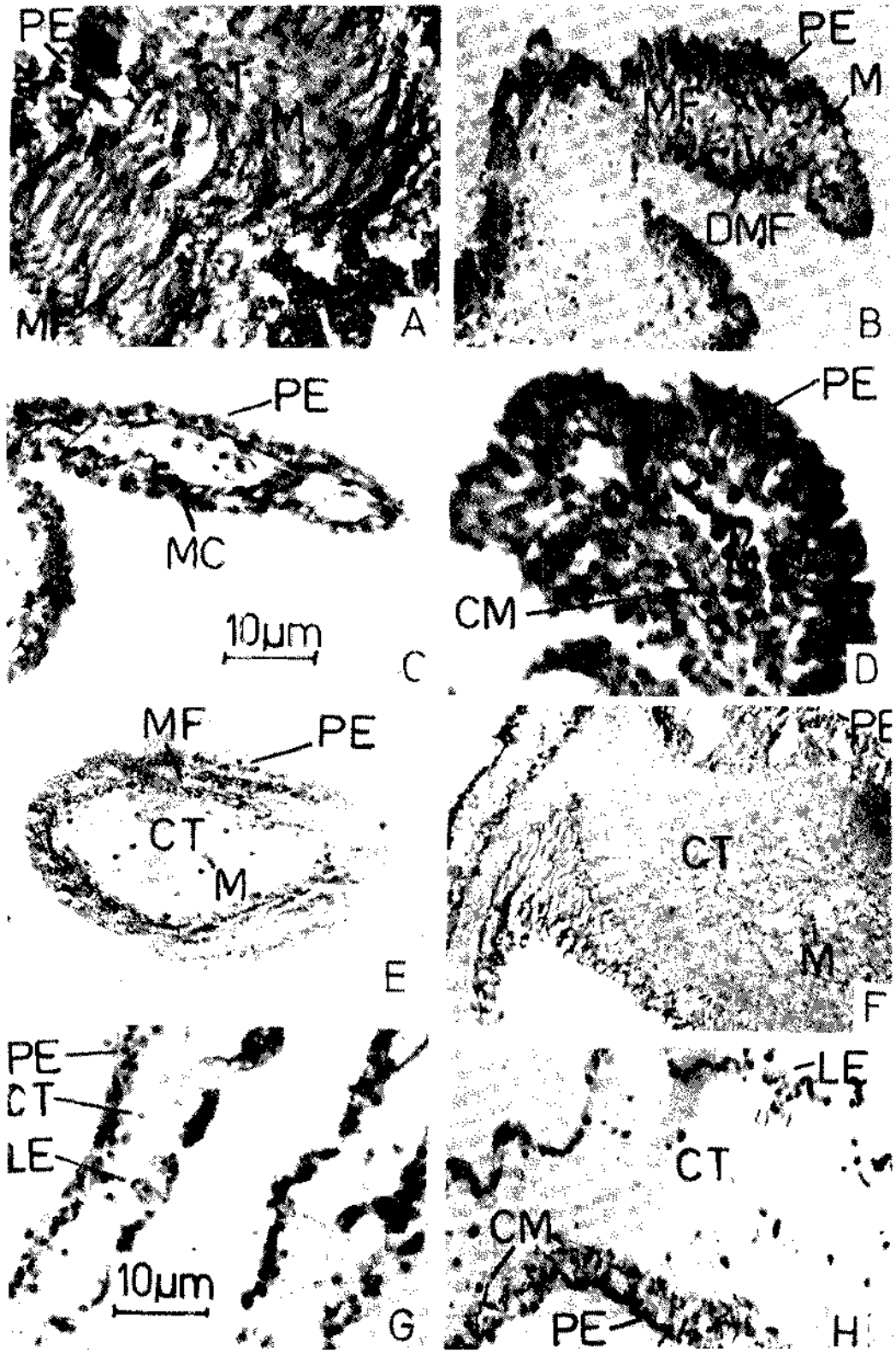

PLATE IV A. T.S. of the ascending small intestine of 32 days regenerate, showing that all the layers have attained the normal structure X 80 (Heidenhain's iron haematoxylin); B. T.S. of the closed end of the ventral haemal vessel showing the outer peritoneal epithelium (PE) and connective tissue (CT) with morula cells (M) and muscle fibres (MF) X 320 (Mallory's triple strain); C. T.S. of the ventral haemal rudiment after 3 days regeneration showing an outer peritoneal epithelium (PE) and inner mesenchyme (MC) with morula cells (M) and de-differentiated muscle fibres (DMF) X 320 (Heidenhain's iron haematoxylin); D. T.S. of the dorsal haemal rudiment after 5 days of regeneration showing the mitotic activity (arrow mark) in peritoneal epithelium (PE) and mesenchyme cells (MC) X 320 (Heidenhain's iron haematoxylin); E. T.S. of the dorsal haemal vessel after 8 days of regeneration showing that the outer peritoneal epithelium (PE), thin circular muscle layer (CM) and inner connective tissue with morula cells (M) and amoebocytes (A) X 320 (Heidenhain's iron haematoxylin); F. T.S. of the dorsal haemal vessel after 12 days of regeneration showing that the haemal vessel has attained the normal structure. X 80 (Heidenhain's iron haematoxylin); G. T.S. of the closed end of the respiratory tree, 24 hours after evisceration showing outer peritoneal epthelium (PE) and inner connective tissue (CT) with muscle fibres (MF) and morula cells (M) X 80 and H. T.S. of the respiratory tree rudiment (RTR) 48 hours after evisceration showing an outer peritoneal epithelium and an inner mass of connective tissue (CT) with morula cells (M) X 80 (Mallory's triple stain).

Regeneration of haemal system : Although investigations have been carried out on the regeneration of different organ systems of holothurians our knowledge of regeneration of haemal plexus is scanty. The earlier observations made by Kille (1935) reveal that this system is formed as a crescent-shaped membrane within the first major loop of the intestine. Even though the above author has not clearly traced out the origin, it can be said from his interpretation that it originates from the intestine. But later Dawbin (1949) has shown in the holothurian, *S. mollis* that the origin of haemal plexus is from the mesentary. Results obtained during the present studies support the view of Dawbin (1949). It is interesting to note both in *Stichopus mollis* and *Holothuria (Metriatyla) scabra* that the ventral haemal vessel appears first as an outgrowth from the mesenterial thickening while the dorsal haemal vessel appears later at the point where the mesentery attaches with the alimentary canal. However, there is a slight difference in that the formation of this system takes a longer time in *S. mollis*.

Regeneration of respiratory tree : Both the alimentary canal and respiratory tree undergo wound healing on the first day itself. The gut takes its origin from the mesentery, the respiratory tree on the other hand starts regeneration from the already existing rudiment of original one. This is because the intestine is lost as a whole during evisceration, whereas, the respiratory tree found on the left side alone is usually expelled out along with the alimentary canal. But for these differences, the other process of de-differentiation and re-arrangement of tissue has been observed to be the same in the gut and in the respiratory tree. The respiratory tree starts regeneration only after 25 days in *S. mollis* (Dawbin, 1949) whereas *H. (M.) scabra* the regeneration starts even after the first day as soon as the wound healing is completed.

Role of coelomocytes : The role of coelomocytes during wound healing has been shown in the different groups of echinoderms (Nusbaum and Oxner, 1915; Cuenot, 1906; Kindred, 1921; Bookhout and Greenburg, 1940; Reichenberger, 1912; Dawydaff, 1901 - as referred by Hyman, 1955). It has been reported that in all these animals the wound is first covered by a delicate membrane, consisting of a layer of epithelium and an underlying connective tissue with coelomocyts. The occurrence of coelomocytes facilitating wound healing has been demonstrated in the echinoid *Psammechinus milaris* by Willi (1966). In the holothurian *S. tremulus* it has been shown by Rollefsen (1965) that a special type of coelomocyte, 'morula cells' play a distinct role in the wound healing process. The accumulation of such morula cells in large numbers at the anterior as well as posterior remnants and closed ends in *H. (M.) scabra* suggests their important role in the process of would healing. Since the major portion of the coelomic fluid is lost during the process of evisceration, with consequent reduction in the number of free coelomocytes in the coelom, the coelomocytes already present in the tissues may have moved to the region of the healing sites. Such a movement of coelomocytes from the tissue, during regeneration has been reported for the asteroid *Henricia leviuscula* by Anderson (1962) and for the holothurian *Leptosynapta crassipatina* by Smith (1971).

It has been shown that in echinoderms, the nutritive materials for the regenerating structure are transported by the amoebocytes (Anderson, 1962; Hyman, 1955). In the holothurians, however, it is thought that this function is taken up by the morula cells. Dawbin (1949) after observing these morula cells (which he calls as wandering cells), in the mesenchyme of regenerating alimentary canal of *S. mollis*, also has concluded that these cells are the carriers of nutritive reserves which are available in the body wall. Since the amoebocytes and morula cells are found accumulated in the regenerating tissues of *H. (M.) scabra*, it is presumed that these may be the carriers of energy source for the maintenance of metabolism during the period of enforced starvation.

Rate of regeneration : The rate of regeneration varies in differentt species of holothurians ranging from 15 to 120 days. Bertolini (1930 b) states that in *S. regalus* the whole process is completed within 15 days. However, a longer period of 25 - 27 days for the completion of regeneration has been shown by Mosher (1956 for the holothurian *A. agassizi*. In *Thyone briareus* regeneration is completed within 32 - 40 days (Scott, 1914; Kille, 1935). Among the

available reports, *S. mollis* is the only form which is known to take 110 days for the regeneration of lost parts. When compared to other species regeneration of lost parts is very rapid in *H. (M.) scabra* in that the animals start feeding even after seven days of regeneration. The high temperature prevailing in Pamban area (27° C) from the where the animals were collected could have induced the process of regeneration quickly.

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