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Echinoid metamorphosis: retraction and resorption of larval tissues

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1 ABSTRACT

During the first fifteen minutes of metamorphosis in Arbacia punctulata and Dendraster excentricus, larval tissues are retracted and resorbed into the aboral surface of the juvenile. Ultrastructural observations of the sequence indicate that there is an immediate contraction of the epidermis mediated by actin-like filaments. Within five minutes, the epidermis begins to histolysize and undergoes necrosis. Metamorphosis can be induced by brief, electrical stimulation of either of two regions of neuropile. Catecholamine neurotransmitters will initiate the contraction-histolysis-necrosis sequence in isolated larval arms. Treatment with the ionophores A23187 or X537A will induce only contraction, whereas histolysis without contraction can be induced by treatment with an extract of histolysizing larval tissues. It is proposed that the nervous system controls initiation of the retraction and resorption sequence during echinoid metamorphosis.

2 INTRODUCTION

It has been reported for several species of echinoids that certain environmental cues will stimulate competent larvae to metamorphose (Strathmann, 1978 for review). Presumably, the environmental cues act as stimuli for receptors on the larva. The receptors, in turn, communicate with the tissues that are the effectors of metamorphosis. The developmental processes that transform or activate these tissues are the response, metamorphosis. Evidence for such a hypothesized stimulus-response mechanism is not complete. Environmental cues have been demonstrated but not fully characterized (Cameron and Hinegardner, 1974; Highsmith, 1977). It is not certain how larvae perceive these cues, though sensory receptors have been identified which may be involved in the perception of tactile components (Burke, 1980). There are several accounts of the fates of larval and adult tissues at metamorphosis (MacBride, 1903; Chia and Burke, 1978) and several of the developmental processes of metamorphosis have been identified (Cameron and Hinegardner, 1978; Chia and Burke, 1978).

The purpose of this report is to present preliminary findings of studies of echinoid metamorphosis in which the main objectives are to characterize developmental processes of metamorphosis and determine how these processes are initiated and controlled. This paper describes the sequence of developmental events that take place in the larval arms during metamorphosis. The arms are specialized larval structures which degenerate during metamorphosis. Results are presented that suggest the retraction and resorption of larval tissues may be controlled in part by the larval nervous system.

3 MATERIALS AND METHODS

All larvae were cultured using the standard procedures outlined by Hinegardner (1969) and Strathmann (1968). Adult Dendraster excentricus were collected intertidally at Sidney Island, B.C. Canada, and adult Arbacia punctulata were collected subtidally at St. Lucie Inlet, Fla. USA.

Larvae were prepared for transmission electron microscopy (TEM) by initially fixing in a solution containing 2.5% glutaraldehyde, 0.2 M phosphate buffer, and 0.14 M NaCl. Specimens were post-fixed for 1 hr at room temperature in 2% osmium tetroxide in 1.25% sodium bicarbonate. Dehydration was by alcohol exchange and specimens were

infiltrated and embedded in Epon. Sections were stained with 50% ethanol saturated with uranyl acetate and lead hydroxide chelated with sodium citrate. Observations and micrographs were made using a Zeiss EM 9S-2.

For experiments with the ionophores A23187 and X537A (courtesy of W.E. Scott, Hoffman-LaRoche Ltd.), and neurotransmitter substances, individual larval arms were dissected from competent larvae with fine tungsten needles and placed in test solutions in depression slides or small watch glasses. The ionophores are not readily soluable in sea water, so dimethyl sulfoxide (DMSO) was used to prepare stock solutions. Final test solutions contained 2% DMSO. To test the effects of electrical stimulation, competent larvae were attached to a fine tipped, polyethylene suction electrode (tip diameter 40-60 µm) and stimulated with either a D.C. square wave stimulator or a regulated D.C. source.

4 RESULTS

4.1 Larval arms

Competent echinoplutei have eight larval arms which in the case of Arbacia punctulata may be up to 800 µm long. The arms are outlined by a dense band of cilia and function as locomotory and feeding organs throughout the life of the larva. In Arbacia punctulata the arms are circular in cross section (Fig. 1a). The exterior surface of the arm is comprised of squamous epithelium 1 to 2 µm thick and the surface of the arm directed towards the larval mouth is columnar epithelium 4 µm thick. The ciliary bands are thickenings of the epidermis that are elliptical in cross section and are comprised of spindle shaped cells each of which has a single cilium at its apical surface. A single tract of axons, 1 to 2 µm in diameter lies in a central position embedded in the base of the ciliary band. Each arm is supported by a single skeletal rod which is located in the central space of the arms and has associated with it numerous mesenchymal cells.

Metamorphosis begins with the eversion of an adult rudiment from an inpocketing on the left side of the larva (see Hyman, 1955 for review). The eversion is usually completed in about 3 to 5 minutes. Beginning at the same time as the eversion, the larval arms are retracted into what will become the aboral surface of the juvenile. The retraction requires about 20 minutes to be completed. It begins with the skeletal rod piercing the tip of the arm and the tube of epidermis collapsing and folding as it is drawn towards the base of the arm. The skeletal rod is eventually dropped from the aboral surface of the juvenile.

The structural changes accompanying the retraction of the larval arms are first apparent in tissues fixed thirty seconds after retraction begins. Bundles of microfilaments (5 to 7 nm in diameter) which are not apparent in competent larvae occur in the cytoplasm of cells of the epidermis and the ciliary bands (Fig. 2). In the epidermal cells, the microfilament bundles occur throughout the cytoplasm and seem most numerous in the cells adjacent to the ciliary bands (Fig. 3). The filaments are usually oriented along the long axis of the arm. Filaments within the ciliary bands occur in both apical and basal portions of the ciliated cells. In the apical regions of the ciliated cells the filaments are oriented at right angle to the long axis of the arm (Fig. 5), whereas in the basal portions of the cell the filaments are oriented along the long axis of the cells.

In larvae fixed two minutes after the beginning of arm retraction, the epidermis is pleated and folded as the arm contracts, and the ciliary bands fold inwards toward the center of the arm. Filament bundles are more numerous in the cells of the epidermis than after 30 seconds and are also apparent within the mesenchymal cells associated with the skeletal rod (Fig. 4). The ciliary bands become disorganized as they fold inward, the cells detach and begin to round up (Fig. 1b). Within the cytoplasm of the ciliated cells axonemes and fibrils of cilia may be found (Fig. 6).

After five minutes the arms have retracted to about one quarter of their original length. The arms are now a thin cuboidal epithelium surrrounding a core of loosely associated individual cells (Fig. 1c). The cells within the core are the cells of the ciliary bands, the axonal tracts, and the mesenchyme. Filaments can still be observed within the covering epithelium and some of the cells within the core. However, ciliary band cells in the core of the retracted arms are rounded-up, have lost their apical-basal polarity, and have nuclei that are more granular and electron dense than at previous stages (Fig. 7). Cytoplasmis debris is scattered throughout the core of the arms, and the cells can be seen to have fragmented.

After ten minutes the larval arms have retracted to small knobs on the aboral surface. The cuboidal epithelium covering the arms has fewer filaments than at previous

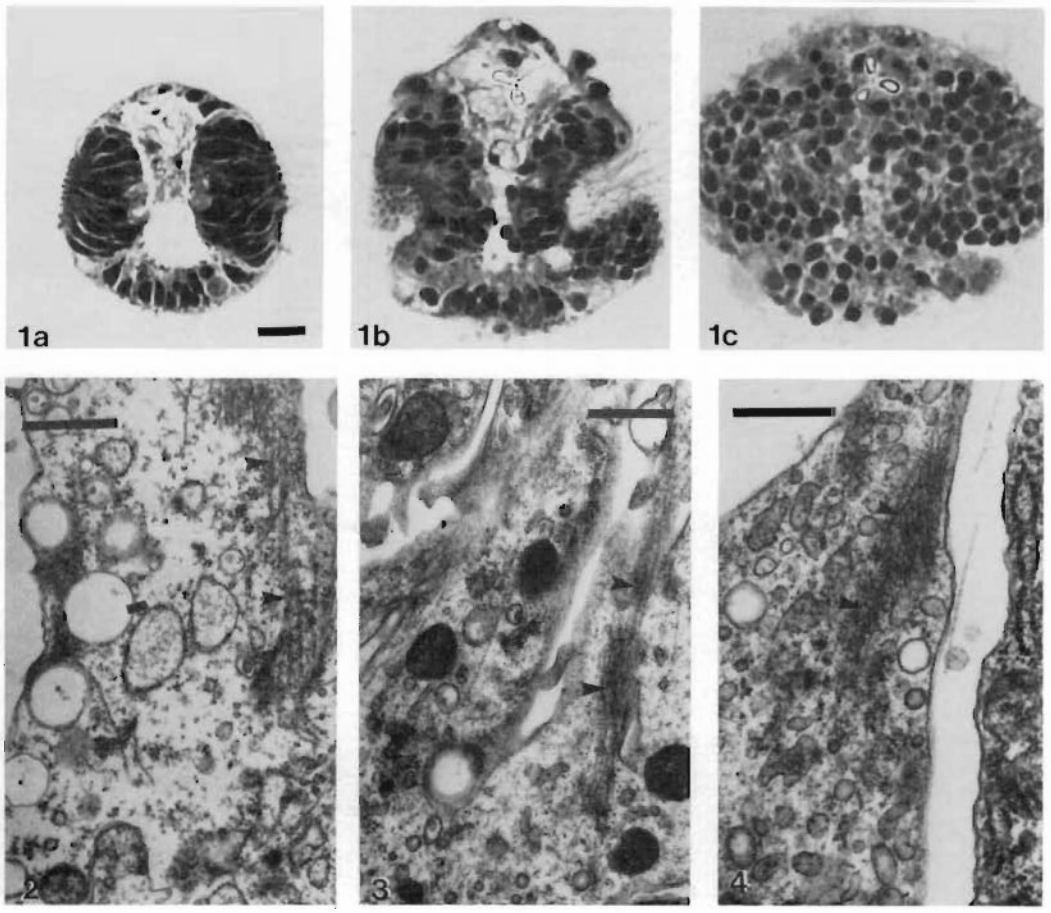

Fig. 1. Light micrographs of cross sections of larval arms of Arbacia punctulata a) prior to metamorphosis, b) two minutes after the beginning of arm retraction, c) five minutes after the beginning of arm retraction. Bar - 10 µm.
Fig. 2. Transmission electron micrograph of epithelial cell of larval arm of Arbacia punctulata fixed thirty seconds after the beginning of arm retraction. Arrows indicate filaments. Bar - 0.5 µm.
Fig. 3. Longitudinal section through cells adjacent to the ciliary band in larval arm of Arbacia punctulata fixed thirty seconds after the beginning of arm retraction. Arrows indicate filaments. Bar - 0.5 µm.
Fig. 4. Longitudinal section through portion of mesenchymal cell associated with larval skeleton in arm of Arbacia punctulata fixed two minutes after the beginning of arm retraction. Arrows indicate filaments. Bar - 0.5 µm.

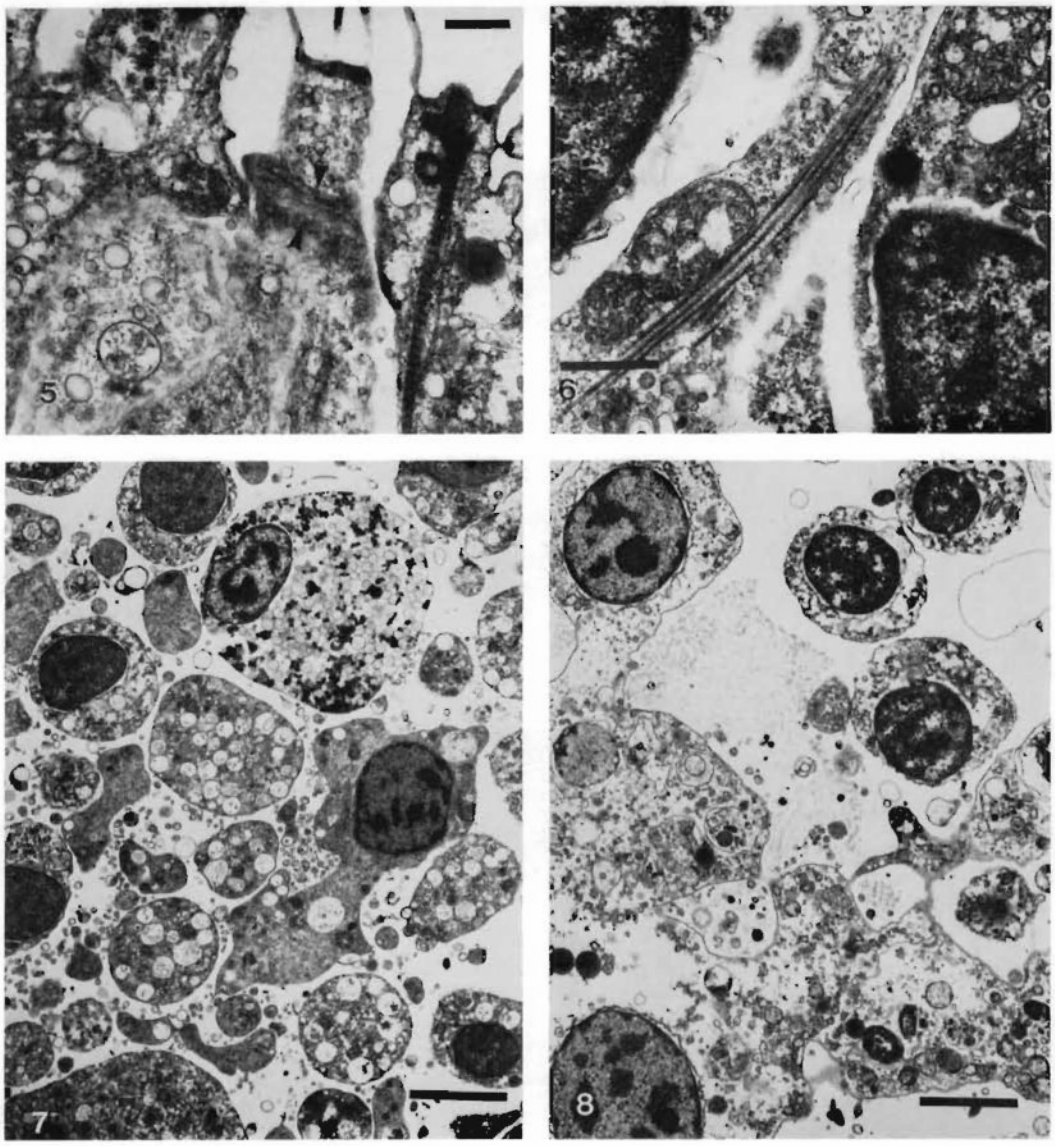

Fig. 5. Cross section of larval arm showing apical region of ciliated cells of ciliary band two minutes after the beginning of arm retraction. Arrows indicate filaments. Bar - 0.5 µm.

Fig. 6. Basal region of ciliated cells of the ciliary band of larval arm of Arbacia punctulata fixed two minutes after the beginning of arm retraction. Note axoneme and fibrils of retracted cilium. Bar - 0.5 µm.

Fig. 7. Core region of retracted arm ten minutes after the beginning of arm retraction. Bar - 1 µm.

Fig. 8. Core region of retracted arm of Arbacia punctulata twenty minutes after the beginning of arm retraction. Large multinucleate mesenchymal cell shows evidence of phagocytotic activity. Bar - 1 µm.

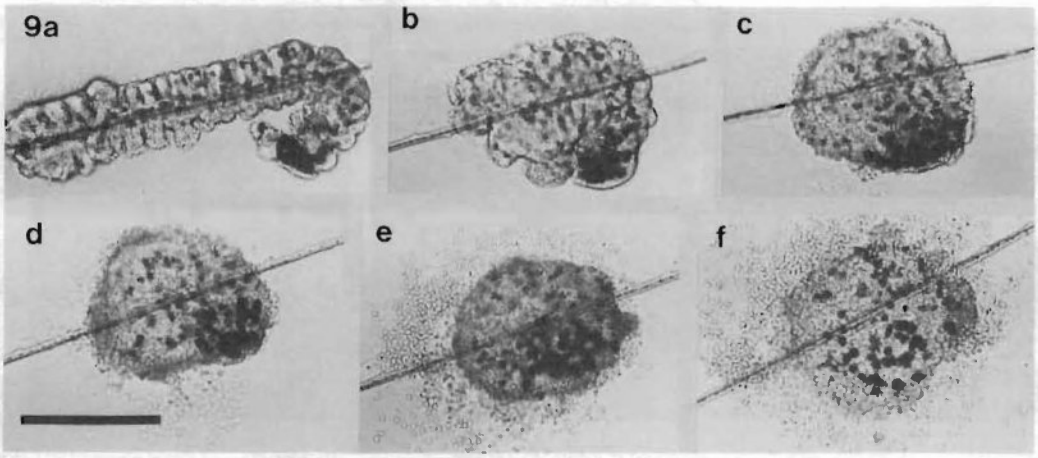

Fig. 9. Larval arm of Arbacia punctulata excised immediately after the beginning of arm retraction. a) 3 minutes after the beginning of ar retraction, b) 5 minutes, c) 10 minutes, contraction is completed, d) fifteen minutes, note beginnings of histolysis, e) 20 minutes, f) 25 minutes. Bar - 50 µm.

stages. The core is largely composed of rounded cells usually containing the remnants of a cilium and a condensed, pyknotic nucleus. Axonal tracts, although greatly disorganized can be identified within the core. As well, there are cells which appear to have been mesenchyme, identifiable by their ultrastructural appearance and their multinucleate condition, scattered throughout the core. In specimens fixed 20 minutes after the beginning of arm retraction these multinucleate cells have begun phagocytizing the necrotic ciliated cells and the cytoplasmic debris of the core (Fig. 8).

4.2 Isolated larval arms

Arms of larvae can be dissected from the body by cutting through the base of the arm with a fine tungsten needle. If an arm is removed from a competent larva not only does the larva seem to be unaffected by its absence, but the isolated arm is able to swim by means of its cilia for periods of up to 48 hours after it has been removed. Isolated arms fixed one hour after excision are ultrastructurally indistinguishable from arms that have not been removed.

Arms removed from metamorphosing larvae that have just begun the retraction of the larval epidermis will complete the entire sequence of contraction and histolysis that occurs in arms that have not been excised (Fig. 9). Ultrastructural characteristics of the sequence in excised arms are identical to those described for intact larvae. Contraction draws both ends of the arms towards the middle, presumably because neither end of the tube of epidermis is attached. After fifteen minutes the arm is a ball of cells pierced with the skeletal rod. Beginning at about the time that the arm is fully contracted, the cells dissociate. After thirty minutes the arm becomes a mass of individual cells.

Isolated larval arms treated with the ionophores A23187 (2×10^{-4}, 2×10^{-5}) or X537A (5 µg/ml, 10 µg/ml) undergo contraction of the larval epidermis 10 minutes after the beginning of treatment (Table 1). Arms rarely contracted to less than $^2/_3$ of their original length and histolysis does not follow contraction. Treatment with 2% DMSO which is used as a solubilizing

Table 1. Summary of experiment with ionophores using excised arms of Arbacia punctulata. Results are from 3 trials of each treatment, 3 arms per trial.

	$2X10^{-4}$M A 23187	10 µg/ml X 537A	2% DMSO
Reversibility (after 15 min)	reversible	not reversible	no effect
10^{-2}M EDTA	no contraction	no contraction	no effect
10^{-4} lanthanum nitrate	no contraction	no contraction	no effect

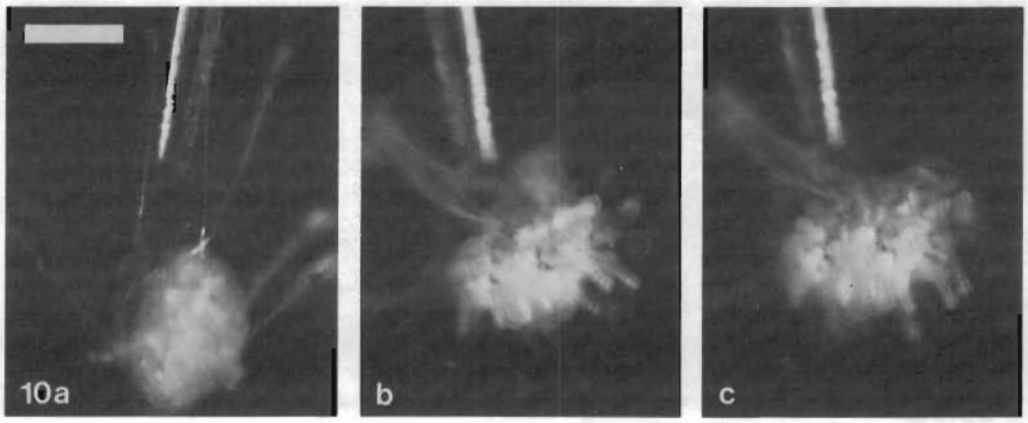

Fig. 10. A competent larva of Dendraster excentricus attached by its apical neural plexus to a suction electrode. a) prior to stimulation, b) 30 seconds after stimulation, the adult rudiment is beginning to evert, c) 45 seconds. Bar - 100 µm.

agent, has no effect on the larval arms. The effects of A23187 can be reversed if the arms are rinsed and put into sea water within 15 min. of treatment with the ionophore, whereas treatment with X537A is not reversible. If either ionophore is used in conjunction with 10^{-2}M EDTA or 10^{-4}M lanthanum nitrate contraction does not occur.

Arms excised from competent larvae that are put into a small volume (50 µl) of sea water in which five larval arms have undergone the contraction and histolysis sequence will also undergo histolysis. After 15 minutes cilia cease beating and the cells dissociate. There is neither apparent retraction of cilia nor contraction of the larval epidermis prior to dissociation.

Excised arms were also exposed to various neurotransmitter substances for periods of up to several hours (Table 2). Arms treated with 10^{-3}, 10^{-4} M noradrenaline, 10^{-3}, 10^{-4} M dopamine, or 10^{-3}, 10^{-4}, 10^{-5} M adrenaline undergo a sequence of contraction and histolysis. The response followed a time course similar to that occurring during metamorphosis of intact larvae, though the arms would not begin to contract until 60 to 120 minutes after treatment. Cells with meshworks of 5 to 7 nm filaments and retracted cilia and pyknotic nuclei, were observed in arms that had been treated with catecholamines and begun contraction. If whole, competent larvae are treated with catecholamines the contraction and histolysis of larval epidermis can be induced, though the adult rudiment would not become activated, nor would it be everted.

4.3 Electrical stimulation

Individual larvae that were competent to metamorphose were stimulated with electrical pulses of 6 to 15 Volts D.C. and 1 msec to 1 sec in duration. Stimuli were administered singly or repetitively for 10 to 15 sec at a rate of 5 to 10 sec^{-1}. If the electrode was attached to any region of the ciliary band, epaulettes, or general body epidermis, each stimulus would produce immediate and coordinated reversals of the

Table 2. Effects of neurotransmitter substances on isolated larval arms of Arbacia punctulata. Two trials of 5 arms per treatment; + indicates contraction and histolysis resulted.

	10^{-3}M	10^{-4}M	10^{-5}M
Acetylcholine chloride	-	-	-
Acetylthiocholine iodide	-	-	-
Acetylcholine iodide	-	-	-
Succinylcholine chloride	-	-	-
γ-Aminobutyric acid (gaba)	-	-	-
5-Hydroxytryptamine (serotonin)	-	-	-
Norepinephrine (noradrenaline)	+	+	-
L-epinephrine (adrenaline)	+	+	+
3-hydroxytyrosine (dopamine)	+	+	-

direction of ciliary beat, and twitch responses of muscles. In the case of Arbacia punctulata, an activation of the adult rudiment as indicated by movements of the spines and tube feet would occur after 5 to 10 stimuli of 100 msec each. For either species if the electrode is attached to the oral surface of the adult rudiment, the larva responds to repeated stimulation by undergoing metamorphosis. In some cases as few as 2 stimuli of 100 msec, or 20 1 msec stimuli delivered in 5 sec would result in metamorphosis. Dendraster excentricus could also be induced to metamorphose by attaching the electrode to a region of the oral hood of the larva between the pre-oral and anterolateral arms (Fig. 10). Metamorphosis begins within the first minute after stimulation, and follows the usual sequence of metamorphic events. Juveniles appear normal and live indefinitely.

The two regions that when stimulated would elicit metamorphosis are associated with dense accumulations of axons. The adult nerve ring is located in the oral surface of the adult rudiment, lying immediately beneath the 5 to 10 µm thick oral epithelium, and a plexus of several hundred axons lies beneath the 3 to 5 µm thick larval epidermis on the preoral hood (Burke, 1978).

5 DISCUSSION

Cameron and Hinegardner (1978) observed bands of dense fibrous material in the apical regions of cells of the larval epithelium of Lytechinus pictus during metamorphosis, and report that the retraction of the larval epithelium is reversibly inhibited with cytochalasin B. They suggest that the retraction of the larval epithelium may be facilitated by autonomously controlled changes in the shape of the cells of the larval epidermis. Several observations reported here for D. excentricus and A. punctulata support such a hypothesis. Within the epidermal cells, ciliary band cells, nerve cells, and mesenchymal cells, 5 to 7 nm filaments with the ultrastructural characteristics of actin were observed during retraction of the larval arms. The time of appearance and orientation of the filaments are consistent with the hypothesis that they produce the contractile forces responsible for the retraction of the larval arms. The ability of arms to contract independently of other larval tissues indicates that the contractile mechanism resides within the arms. The retraction does not appear to simply involve a contraction of the epidermis throughout its length, but apparently additional forces are involved in causing involution of the ciliary bands.

Chia and Burke (1978) reported that during metamorphosis the larval epidermis of Dendraster excentricus becomes necrotic and is phagocytized by cells of the larval gut. The observations reported here support this idea and also show that mesenchyme is involved in the phagocytosis of the histolysized and necrotic tissues of the larval arms. Apparently the epidermis of the larval arms first contracts and about two minutes later histolysis and necrosis begin.

The calcium ionophores A23187 and X537A are a means of elevating the intracellular calcium concentration of cells and have been used in a variety of biological systems (Pressman, 1976). It has also been shown that elevated levels of intracellular calcium are associated with contracting non-muscle cells (Lee and Auersperg, 1980) and it has been suggested that an influx of calcium may be required to initiate contraction (Hitchcock, 1977). The experiments reported here provide indirect evidence that such a hypothesis is correct. The calcium ionophores were able to induce contraction of the larval arms except when used in conjunction with a calcium chelating agent (EDTA) or an inhibitor of calcium flux (lanthanum) (Weiss, 1974).

The ability of larval arms undergoing histolysis to induce dissociation of excised larval arms that have not been stimulated to undergo contraction and histolysis suggests that there is some substance released from the histolysizing arms that causes histolysis. Although further work is required to substantiate such a claim it is notable that contraction and histolysis can occur independently of each other. These experiments suggest that the developmental processes of contraction and histolysis both lie innately programmed within the tissues of the larval arms, and during metamorphosis they are elicited in such a manner as to result in the retraction and resorption of the larval arms.

It has been previously reported that Arbacia punctulata larvae would metamorphose in response to electrical stimulation of 150 Volts delivered in pulses of 1 msec (Cameron and Hinegardner, 1974). They did not note any specific locations for the placement of electrodes. The results reported here differ from those of Cameron and Hinegardner (1974) in that only very low voltages were used, and subsequently, placement of the electrode was critical to stimulating metamorphosis. Because the two locations used are intimately associated

with regions of neuropile it is presumed that these locations were effective because direct stimulation of nervous tissues is necessary to elicit metamorphosis.

These observations are consistent with the hypothesis that the nervous system mediates between perception of cues and the coordinated metamorphic responses of the larval tissues. Presumably the electrical stimulation of neuropile activates nervous elements which act either directly or indirectly to initiate metamorphosis. Because catecholamines appear to initiate developmental responses of metamorphosis, and have been localized histochemically to the axonal tracts of the ciliary bands (Ryberg, 1974; Burke, unpublished observations), they may act as a chemical intermediary between nervous tissues and the tissues of the larva which respond during metamorphosis.

6 ACKNOWLEDGEMENTS

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