International Journal of Invertebrate Reproduction, 2 (1980) 1—12

Morphogenesis of the digestive tract of the pluteus larva of *Strongylocentrotus purpuratus*: sphincter formation

ROBERT D. BURKE [1] and F.S. CHIA

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

(Received 7 June 1979)

In the developing pluteus larva of *S. purpuratus*, the initial morphogenetic event in the formation of a functional gut is the appearance of two constrictions in the archenteron. These two constrictions become the cardiac and pyloric sphincters. During the 2 h in which the constrictions form, the sphincter cells change from cuboidal to wedge-shaped, and the apical ends of the sphincter cells develop an electron-dense region in which microfilaments can be resolved. Constriction of the archenteron was reversibly inhibited by cytochalasin B, although cytochalasin B had no effect once the constrictions had fully formed. Neither the electron-dense region nor the microfilaments were observed after cytochalasin B treatment. It is suggested that sphincter formation is initially accomplished by a microfilament-mediated contraction of the apical ends of the sphincter cells, which changes their shape and constricts the archenteron.

morphogenesis; pluteus larva; microfilaments; cytochalasin B

INTRODUCTION

It has been recognized for some time that a change in the shape of the cells within an epithelial sheet alters the form of the sheet [1]. The thickening, thinning, folding or pocketing of an embryonic epithelium is often attributed to this morphogenetic mechanism [2]. Microfilaments have been found to be associated with a variety of contractile forces that change the shape of cells, thus altering the form of epithelia.

Gustafson and Wolpert [7,8], in reviewing their descriptions of early sea urchin development, concluded that alterations in the form of cell sheets was a major morphogenetic mechanism involved in the formation of the pluteus larva. They further hypothesized that the forces responsible for the changes in cell shape are produced by changes in adhesion between adjacent cells and supporting layers, as well as variations in the tension of cell membranes. There is evidence of microfilament-mediated processes in echinoid embryos during cleavage of the egg and migration of mesenchyme cells

[1] *Current address:* Smithsonian Institution, Fort Pierce Bureau, R.R. 1, Box 194-C, Fort Pierce, FL 33450, U.S.A.

[9—11]. The objective of the research reported here was to examine the role of microfilament-mediated contractility in cell shape changes during morphogenesis of the digestive tract of the pluteus larva.

Morphogenesis of the larval digestive tract in echinoids is essentially the transformation of a hollow tube, the archenteron, into a functional, tripartite gut (esophagus, stomach and intestine). The simple epithelial tube is constricted, shaped and bent by a characteristic sequence of morphogenetic events that are amenable to experimental investigation.

MATERIALS AND METHODS

Culture methods. All embryos were reared from fertilized eggs according to the standard procedures outlined by Strathmann [12]. Adult *S. purpuratus* were obtained from Pacific Biomarine Supply, Venice, CA, or collected intertidally from San Juan Island, WA. Spawning was induced by intracoelomic injection of 0.55 M KCl. Embryos developed normally in either Millipore-filtered sea water (MFSW) or artificial sea-water made from synthetic sea-salts (Instant Ocean, Inc.). Cultures were maintained at seatable temperatures at Friday Harbor Laboratories (10—15°C) or in a controlled environment chamber (15°C) at the University of Alberta campus. When embryos hatched from their fertilization envelopes and began to swim (ca. 30 h), the first embryos to rise to the surface were subcultured and used for experiments.

Microscopy. Nomarski differential interference contrast micrographs of live, unstained specimens were made using the techniques outlined by Allen et al. [13]. Specimens were immobilized by entrapment beneath a supported coverslip.

For transmission electron microscopy (TEM), embryos were fixed for 1 h at room temperature in 2.5% glutaraldehyde in 0.2 M phosphate buffer [14] made isosmotic to sea-water with sodium chloride [15], and postfixed for 1 h at room temperature in 2% OsO_4 either in 1.25% sodium bicarbonate [15] or 0.2 M phosphate buffer [14]. After fixation, specimens were rinsed in distilled water, dehydrated in increasing concentrations of ethanol, and embedded in Epon according to the methods of Luft [16]. Thin sections were mounted on parlodion-coated copper grids and stained with 50% ethanol saturated with uranyl acetate [17] and lead hydroxide chelated with sodium citrate [18]. Sections were observed and photographed with either a Philips EM 201 or EM 300.

Cytochalasin B (CB). Embryos, at appropriate stages of development, were cultured in MFSW containing either 1 μg/ml CB in 0.5% DMSO, or 10 μg/ml in 1% DMSO. Control cultures contained either 1% DMSO or MFSW alone. Individual embryos were isolated from control and experimental cultures, and put into depression slide wet chambers for repeated observations. CB-treated embryos were fixed for TEM as stated above.

RESULTS

General observations. Fertilized eggs of *S. purpuratus* begin to cleave 3 h after fertilization (15°C). Subsequent divisions occur more rapidly and, by 24 h, a morula has formed. The ensuing mesenchyme-blastula stage hatches from the protective fertilization envelope at about 30 h. The initial invagination of the archenteron begins at about 38 h, and gastrulation is completed by 44 h.

At the completion of gastrulation the embryo is roughly ovoid in shape, although the ventral surface is slightly flattened. The archenteron is a blind tube lying along the anterior—posterior axis. The tip of the archenteron is flexed slightly toward the ventral surface and apposed to the prospective stomodeum (Fig. 1).

The epithelium of the archenteron, in regions where the cardiac and pyloric sphincters are to form, begins to thicken by 54 h. The single layer of cells increases from about 3 μm to between 5 and 7 μm in thickness. The cardiac sphincter begins to form at 58 h, appearing initially as a constriction in the archenteron, 20—25 μm from its anterior end. The complete formation of this constriction takes about 2 h. The pyloric sphincter begins to form shortly after the cardiac sphincter and also requires about 2 h to complete formation (Fig. 1).

As a result of the sphincter constrictions, the regions of the archenteron that will form the larval esophagus, stomach and intestine become distinct by about 64 h (Fig. 1). Between 60 and 72 h the larval stomach changes

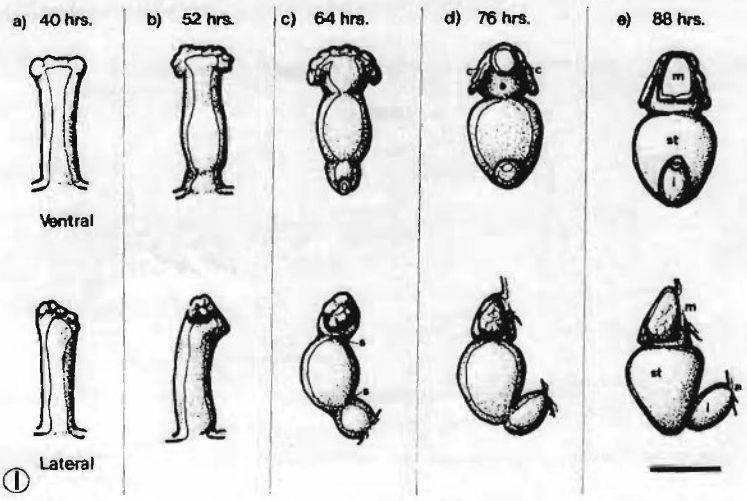


Fig. 1. Scale drawings of the transformation of the archenteron into the larval digestive tract of *S. purpuratus*. a: anus; c: coelom; e: esophagus. i = intestine; m = mouth; s = sphincter; st = stomach. Bar = 50 μm.

from a straight-walled tube to a sphere. By 76 h the larval stomach has expanded from its initial diameter of 20 μm to a diameter of 50 μm. During this period of time, the esophagus enlarges to 40 μm at its greatest diameter and the intestine becomes 30 μm at its greatest diameter. As well, between 72 and 86 h there is an apparent thinning of the epithelium of the esophagus, stomach and intestine.

Between 64 and 76 h the digestive tract, which began as a relatively straight tube, bends to form a J-shape, with the intestine curving anteriorly from the pyloric sphincter.

The mouth forms between 60 and 72 h appearing initially as a small opening which gradually enlarges to 15 μm in diameter when the larva begins to feed (88 h after fertilization, 15°C).

Transmission electron microscopy. Prior to the formation of sphincters, the archenteron is comprised of simple cuboidal epithelium. The cells, which measure about 4 μm on each side, are connected near the luminal surface by zonulae adhaerentes, and a thin basal lamina lines the blastocoelar surface. Each cell contains numerous yolk vesicles, clear vacuoles, cisternae of rough endoplasmic reticulum, clumps of ribosomes, mitochondria, and a centrally located nucleus (Fig. 2a). As well, each cell bears a single cilium on its luminal surface. Between 54 and 58 h, the cells in the region of the presumptive sphincter become columnar and measure 7 μm in height.

During the constriction of the archenteron, the cells that participate in the formation of the sphincter change from columnar to wedge-shaped (Fig. 2b). In specimens fixed during the constriction of the archenteron, there is an electron-dense region, 0.1 μm thick, immediately beneath the

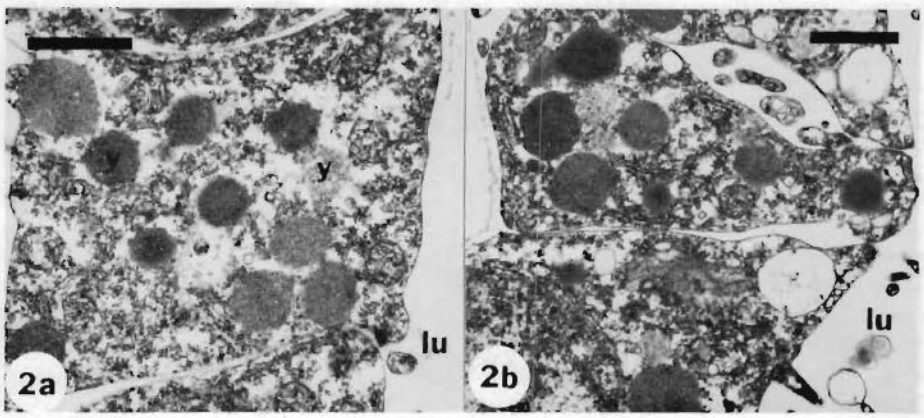


Fig. 2. a: TEM of cells in the presumptive sphincter region of the archenteron of a *S. purpuratus* embryo prior to the formation of the constriction. b: wedge-shaped cells of the presumptive sphincter region of an embryo fixed during the constriction of the archenteron. lu = lumen of the archenteron; y = yolk. Bars = 1 μm.

plasmalemma on the luminal surface of the wedge-shaped sphincter cells (Fig. 3a, b, c). The electron-dense region consists of a fine granular matrix in which microfilaments can be discerned. The microfilaments within the sphincter-forming cells appear to be aligned circumferentially (Fig. 3b, c, d). In some sections the microfilaments are sparse and short, while in others they are more abundant, and occur in lengths up to 0.7 μm. The luminal surface of the wedge-shaped sphincter cells also appears to have a greater density of 0.5 μm-long microvilli than adjacent cells.

Sphincter cells in embryos fixed after the formation of the constriction are also wedge-shaped, although neither electron-dense regions nor microfilaments were observed. The cytoplasmic constituents of the cells appear unchanged from their condition prior to sphincter formation.

Cytochalasin B experiments. The first of 3 sets of experiments was conducted on embryos in which the epithelium of the presumptive sphincters had thickened but had not yet formed sphincters (54 h after fertilization) (Fig. 4a). After 1 h, embryos treated with 1 μg/ml CB maintained the same thickened epithelium in the presumptive sphincter region, and some individuals showed the onset of constriction. Embryos treated with either 5 μg/ml or 10 μg/ml CB developed no constrictions in the archenteron, whereas control embryos incubated in either MFSW alone or 1% DMSO formed constrictions in the archenteron.

After 2 h, the control embryos had completed constriction of the archenteron (Fig. 4c). Embryos treated with 1 μg/ml CB showed retarded development of sphincters with respect to control embryos, as the sphincters were at a stage comparable to that of the untreated embryos after 1 h. In embryos treated with 5 μg/ml, the endodermal cells appeared to be rounded, and no constrictions were evident (Fig. 4b). Embryos treated with 10 μg/ml also showed no constrictions, and there was extensive rounding and dissociation of endodermal cells.

When the experimental embryos (after 2 h treatment) were rinsed and returned to MFSW, the effects abated. Those previously treated with 1 μg/ml CB responded quickly (within 1 h) and completed the formation of the sphincter. The embryos that had been treated with 5 μg/ml CB developed recognizable sphincters within 2 h (Fig. 4d). The 10 μg/ml treatment was not so readily reversed: the dissociation and rounding of endodermal cells in most cases remained even after 6—8 h in MFSW.

The second set of experiments involved treating embryos in which the constriction in the archenteron had begun to form (59 h after fertilization) (Fig. 5a, b). Treatment with 5 or 10 μg/ml CB removed the constrictions within 1 h (Fig. 5b). The 1 μg/ml CB treatment did not obviously affect the constriction process. Embryos in both of the control cultures developed normally.

Removal of the embryos from the 5 μg/ml CB treatment after 1 h

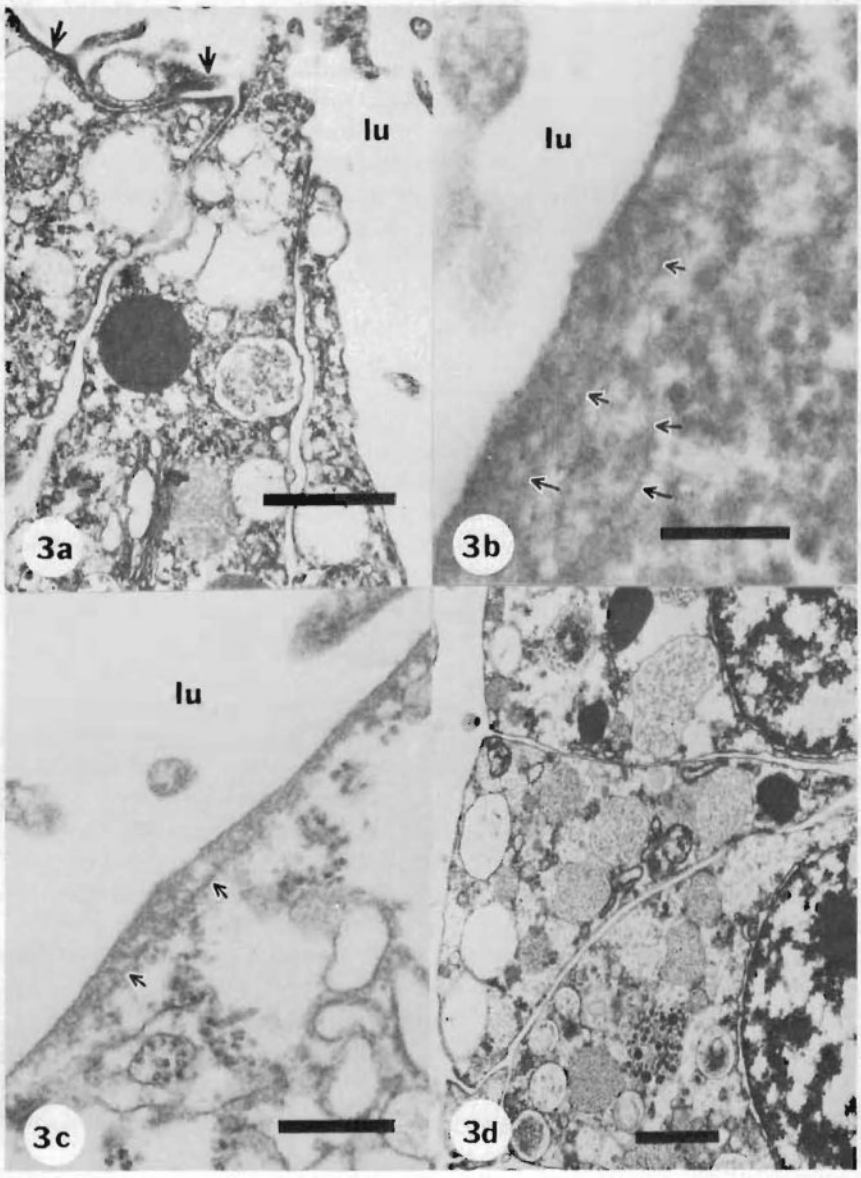


Fig. 3. a: TEM of a section cut obliquely through the forming cardiac sphincter of a 60-h *S. purpuratus* embryo. The arrows indicate a juxtaluminal electron-dense region. Bar = 1 μm. b: the luminal regon of a sphincter-forming cell sectioned at right angles to the long axis of the archenteron. The arrows indicate the filamentous nature of the electron-dense region. Bar = 0.1 μm. c: the luminal region of a sphincter-forming cell. The arrows indicate filaments associated with the apical plasmalemma. Bar = 0.25 μm. d: TEM of cells in the region of the presumptive sphincter of embryos that were treated with 5 μg/ml CB for 2 h prior to fixation. Bar = 1 μm. lu = lumen of the archenteron.

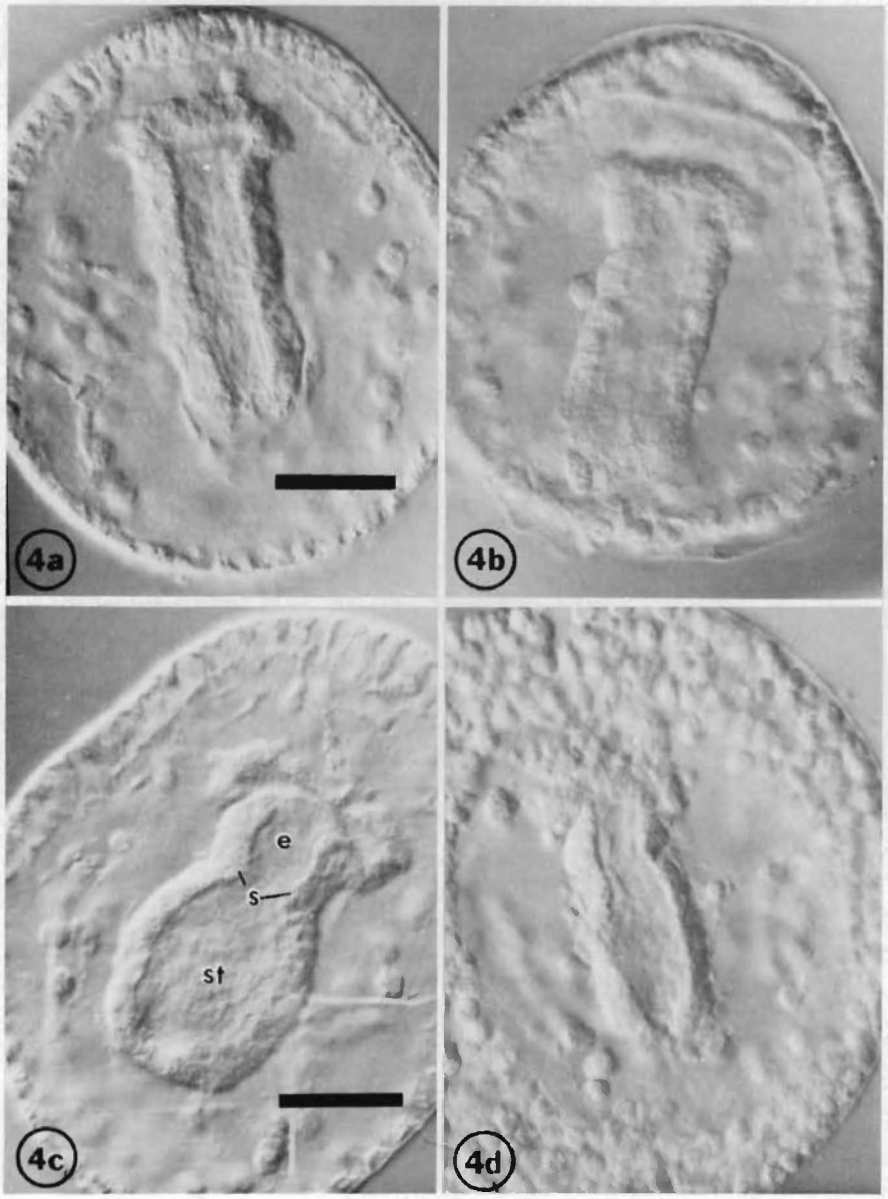


Fig. 4. *S. purpuratus* embryos between 59 and 60 h of development used in an experiment to test the effects of cytochalasin B (CB) on sphincter formation. a: a 59-h embryo prior to formation of the sphincter, representative of embryos at the beginning of the experiment; b: an embryo after 2 h of treatment with 5 μg/ml CB; c: a control embryo after 2 h in sea-water containing 1% DMSO; d: an embryo that had been treated with 5 μg/ml CB for 2 h and then returned to sea-water for 2 h; note that a constriction has begun to form. e = esophagus; s = sphincter; st = stomach. Bar = 20 μm.

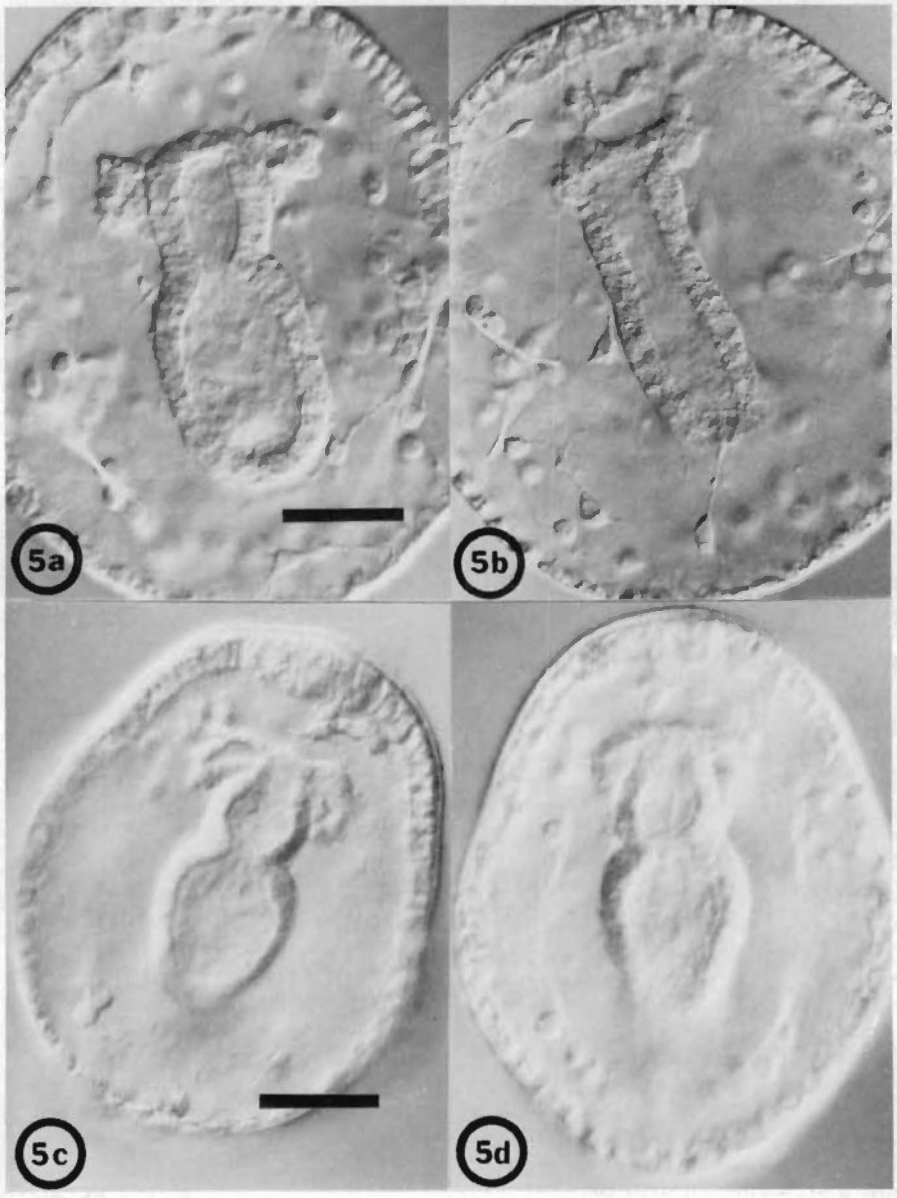


Fig. 5. a: a 60-h *S. purpuratus* embryo during the formation of the sphincter constriction. b: at this stage embryos treated with 5 μg/ml CB rapidly lost the constriction. c: a 62-h *S. purpuratus* embryo after the constriction of the archenteron has fully formed. d: treatment with 5 μg/ml CB for up to 4 h was incapable of removing the constriction. Bar = 20 μm.

returned the sphincters to a state comparable to the control cultures. The 10 μg/ml CB treatment was also reversible; however, embryos that had undergone dissociation of the endoderm were not always able to return to their previous condition.

In a third set of experiments, embryos were treated with CB after the constrictions of the archenteron had fully formed (Fig. 5c, d). The sphincters were unaffected by all 3 concentrations of CB, even when treated for up to 4 h (Fig. 5d).

In embryos treated with 5 μg/ml CB throughout the period of sphincter formation, the cells of the presumptive sphincter remained columnar, though somewhat rounded. There were no apparent elaborations of the luminal surface (Fig. 3d), and neither electron-dense regions nor microfilaments were observed. All other ultrastructural characteristics of the CB-treated embryos were essentially the same as both groups of control embryos.

DISCUSSION

Sphincters in the digestive tracts of echinoid embryos first appear as thickenings and then constrictions of the archenteron. Intuitively, this change in form can most easily be accomplished by a contractile force acting circumferentially. Moore [19] noted that sphincters formed in the archenterons of exogastrulae and gastrulae from which the animal hemisphere had been removed. He concluded that the formation of the tripartite gut (sphincter formation) was an autonomous process occurring without the influence of other tissues.

Microfilaments were observed near the luminal surface of the sphincter cells of embryos fixed during the constriction of the archenteron. Microfilaments were never observed in sphincter cells of embryos fixed prior to, or after, sphincter formation. This transient presence of the microfilaments during the constriction of the tube allies them with the observed change in cell shape. The location and orientation of the microfilaments (circumferential) is also consistent with their participation in sphincter formation.

The appearance of additional microvilli on the luminal surface, coincidental with sphincter formation, can be interpreted as a result of contraction of the apical cytoplasm of the sphincter cells. Presumably the contraction of the cytoplasm beneath the plasmalemma results in the plication of the membrane. Cloney [5] and Baker and Schroeder [6] noted similar folding of membranes associated with contraction of cell surfaces.

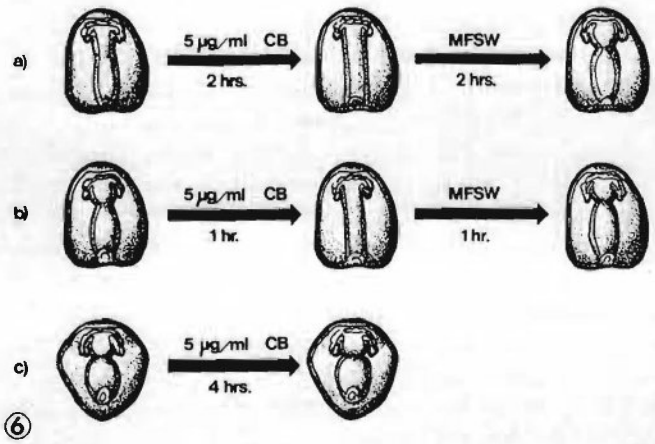
Numerous non-muscle cells have been demonstrated to contain the contractile proteins actin and myosin [3,4,20]. Filaments 5—7 nm thick have been shown to be associated, both temporally and geometrically, with contractile events in many cells. Microfilaments have the ultrastructural characteristics of actin, and under specific conditions have been demonstrated to bind heavy meromyosin, as does actin [21].

It is suggested that the formation of both the cardiac and pyloric sphinc-

ters is initially accomplished by an active contraction of the apical ends of the sphincter cells, altering their shape and resulting in the constriction of the archenteron (Fig. 7). Also, it is suggested that the force required is produced by the contraction of microfilaments associated with the apical plasmalemma of the sphincter cells.

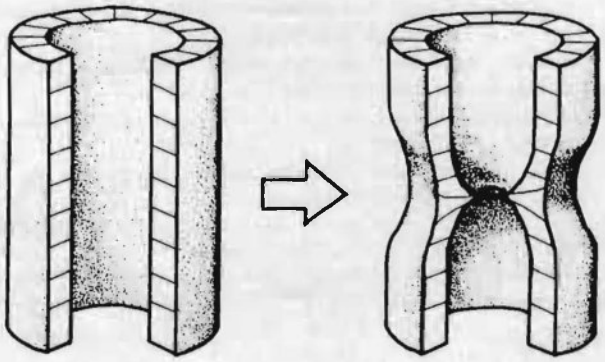
Embryos treated with CB prior to, or during, sphincter formation were unable to form sphincters (Fig. 6). The effects of the CB could be reversed by returning the embryos to MFSW. CB is known to inhibit numerous contractile processes in non-muscle cells and disrupt microfilaments associated with contraction [22]. Little is known of the means by which it interferes with either the contractility or the stability of the filaments. Although CB has been shown to affect sugar transport in some types of cells [23,24], it is assumed that the drug also acts with some specificity for contractile processes [25]. The reversible inhibition of sphincter formation by CB, and the absence of microfilaments in sphincter cells treated with CB, supports the idea that sphincter formation is dependent upon a microfilament-mediated contractile process.

CB treatment of embryos in which the sphincters had fully formed was unable to alter sphincter constrictions (Fig. 6). If CB sensitivity is a reliable indication of the functioning of contractile microfilaments, then these results may be interpreted as evidence that the contractile processes are functioning only for a brief period of time (1—2 h) during the initial phase of sphincter formation. Alternatively, there may be factors that stabilize the newly formed sphincters which are refractory to CB. Examination of changes in cell junctions, glucosaminoglycans and collagen may provide further evidence of stabilizing factors.



⑥

Fig. 6. A graphical summary of the experiments performed to examine the effects of CB on sphincter formation in *S. purpuratus* embryos. a: treatment throughout formation of the constriction; b: treatment during formation; c: treatment after formation.



⑦

Fig. 7. A graphical presentation of the model proposed to account for the formation of sphincter constrictions by the change in shape, from cubes to wedges, of the sphincter-forming cells.

Gustafson and Wolpert [7] suggested increases in the lateral adhesion between sphincter cells as a possible mechanism for the formation of sphincter constrictions. They regard this process as being fundamentally different from the active contraction of one end of a cell [8]. There is an apparent increase in the lateral adhesion between sphincter cells prior to and during sphincter formation. However, it is difficult to determine the extent to which the increase in adhesion is an active process causing the constriction or whether it is a passive effect of the contraction of the apical ends of the sphincter cells. It remains possible that some combination of cellular contractility and cellular adhesion results in the formation and stabilization of sphincters.

ACKNOWLEDGEMENTS

We thank Dr. A.O.D. Willows, Director of the Friday Harbor Laboratories, for providing facilities. This research was supported by a National Research Council of Canada grant to F.S. Chia and a pre-doctoral fellowship to R.D. Burke.

REFERENCES

1 Rhumbler, L., Zur Mechanik des Gestrulationsvorganges, Wilhelm Roux' Arch. Entwicklungsmech. Org., 14 (1902) 401—438.
2 Trinkaus, J.P., Mechanisms of morphogenetic movements. In: H. Upsrung (ed.), Organogenesis, Holt, Rinehart and Winston, New York, 1965, pp. 55—104.
3 Inoué, S. and Stephens, R.E. (ed.), Molecules and Cell Movement. In Soc. Gen. Physiol. Ser., Vol. 30, 1975.

4 Goldman, R., Pollard, T. and Rosenbaum, J. (ed.), Cell Motility, Book A: Motility, Muscle and Non-muscle Cells, Academic Press, New York, 1976.
5 Cloney, R.A., Cytoplasmic filaments and cell movements. Epidermal cells during ascidian metamorphosis, J. Ultrastruct. Res., 14 (1966) 300—328.
6 Baker, P.C. and Schroeder, T.E., Cytoplasmic filaments and morphogenetic movement in the amphibian neural tube, Dev. Biol., 15 (1967) 432—450.
7 Gustafson, T. and Wolpert, L., The cellular basis of morphogenesis and sea urchin development, Int. Rev. Cytol., 15 (1963) 139—214.
8 Gustafson, T. and Wolpert, L., Cellular movement and contact in sea urchin morphogenesis, Biol. Rev., 42 (1967) 442—498.
9 Schroeder, T.E., The contractile ring, I: determining its brief existence, volumetric changes, and its vital role in cleaving *Arbacia* eggs, J. Cell Biol., 53 (1972) 419—434.
10 Gibbins, J.R., Tilney, L.G. and Porter, K.R., Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata*, I: distribution of microtubules, J. Cell Biol., 41 (1969) 201—226.
11 Tilney, L.G. and Gibbins, J.R., Microtubules and filaments in the filopodia of secondary mesenchyme cells of *Arbacia punctulata* and *Echinorachnius parma*, J. Cell Sci., 5 (1969) 195—210.
12 Strathmann, M., General procedures and Echinodermata-Echinoidea. In Methods in Development Series, Vol. 1, Friday Harbor Laboratories, Friday Harbor, WA, 1968.
13 Allen, R.D., David, G.B. and Nomarski, G., The Zeiss—Nomarski interference equipment for transmitted light microscopy, Z. Wiss. Mikrosk., 69 (1969) 192—221.
14 Millonig, G., Advantages of a phosphate buffer for OsO_4 solutions in fixation, J. Appl. Phys., 32 (1961) 1637—1646.
15 Cloney, R.A. and Florey, E., Ultrastructure of cephalopod chromatophore organs, Z. Zellforsch., 89 (1968) 250—280.
16 Luft, J.H., Improvement in epoxy resin embedding methods, J. Biochem. Biophys. Cytol., 9 (1961) 409—414.
17 Watson, M.L., Staining of tissue sections for electron microscopy with heavy metals, II. Application of solutions containing lead and barium, J. Biophys. Biochem. Cytol., 4 (1958) 475—479.
18 Reynolds, E.S., The use of lead citrate at high pH as an electron opaque stain in electron microscopy, J. Cell Biol., 17 (1963) 208—212.
19 Moore, A.R., Self differentiation of the intestine in larvae of the sand dollar, *Dendraster excentricus*, Proc. Soc. Exp. Biol. Med., 25 (1927) 40—41.
20 Pollard, T.D. and Weihing, R.R., Actin and myosin and cell movement. CRC Crit. Rev. Biochem., 2, (1973) 1—65.
21 Ishikawa, H., Buschoff, R. and Holtzer, H., Formation of arrowhead complexes with heavy meromyosin in a variety of cell types, J. Cell Biol., 43 (1969) 312—328.
22 Schroeder, T.E., The contractile ring, I. Fine structure of dividing mammalian (HeLa) cells the effects of cytochalasin B, Z. Zellforsch., 109 (1970) 431—439.
23 Estensen, R.D. and Plagemann, P.G.W., Cytochalasin B: inhibition of glucose and glucosamine transport, Proc. Nat. Acad. Sci., 69 (1972) 1430—1434.
24 Mizel, S.B. and Wilson, L., Inhibition of hexose transport in mammalian cells by cytochalasin B, J. Biol. Chem., 247 (1972) 4102—4105.
25 Wessells, N.K., Spooner, B.S., Bradley, N.D., Luduena, M.D., Wrenn, J.T. and Yamada, K.M., Microfilaments in cellular developmental processes, Science, 171 (1971) 135—143.