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What do dissociated embryonic cells of the starfish, *Asterina pectinifera*, do to reconstruct bipinnaria larvae?

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SUMMARY

The cellular events that take place during reconstruction of larval forms from dissociated embryonic cells of the starfish are investigated by thick and thin sections. Dissociated cells reaggregate, form an external epithelium (ectoderm), internal epithelial vesicles (endoderm), the blastocoel and the mesenchyme. The internal vesicles continue to fuse until there is only one large one suspended in the centre of the blastocoel. Eventually, the ectoderm invaginates at one or more sites and fuses with the endoderm to form blastopore(s).

Special emphasis is placed on the differences in cell behaviour during endoderm-to-endoderm and endoderm-to-ectoderm fusion.

INTRODUCTION

It has been reported earlier that cells dissociated from sea urchin embryos form reaggregates which will eventually develop into 'plutei' (Giudice, 1962; Giudice & Mutolo, 1970). According to Millonig & Giudice (1967) and Millonig (1975), this process includes some characteristic steps such as (1) reformation of the external epithelium with accessory terminal bars and hyaline membrane, (2) formation of intracellular and intercellular cavities which will develop into intestinal lumen and blastocoel, respectively, (3) opening of the intestine to the outside, which may or may not take place according to the species, (4) formation of triradiated spicules, (5) differentiation of pigment cells and (6) elongation of spicules.

Recently, cells dissociated from starfish embryos were also shown to possess the ability to reconstruct the larval form, bipinnaria (Dan-Sohkawa, Yamanaka & Watanabe, 1986). This paper describes electron microscopic and thick sectional observations of cellular events which take place during the process. These events include reaggregation of dissociated cells (stage 1), formation of the external

Key words: echinoderm, dissociated embryonic cell, reconstruction, bipinnaria larvae.

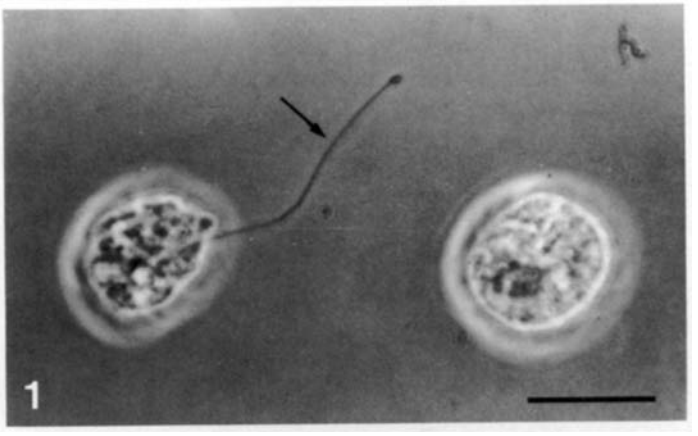


Fig. 1. Phase-contrast photomicrograph of dissociated cells. A great majority of them are actively beating their cilia (arrow). Bar, 10 μm.

epithelium (stage 2), formation of internal structures (stage 3) and the process of 'gastrulation' (stage 4).

The difference in the mechanism of fusion between endodermal vesicles (stage 3) and between endoderm and ectoderm (stage 4) is discussed.

MATERIALS AND METHODS

Late gastrulae of the starfish, *Asterina pectinifera* (see fig. 1D of Dan-Sohkawa *et al.* 1986), were developed, collected and dissociated as described in previous papers (Dan-Sohkawa & Satoh, 1978; Dan-Sohkawa *et al.* 1986). Dissociated cells were allowed to reaggregate, also as described previously (Dan-Sohkawa *et al.* 1986).

Reaggregates and dissociated cells were observed with ordinary or phase-contrast microscopes after fixation with 10% formalin in the artificial sea water Jamarin (Jamarin Laboratory, Osaka, Japan). Reaggregates were fixed at various stages with 2% OsO_4 in 0·1 M-sodium cacodylate in Jamarin (pH 8·0) for 1·5 h at room temperature, and postfixed with 2% glutaraldehyde in 0·1 M-sodium cacodylate buffer (pH 7·4) overnight or for several days at 4 °C, dehydrated, and embedded in epoxy resin. OsO_4 was used as the first fixative since overall shape of the embryo and structure of the septate junctions were preserved in a better state than when glutaraldehyde was used first. Sections, 1 μm thick, were prepared, stained with toluidine blue and observed and photographed with Zeiss Orthoplan microscope. Ultrathin sections were observed with JEM 100-C electron microscope (JEOL) after staining with uranyl acetate and lead citrate.

RESULTS

Dissociated cell (stage 0)

Dissociated cells are spherical (Fig. 1). They usually carry a cilium, which beats actively in the living state.

Early reaggregate (stage 1)

At 3 h, cells forming the reaggregate are packed tightly, but show no particular structure (Fig. 2A). Individual cells of the reaggregate appear identical even

though derived from all three germ layers. Septate junctions are sometimes found between cells at the periphery of the reaggregate (Fig. 2B,C).

Formation of the external epithelium (stage 2)

At 9 h (Fig. 3A), some of the cells situated at the periphery of the reaggregate assume cuboidal form and are bound to neighbouring cells by septate junctions

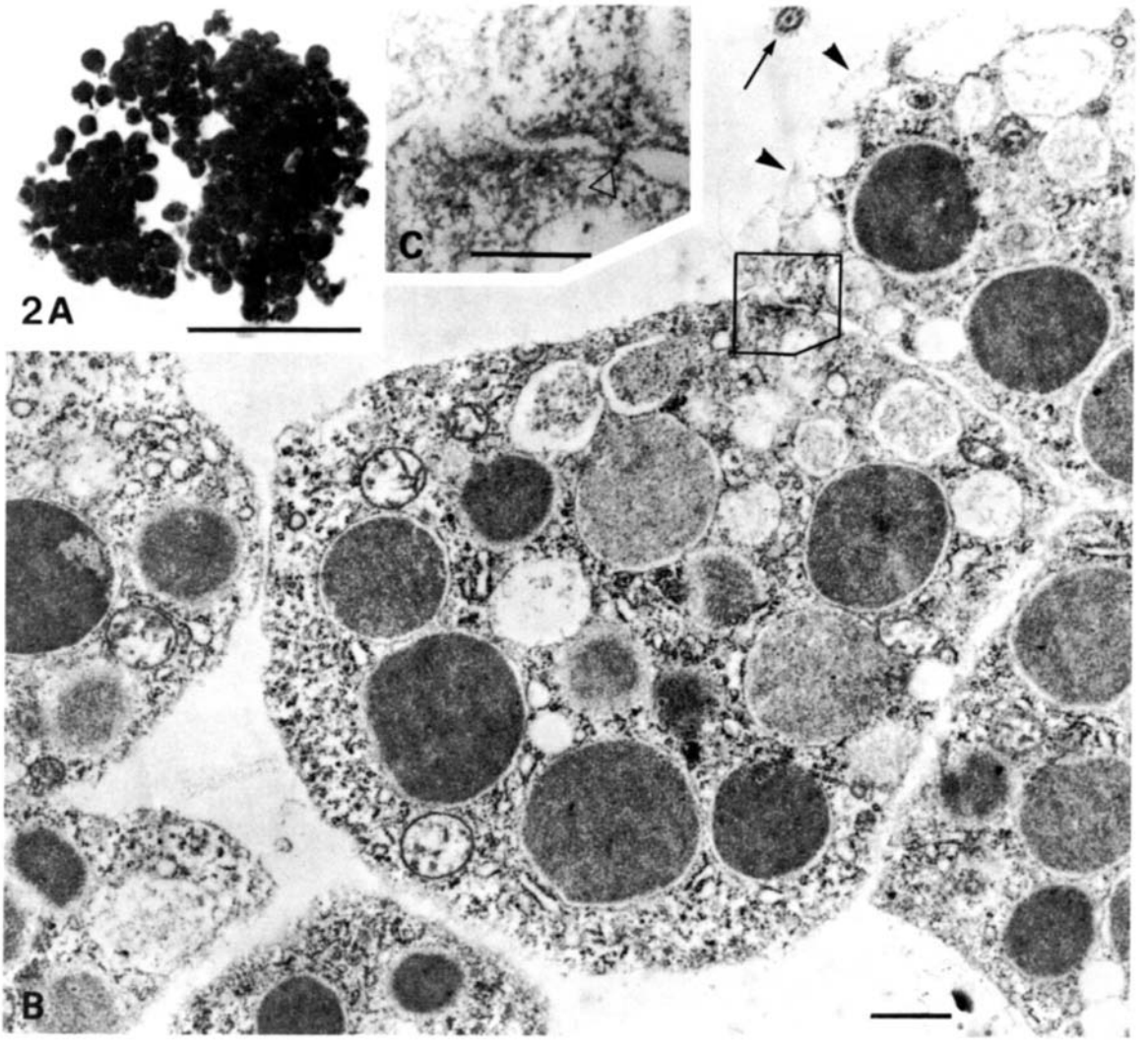


Fig. 2. Reaggregation (stage 1). (A) 1 μm section of a 3 h reaggregate stained with toluidine blue. Round cells are packed tightly into structureless clusters. Cells deriving from different germ layers of the material embryo are not distinguishable. Bar, 50 μm. (B) Electron micrograph of packed cells. Hyaline membrane (arrowheads) and a cilium (small arrow) are observed over one of the cells. Bar, 1 μm. (C) A septate junction is shown at a higher magnification. Only one septum is seen (open arrowhead). Bar, 0·5 μm.

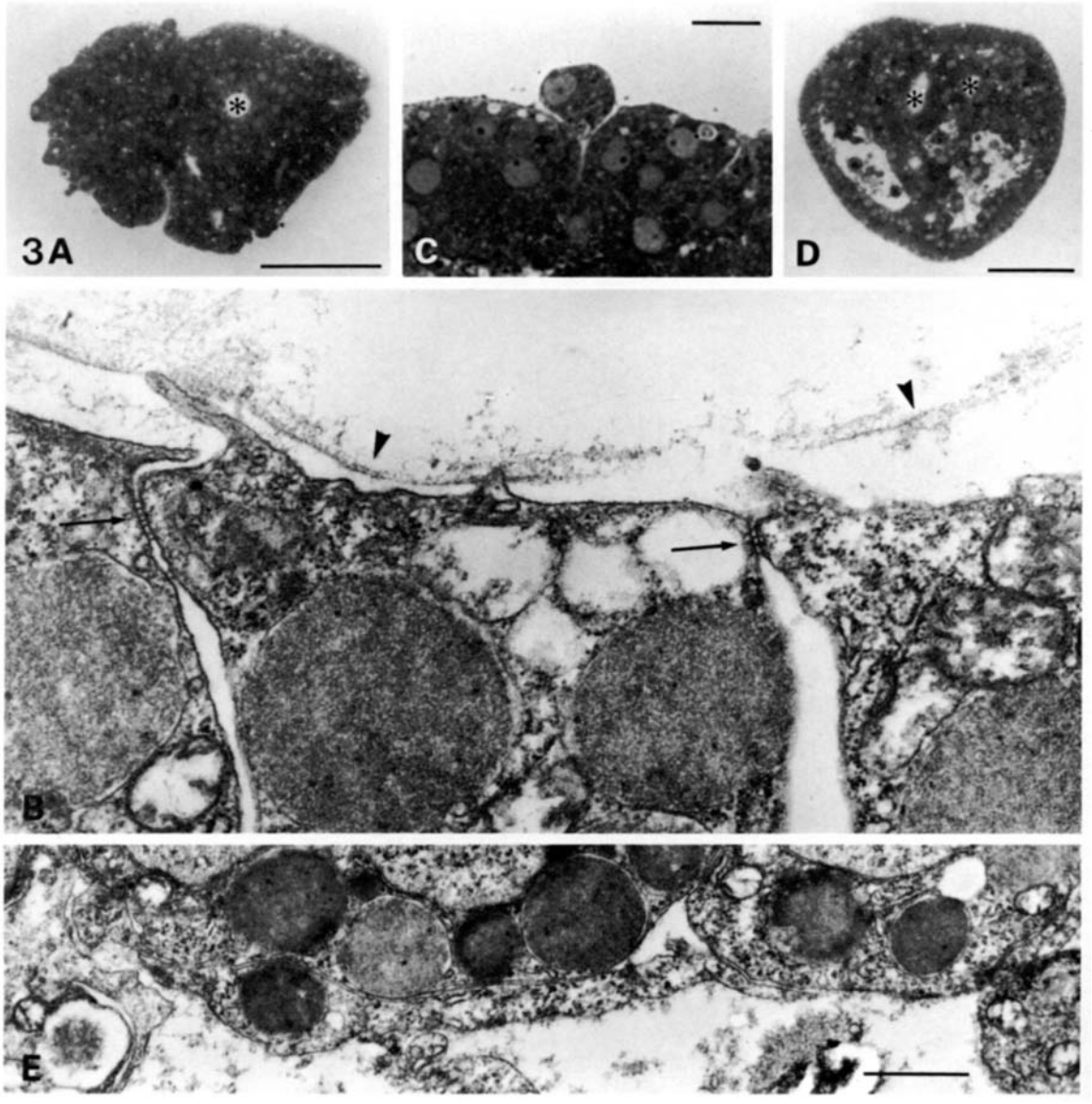


Fig. 3. Formation of external epithelium (stage 2). (A) Thick section of a 9 h reaggregate. More than half of the surface area is smooth. Spaces begin to appear within the interior mass of cells (see (D) for the asterisk). Bar, 50 μm. (B) Electron micrograph of the smooth surface of a 9 h reaggregate. Cells are covered by a thin hyaline membrane (arrowheads) and fastened to neighbouring cells by septate junctions (arrows). The number of desmosomal septa is still fewer than usual. Bar, 0·5 μm. (C) Thick section of a 9 h reaggregate. A cell is about to drop out. Bar, 10 μm. (D) Thick section of a 26 h reaggregate. The surface is completely smooth. Blastocoels and lumina of endodermal vesicles (asterisks) have grown in size. Bar, 50 μm. (E) Electron micrograph of the basal surface of the external epithelium of a 26 h reaggregate. Basement membrane is not formed. Bar, 0·5 μm.

(Fig. 3B). A hyaline membrane usually accompanies the cells in these places. In other places, however, peripheral cells remain spherical. Some of these cells will eventually drop out of the reaggregate in ones or in groups (Fig. 3C). Small spaces begin to form deeper in the reaggregate (Fig. 3A).

The external epithelium is well-established by 26 h (Fig. 3D). It consists of a single layer of columnar cells with basal nuclei. Cells are bound together by well-established septate junctions, often consisting of supernumerary septa separated by uneven spaces (photograph not shown). Cells are overlain by a hyaline membrane at their apical surfaces, and are separated from internal cells by a narrow blastocoel (Fig. 3D). Basement membrane, however, is lacking at this stage (Fig. 3E). It only appears around 45 h and is not continuous.

Development of the internal structures (Stage 3)

Blastocoel and mesenchymal cells

The blastocoel makes its appearance as small, structureless, intercellular cavities at about 18 h. Bipolar or irregularly shaped mesenchymal cells migrate into it as it enlarges (Fig. 4A).

Endodermal vesicles

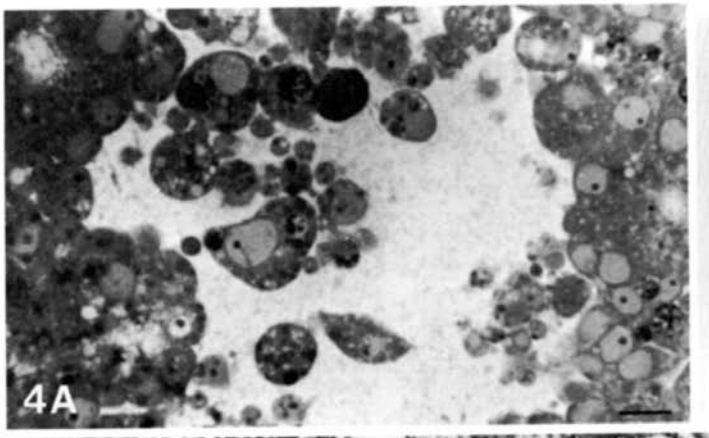
Small vesicles consisting of epithelial cells with well-structured central lumina are present in the interior of the reaggregate as early as 9 h (see Fig. 3A,D, asterisks). The cells constituting these vesicles are bound to each other by septate junctions which are formed near the surface of the central lumen (Fig. 4B, filled arrowheads). The lumen, on the other hand, contains numerous cilia (arrows) and microvilli as well as a prominent hyaline membrane (open arrowheads).

Although the endodermal nature of these cells is not recognizable at this stage, it becomes clear by 38 h (Fig. 4C). They become columnar in shape and their cytoplasm stains heavily with toluidine blue, while ectodermal cells are cuboidal in shape and stain lightly.

These vesicles fuse until there is only one large one suspended at the centre of the blastocoel. Fig. 5A–C shows the sites of such fusion at 68 h. Cells constituting the common wall of fusing vesicles are oriented in two opposite directions (Fig. 5B, asterisks), unlike those constituting other parts of the vesicular wall. Walls are often seen to stick out into the lumen with only one end attached to the vesicular wall (Fig. 5C).

'Gastrulation' (stage 4)

At 68 h, endoderm and ectoderm are also fusing actively with one another. In contrast to the above described fusion between endodermal vesicles, during endodermal–ectodermal fusion a wedge-shaped front is seen to cut into the endodermal wall (Fig. 6B) at the site of ectodermal invagination (Fig. 6A) (also see Dan-Sohkawa *et al.* 1986). A small pore, not greater than 2 μm in diameter, is sometimes found in the ectodermal domain of the fusion site (Fig. 6B).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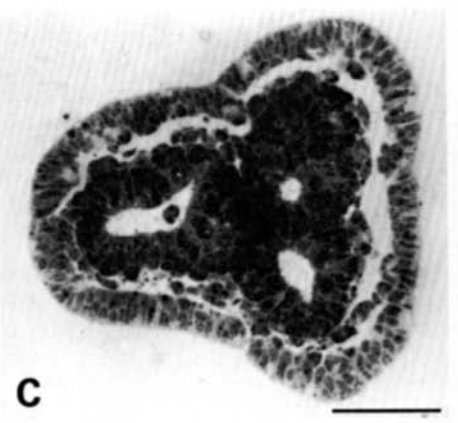
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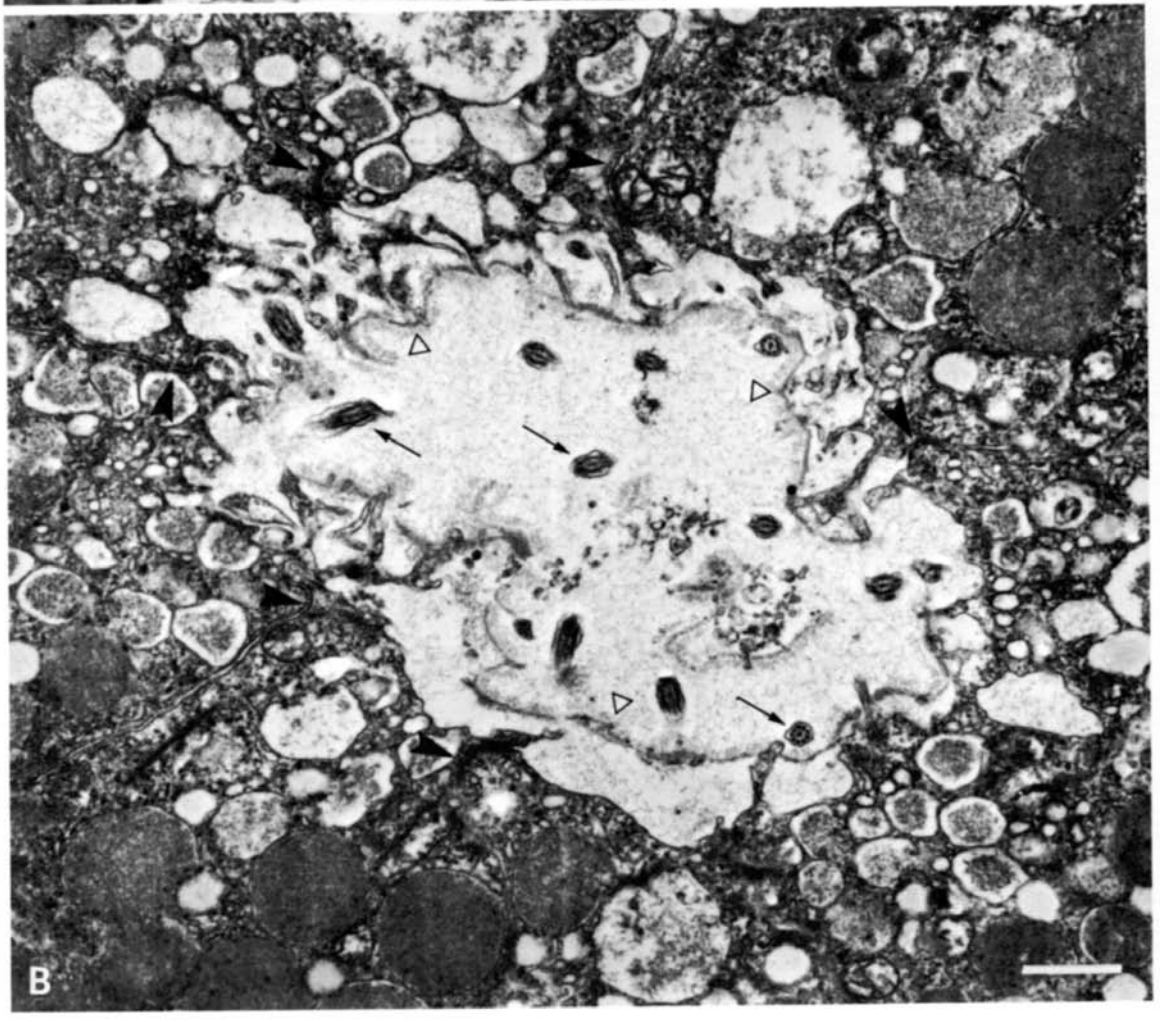
B

DISCUSSION

These observations show that the process of reconstruction of the larval form from dissociated embryonic cells of the starfish includes such cellular events as (1) reaggregation, (2) formation of the external epithelium by superficial cells,

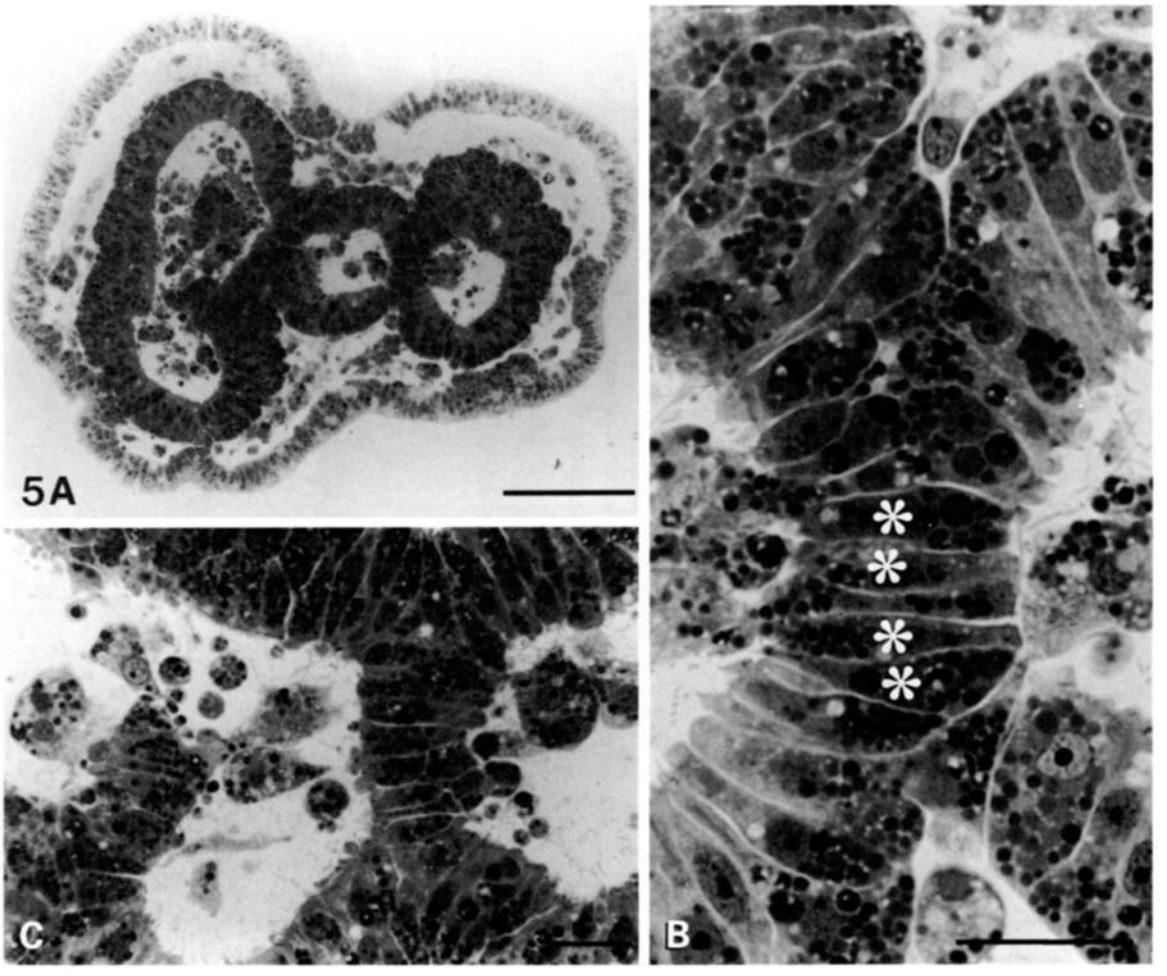

Fig. 5. Fusion of endodermal vesicles. (A–C) Thick sections of 68 h reaggregate. Cells at the sites of fusion look as if they are sliding into the reciprocal walls. (A) Three vesicles are fusing at the same time. Bar, 50 μm. (B) Fusion site at a higher magnification. Cells are oriented in opposite directions (asterisks). Bar, 10 μm. (C) Fusion of three vesicles. The common wall to the left has broken away at one end. Bar, 10 μm.

Fig. 4. Formation of internal structures (stage 3). (A) Thick section of a 26 h reaggregate. Blastocoel appears and enlarges as structureless intercellular cavities. Mesenchymal cells migrate into them. Bar, 10 μm. (B) Electron micrograph of the central portion of an epithelial vesicle at 26 h. The lumen is structured by a prominent hyaline membrane (open arrowheads), microvilli and cilia (arrows). The cells are bound together by septate junctions (filled arrowheads). Bar, 1 μm. (C) Thick section of a 38 h reaggregate. Three germ layers are established. Bar, 50 μm.

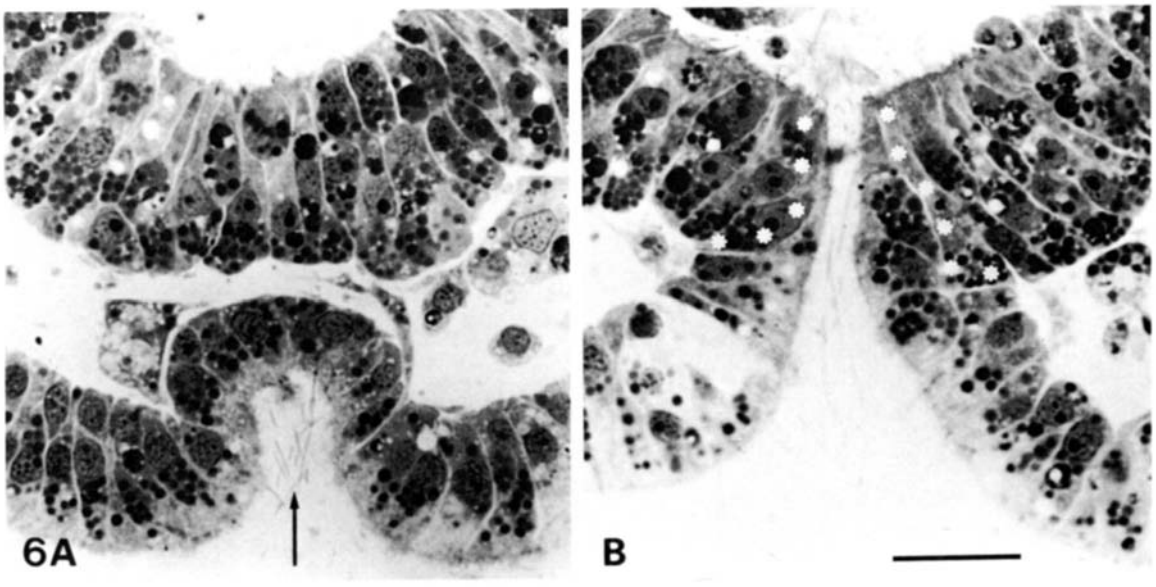

Fig. 6. 'Gastrulation' (stage 4). (A,B) Thick sections of 68 h reaggregate. Bar, 10 μm. (A) Site of invagination of the ectoderm (arrow). Endoderm is shown above. (B) A small pore of about 2 μm in diameter is formed within the ectodermal wedge (border shown by white dots).

(3) expansion of blastocoel, which originates as structureless, intercellular cavities, (4) migration of mesenchymal cells from the internal mass of cells into the blastocoel, (5) formation of the internal epithelial vesicles, their growth by mutual fusion, and their differentiation into endoderm, and (6) fusion between endodermal and ectodermal epithelia ('gastrulation').

The most interesting aspect of cell behaviour during reconstruction is the difference in the process of fusion between the two epithelial cell sheets, i.e. fusion between endodermal vesicles and that between endoderm and ectoderm. Figs 7, 8 show diagramatically the two ways of fusion. In the process of fusion between two endodermal vesicles, cells constituting the walls of each of the vesicles invade each other (Fig. 7A–C) so as to form a common, monolayered wall of loosely bound cells oriented in opposite directions. This wall breaks open at a point as the fused, 8-shaped vesicle starts to round up to form a sphere (Fig. 7D–F). We do not know at present whether all or only part of the cells forming the common wall are finally incorporated into the wall of the resultant vesicle or whether they are left behind to degenerate in the lumen.

In contrast to their behaviour during endoderm-to-endoderm fusion, endodermal cells seem to act passively in the process of fusion with the ectoderm. This process starts as an ectodermal invagination at a site determined by unknown factor(s) (Fig. 8B). As the basal tip of invaginating ectoderm touches the basal surface of the endodermal epithelium, it forms a wedge-shaped front and pushes into the endodermal wall until its tip reaches the central lumen (Fig. 8C). A small

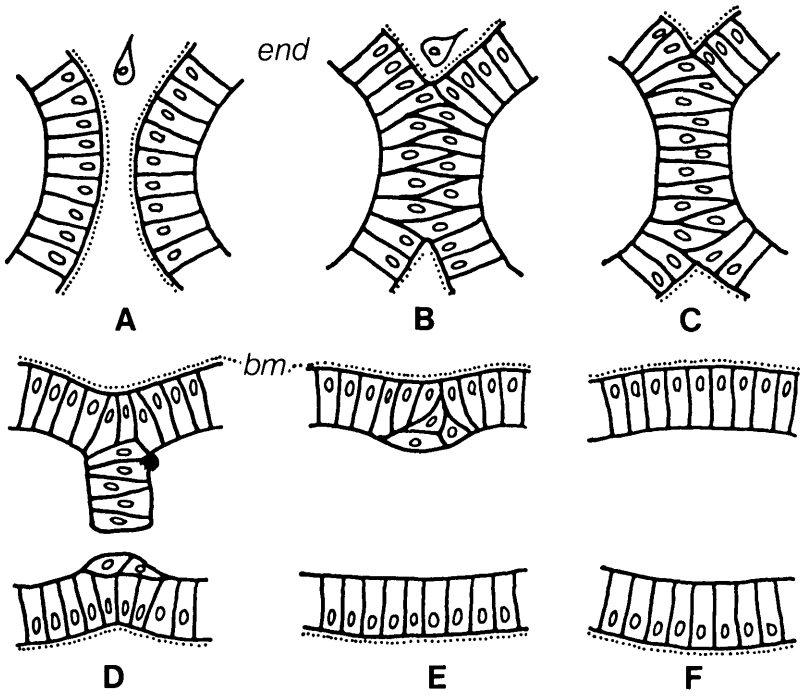

Fig. 7. Schematic drawings of the process of endoderm-to-endoderm fusion. Two endodermal vesicles (*end*) approach each other by their basal surfaces (A). The basement membrane (*bm*) is drawn as dotted lines. When the basal surfaces touch each other, cells begin to slide between (B) to form a common, monolayered wall (C). Cells constituting the common wall are facing in opposite directions. The wall breaks at a point (D) and the cells are either incorporated into the peripheral walls (E) or abandoned. Peripheral walls of the fused vesicles round up to form one larger vesicle (F).

pore about the size of a single cell opens within the ectodermal wedge by an unknown mechanism, and connects the central lumen to the outside (Fig. 8D). The epithelial structure of the junction site is resumed as the two epithelia interconnect to form one continuous epithelium (Fig. 8E), and the canal widens eventually.

Our observations so far give no evidence for or against the ability of these cells to sort themselves out according to their original positions in the embryo. It was reported in the sea urchin that cells dissociated from the mesenchymal blastula of two different species segregate to form separate reaggregates within a few hours (Giudice, 1962). Micromeres deriving from 16-cell-stage embryos also recognize one another (Spiegel & Spiegel, 1978). We were not able, however, to distinguish any differences among cells dissociated from the late gastrula, which comprises three definite germ layers (see Fig. 1D, Dan-Sohkawa *et al.* 1986). Furthermore, there was no fundamental difference in the reconstruction process or its time course between undifferentiated cells of the hatched blastula and differentiated cells of the late gastrula (Dan-Sohkawa *et al.* 1986). Further experimentation concerning the composition of the reaggregating cell population is necessary to

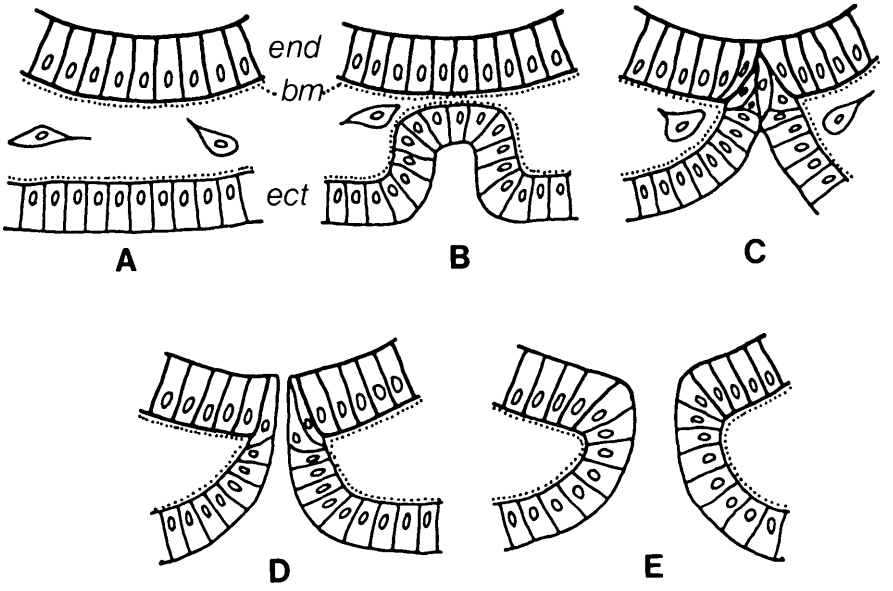

Fig. 8. Schematic drawings of the process of ectoderm-to-endoderm fusion ('gastrulation'). Ectoderm (*ect*) invaginates at one point (A,B) and when it touches the basal surface of the endoderm (*end*) (B), it pushes into the endodermal epithelium like a wedge (C). A small pore of a diameter of about 2 μm opens at the tip of the ectodermal wedge (D) to connect the endodermal lumen to the outside. Endoderm and ectoderm form a continuous epithelium around the widening blastopore (E). Endoderm appears to play only a passive role in the process of 'gastrulation'. *bm*, basement membrane.

decide whether development of reconstructed embryos involves sorting-out or redifferentiation.

The major difference in the cellular aspect of reconstruction between the starfish and the sea urchin lies in the origin of the intestinal lumen. While, in the starfish, it is initiated as a small intercellular space lined by presumptive endodermal cells, that of the sea urchin is reported to grow from an intracellular cavity (Millonig *et al.* 1967; Millonig, 1975). However, the sea urchin intestinal lumen is intercellular and these authors do not describe how and when this cavity becomes intercellular.

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