J. Embryol. exp. Morph. **94**, 47–60 (1986)
Printed in Great Britain © *The Company of Biologists Limited 1986*

Reconstruction of bipinnaria larvae from dissociated embryonic cells of the starfish, *Asterina pectinifera*

M. DAN-SOHKAWA

Department of Biology, Faculty of Science, Osaka City University, Sughimoto-cho, Sumiyoshi-ku, Osaka 558, Japan

H. YAMANAKA AND K. WATANABE*

Kanebo Institute for Cancer Research, 1-5-90 Tomobuchicho, Miyakojima-ku, Osaka 534, Japan

SUMMARY

Cells dissociated from swimming embryos of the starfish are able to reconstruct bipinnaria larvae. This process consists of reaggregation (stage 1), formation of the external epithelium (stage 2), development of the internal cavities which will eventually grow either into the blastocoel or the intestinal lumen (stage 3), gastrulation or fusion of the internal and the external epithelia (stage 4), formation of the mouth (stage 5) and the established bipinnaria (stage 6).

The optimal population of cells to support the process of reconstruction is estimated to fall between 500 and 1000, values corresponding to one eighth and one fourth the total number of cells of a normal embryo, respectively.

Morphogenetic events of reconstruction are discussed in relation to the normal course of starfish development and to the reconstruction process of the sea urchin.

INTRODUCTION

The ability of dissociated embryonic cells to reassemble and to form an aggregate has been demonstrated in a wide range of animal groups including echinoderms (Herbst, 1900; Giudice, 1962), amphibia (Holtfreter, 1938; Lucey & Curtis, 1959), teleosts (Trinkaus, 1963; Yokoya, 1966), avians (Moscona, 1956; Weiss & Andres, 1952) and mammals (Lin & Florence, 1970; Stern, 1972).

In some cases the aggregates develop into more or less normal tissue-like structures (Townes & Holtfreter, 1955; Patricolo, 1967; Moscona, 1956) or into preimplantation blastocysts (Stern, 1972). It is only in the sea urchin, however, that these aggregates develop into an intact larval form (Giudice, 1962).

In the sea urchin, dissociated cells deriving from stages between mesenchymal blastula and early gastrula, after an initial reaggregation process, form tightly

*Present address: Cell and Developmental Biology Laboratory, Faculty of Integrated Arts and Sciences, Hiroshima University, 1-1-89 Higashisenda-machi, Naka-ku, Hiroshima 730, Japan.

Key words: echinoderm, dissociated embryonic cell, reconstruction, bipinnaria larvae, starfish, *Asterina pectinifera*.

packed clumps which are enclosed in an epithelium-like boundary. Internal cells eventually develop into an intestine-like structure which may or may not open to the outside through a 'blastopore'. Triradiated spicules are formed as the reaggregate develops to the 'pluteus' stage (Giudice, 1962; Giudice & Mutolo, 1970).

This paper describes the process of reconstruction of starfish bipinnaria larvae from dissociated embryonic cells obtained from embryos at stages between early blastula and late gastrula.

MATERIALS AND METHODS

Developing embryos of the starfish, *Asterina pectinifera*, were obtained as described elsewhere (Dan-Sohkawa & Satoh, 1978), in which eggs treated with 1-methyladenine (1-MA) (Kanatani, 1969) were inseminated with diluted dry sperm and were allowed to develop in natural sea water at 20°C.

Pretreatment

Swimming embryos up to the late gastrula stage were collected at a desired stage by hand centrifugation and were transferred to a large volume (e.g. more than 100 times the volume of packed embryo) of slightly hypertonic Ca^{2+}-free Jamarin (1·14 times stronger than the standard concentration) (Jamarin Laboratory, Osaka, Japan) supplemented with 1/100 volume of natural sea water. This sea water will be called 1/100 Ca^{2+} Jam×1·14, hereafter. Embryos were allowed to swim about in this sea water for 3 h at 20 °C. A portion of embryos was fixed at the end of the 3 h period to observe the condition of cells at the end of the pretreatment.

Dissociation medium

(a) Pretreated embryos at stages between mesenchymal differentiation and late gastrula (Fig. 1C,D) were dissociated in 5% foetal bovine serum (FBS) in 1/100 Ca^{2+} Jam×1·14 (standard dissociation medium).

(b) Those at stages between hatched blastula and early gastrula (Fig. 1A,B) were dissociated in the standard dissociation medium supplemented with 0·25 M-sucrose. Addition of sucrose was found necessary to protect these young and large cells from rupturing during the dissociation process.

Dissociation

0·2 ml of packed, pretreated embryos was transferred to a centrifuge tube containing 4 ml of dissociation medium (20–23°C) and submitted to 20–30 strokes of a Pasteur pipette. Undissociated bits of embryos were collected using strong hand centrifugation and dissociated once or twice more as above. A small pellet was usually found after the final hand centrifugation, regardless of the stage of the embryo at the time of dissociation. The supernatants of each of the hand centrifugations were pooled. Dissociated cells were collected by centrifugation at 900 to 1100 rev min^{-1} for 5 min.

Reconstruction

Collected cells were resuspended in 15 ml of millipore-filtered sea water containing 4% FBS and were incubated undisturbed in a Petri dish (6 cm in diameter) for 24 h at 14°C. Cells sedimented quickly to the bottom and formed reaggregates of various sizes. Reaggregates were broken down to small pieces by gentle pipetting at the end of this period. Reaggregates were incubated at 20°C, hereafter.

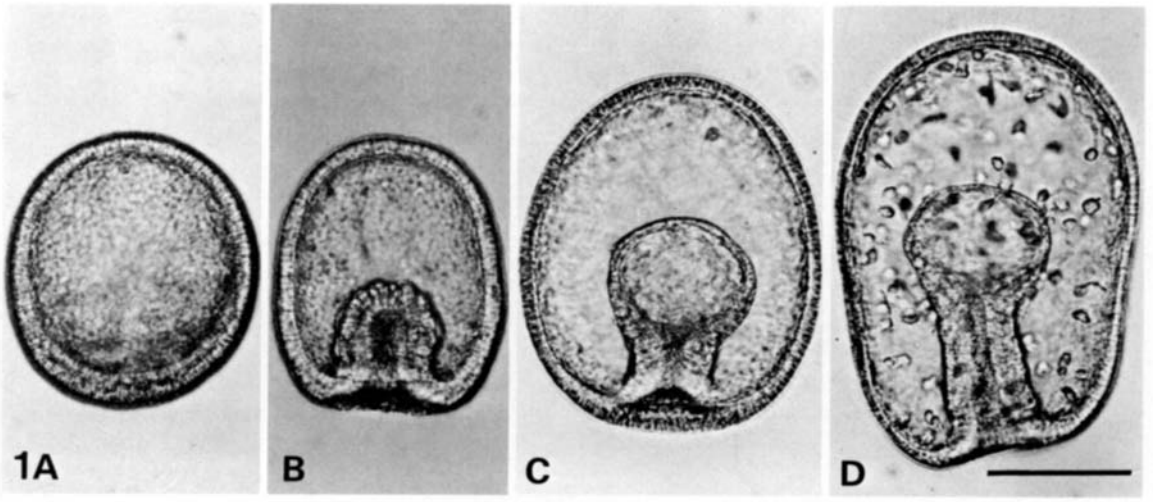


Fig. 1. Different stages of normal development of *Asterina pectinifera*. Bar indicates 100 μm. (A) Blastula shortly after hatching (15·5 h after fertilization at 20°C). (B) Early gastrula (17 h). (C) Gastrula at the mesenchymal differentiation stage (20 h). Mesenchymal cells will start to migrate into the blastocoel from the thin wall at the tip of the archenteron shortly after this stage. (D) Late gastrula (30 h). Mesenchymal cells have migrated to the anteriormost and the posteriormost points of the blastocoel.

Observation

Reaggregates were observed and photographed throughout the process of reconstruction by a light microscope.

Protein measurements

An aliquot of the material was set aside at various experimental steps. Protein contained in each of the samples was solubilized with 1N-NaOH and its concentration was measured by the Protein Assay (Bio-Rad Laboratories, California, USA) in order to monitor the loss of material during the procedure.

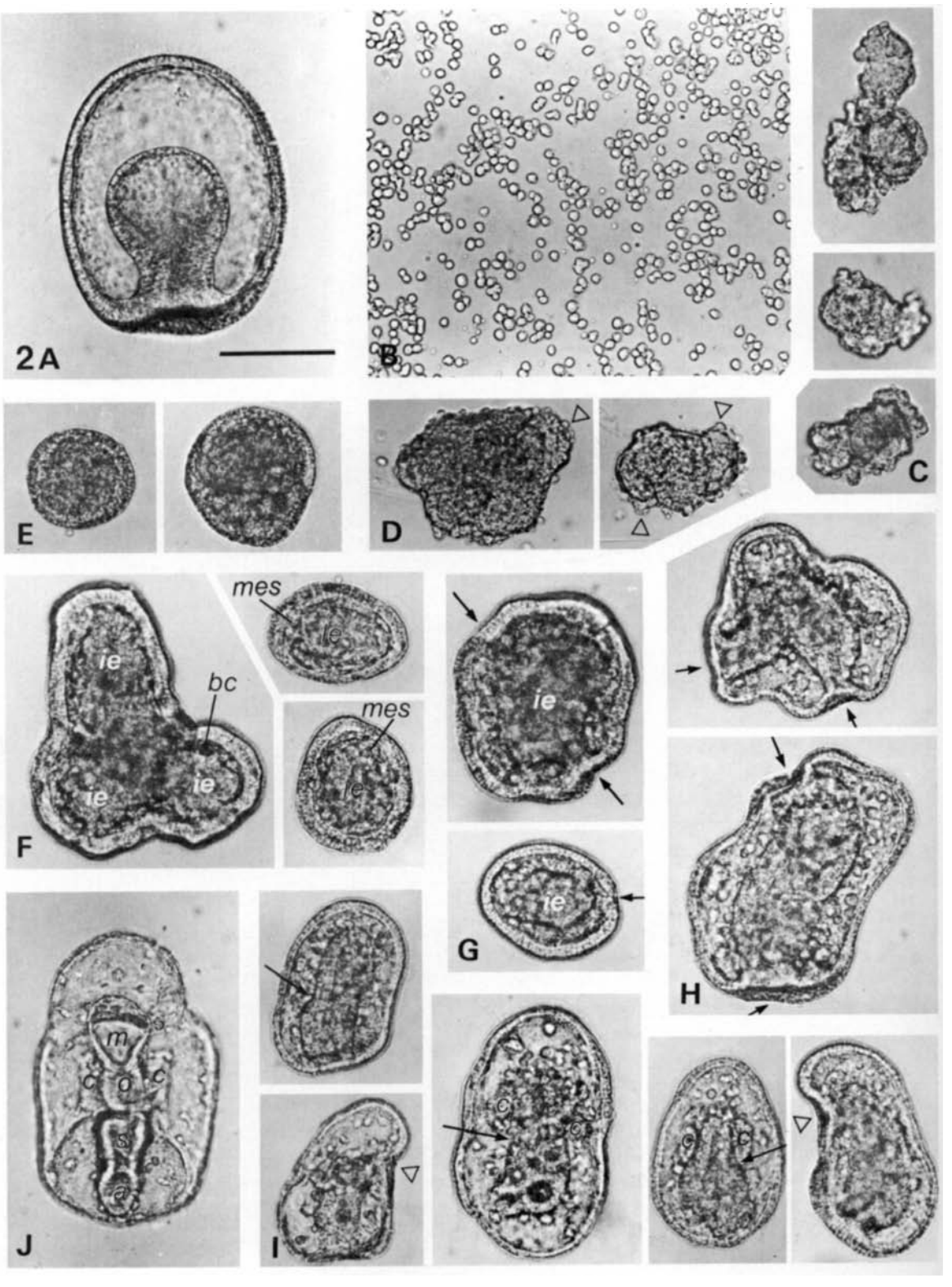
Electron microscopy

Embryos were fixed at the end of the pretreatment, dehydrated, embedded and sectioned by usual methods (Yamanaka, Tanaka-Ohmura & Dan-Sohkawa, 1986) and observed with JEM 100-C electron microscope (JEOL).

RESULTS

Dissociated cells (stage 0)

The present method does not necessarily dissociate the embryos completely into single cells (Fig. 2B). Small clusters of two to ten cells are allowed to remain, since further efforts to dissociate or to remove the clusters either damage the cells too severely or cause the loss of single cells. The majority of dissociated cells are actively beating their cilia.


2A
B
C
D
E
F
ie
bc
mes
G
H
I
J
m
o
c
s
a

Reaggregation (stage 1)

Cells start to reaggregate immediately after they are returned to the sea water containing 4% of FBS. Reaggregates gain size quickly during the first hours by colliding randomly with free cells and with other reaggregates (Figs 2C, 3B).

Formation of the external epithelium, or 'blastulation' (stage 2)

A considerable number of cells drops out from the surface of the reaggregates between 7 to 15 h (Fig. 2D, open arrowheads). The reaggregates assume a transparent, well-established, external epithelium by 25 h identified by the smoothness of the surface (Fig. 2E). This epithelium is separated from internal cells by a narrow gap, which will eventually develop into the blastocoel (Figs 2F, 3D). By about 30 h, the epithelium acquires further stability and transparency and becomes recognizable as the ectoderm (Fig. 2F). Reaggregates now assume a form which resembles, at least externally, the normal blastula.

Development of the internal structures (stage 3)

Expansion of the blastocoel and migration of mesenchymal cells therefrom, which seem to take place concomitantly, leave one or more vesicles of internal epithelium suspended in the blastocoel (Fig. 2F). The actual number of these vesicles per 'blastula' seems to depend on the size of the individual animal. In contrast to the transparent ectoderm, these vesicles retain the orange colour of the egg cytoplasm and are now recognizable as endoderm. They fuse actively with one another until there is only one in the whole blastocoel (Figs 2G, 3E) (also see Yamanaka *et al.* 1986).

'Gastrulation' (stage 4)

At about 44 h, the ectoderm invaginates at one or more sites (Figs 2G, 3F), the actual number of which seems to depend on the size of the 'blastula'. Eventually ectoderm and endoderm fuse together at the points of invagination, turning the 'blastula' into 'mesenchymal gastrula' (Figs 2H, 3G).

One prominent event which accompanies 'gastrulation' concerns the change in the mode of swimming of the reaggregates. Up to this stage, they were either turning slowly (stage 1) or quickly (stage 2 and 3) on the spot, or rolling about in an

Fig. 2. Process of reconstruction. Bar indicates 100 μm. (A) Normal gastrula, from which cells were dissociated. (B) Dissociated cells (0 h). (C) Early reaggregates (stage 1, 3 h). (D) Formation of external epithelium (stage 2, 10 h). Some of the cells are dropping out from the periphery (open arrowheads). (E) 'Blastula'. The external epithelium is established, identified by the smoothness of the surface (stage 2, 25 h). (F) Stage-3 reaggregate (30 h). Blastocoel (*bc*), internal epithelium (*ie*) and mesenchymal cells (*mes*) are distinguishable. (G) 'Gastrulation' (stage 4, 44 h). Arrows indicate the sites of invagination. *ie*, internal epithelium. (H) 'Mesenchymal gastrulae' (stage 4, 60 h). Arrows indicate the blastopores. (I) Formation of the mouth (stage 5). Smaller four 'embryos' are at 55 h. The large one is at 68 h. Constrictions of the archenteron are shown by arrows. Open arrowheads indicate the stomodaeum. (J) A medium size bipinnaria (stage 6, 85 h). *a*, anus; *c*, coelomic pouch; *m*, mouth, *o*, oesophagus; *s*, stomach.

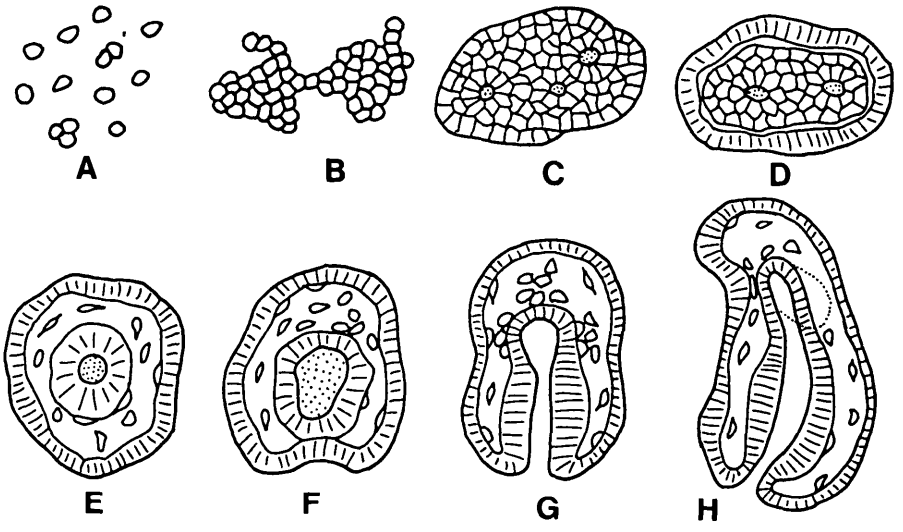


Fig. 3. Diagrammatic representation of the process of reconstruction. Cells dissociated from swimming embryos of the starfish are actively beating their cilia (A). They start to reaggregate as soon as they are returned to the normal sea water (B). Reaggregates gain in size by collision with free cells and with one another. The surface becomes smoother as the peripheral cells arrange themselves into an epithelium (C). Small lumina lined with epithelial cells (epithelial vesicles) are formed inside the reaggregate (C). The blastocoel develops and separates the external epithelium from the interior mass of cells (D). The blastocoelic space grows, into which mesenchymal cells migrate (E). Fusion of epithelial vesicles brings interior lumina together until there is only one large one suspended at the centre of the blastocoel (E). The external epithelium invaginates from one or more points (F) and fuses with the internal epithelium thus connecting the interior lumen with the outside. The reaggregate now assumes the form of mesenchymal gastrula (G). Stomodaeum forms at an anterolateral site of the 'ectoderm' and fuses with the tip of the 'archenteron' (H), which renders the reaggregate the larval form. Dotted line in H indicates the position and the size of the coelomic pouches which bulge from either side of the archenteron tip.

uncoordinated fashion. As the ectoderm invaginates, however, they start to swim in a normal spin-and-advance mode, keeping the invagination site always at the rear end. This change, however, is noticed only in 'gastrulae' which have formed only one invagination site. Those with more than one site of invagination continue to swim in an uncoordinated mode.

Formation of the mouth (stage 5)

The process of reconstruction, hereafter, does not differ essentially from that of normal development. The archenteron is divided into two parts by a constriction formed at about a half to one third of the way down from its tip (Fig. 2I). Stomach and intestine emerge from the lower part, while oesophagus and coelomic pouches are formed from the upper. In smaller 'gastrulae', however, coelomic pouches are either not formed or formed as small clusters of mesenchymal cells (Figs 2I, 3G, 4A). The oesophagus opens, eventually, to the bottom of the stomodaeum which invaginates at an anterolateral site of the ectoderm (Figs 2I, 3H).

Although the time course of reconstruction may vary considerably from experiment to experiment, events up to stage 4 take place synchronously among reaggregates of the same batch. After stage 4 the situation changes; the mouth opens much earlier in smaller reaggregates compared with larger ones of the same experimental batch. In order to describe the situation more precisely, 'gastrulae'

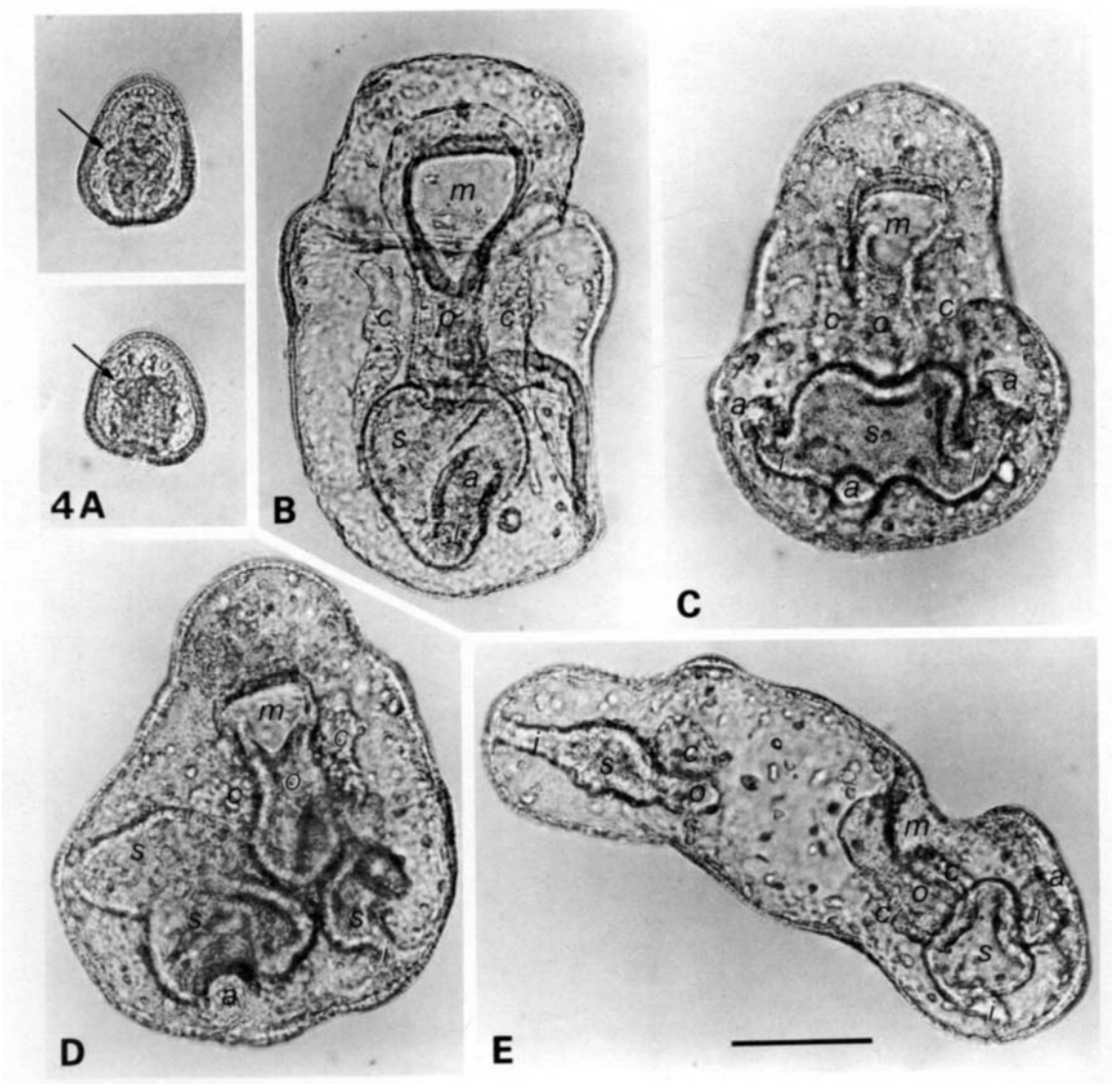
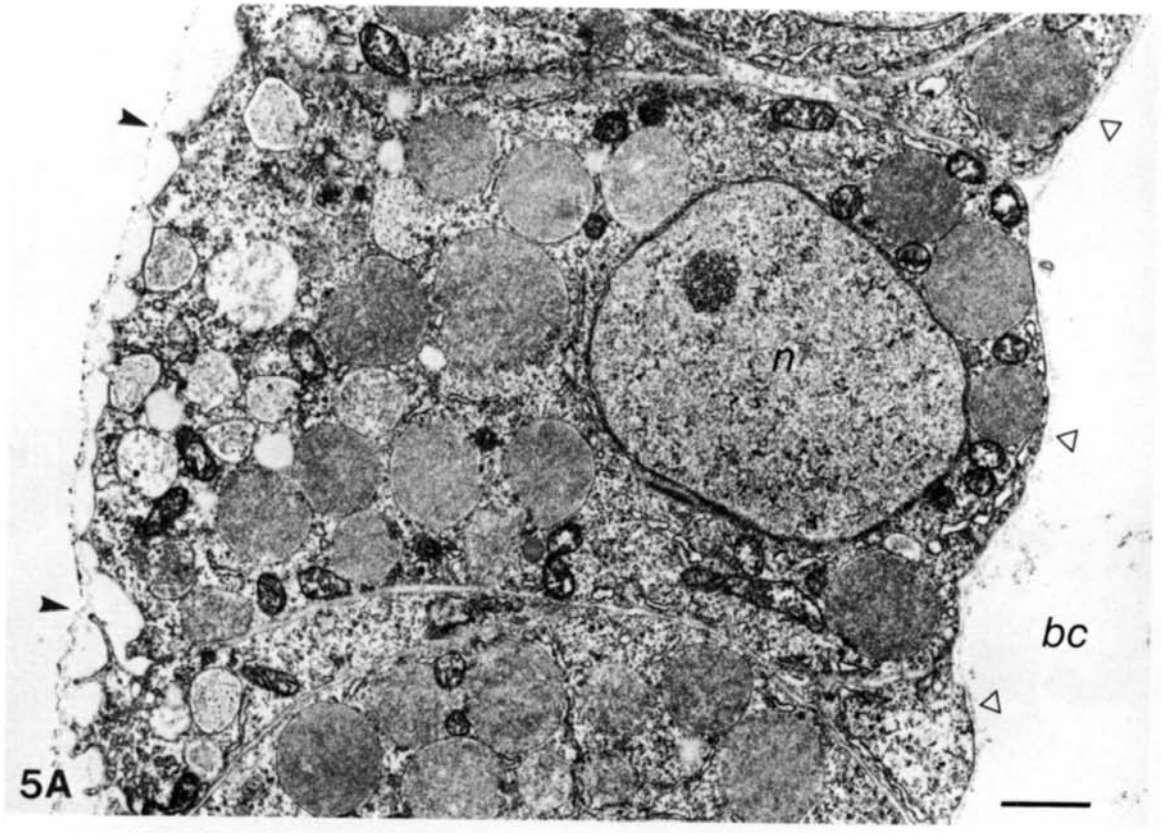
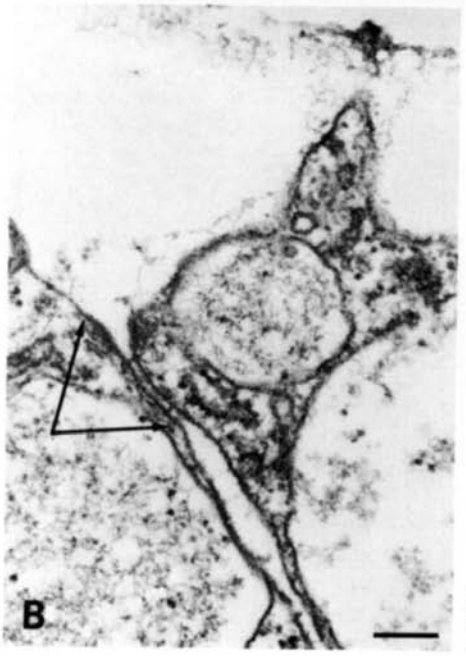
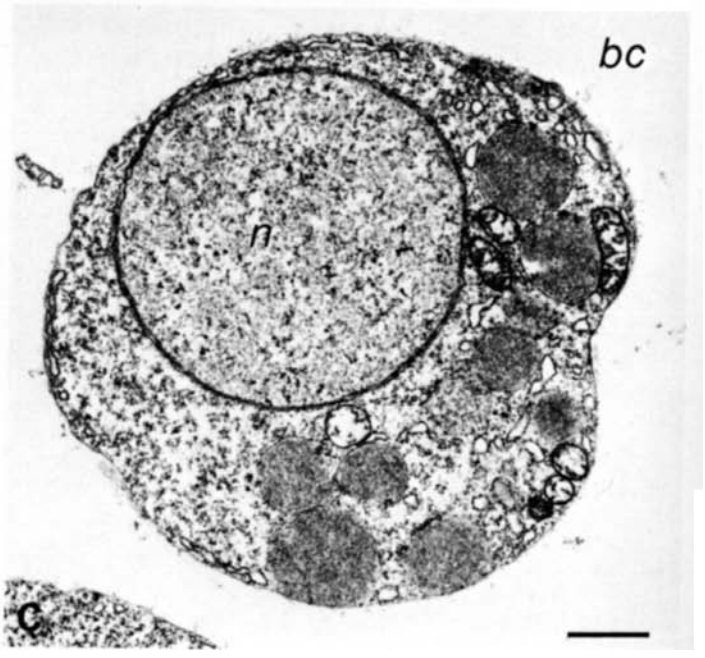


Fig. 4. Reconstructed bipinnariae. Bar, 100 μm. (A) Examples of the smallest reconstructed bipinnaria (arrows indicate the coelomic pouches). (B) The largest 'normal' bipinnaria so far reconstructed. (C) Bipinnaria having three ani, three intestine, one continuous stomach, an oesophagus and a mouth. (D) Bipinnaria consisting of one mouth, one oesophagus and three sets of gut below the stomach level. (E) Bipinnaria with two complete sets of gut; the right one has two intestine and two ani. *a*, anus; *c*, coelomic pouch; *i*, intestine; *m*, mouth; *o*, oesophagus; *s*, stomach.


n
bc
5A


B


bc
n
C

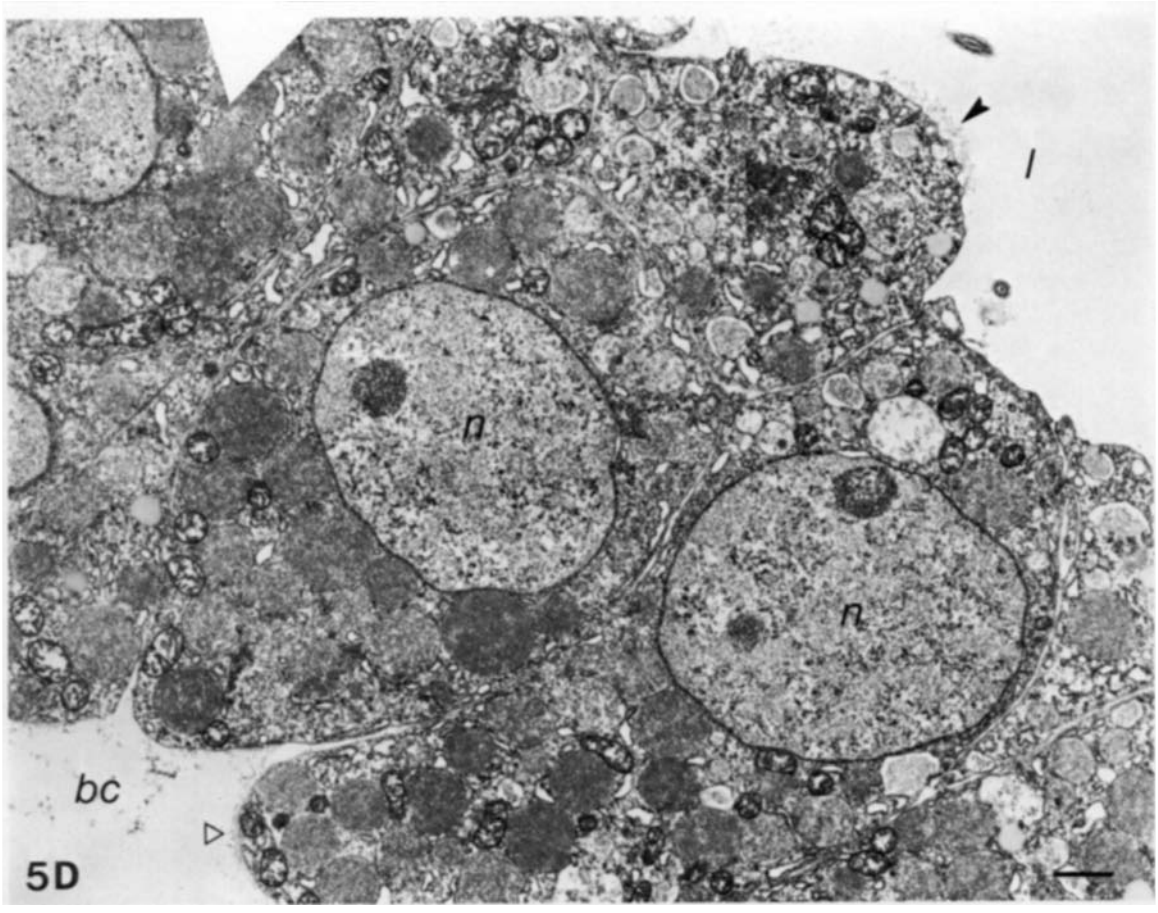


Fig. 5. Electron micrographs of gastrula after being treated with $1/100\,Ca^{2+}$ Jam× 1·14. (A) Ectoderm. Cytoplasmic projections reaching out to the hyaline layer (filled arrowheads) are still present. Basal surface is smooth and is still attached to the basement membrane (open arrowheads). (B) Ectodermal septate junction (between arrows). Some of the septa seem to be lost. (C) A mesenchymal cell. Pseudopods are withdrawn. (D) Endoderm. Cells are rounded. Apical projections are withdrawn. Open arrowheads, basement membrane; filled arrowheads, hyaline membrane; *l*, lumen of the archenteron; *bc*, blastocoel; *n*, nucleus. Bars in A,C,D, 1 μm; in B, 0·1 μm.

were classified into three size groups, small, medium and large. The average body lengths of 'gastrulae' belonging to these groups were about 150, 225 and 300 μm, respectively. Small ones reach the larval form generally around 80 h, the medium ones around 90 h (Fig. 2J) and the large ones around 100 h. Even the largest 'gastrulae' that have formed many 'blastopores' (Fig. 4C–E) acquire the form of bipinnaria by 100 h.

Bipinnaria (stage 6)

The body length of the smallest bipinnaria obtained so far is 94 μm, while that of the largest 'normal' bipinnaria is 390 μm (Fig. 4A,B).

The larger the body size, the greater is the chance of animals forming multiple ani. Examples of multi-anal bipinnaria are shown in Fig. 4C–E. It is noteworthy that multi-anal bipinnariae almost always form only one oesophagus and one mouth, regardless of the number of ani.

State of cells at the end of pretreatment with 1/100 Ca^{2+} sea water

No drastic change was observed in ectodermal cells at the end of 3 h pretreatment with 1/100 Ca^{2+} Jam×1·14 (Fig. 5A). Cytoplasmic projections extending into the hyaline membrane (filled arrowheads) are preserved. The inner surface of these cells becomes somewhat rounded, but stays attached to the basement membrane (open arrowheads). Septate junctions seem to lose some of their septa and become more obscure, but are not lost completely (Fig. 5B). Mesodermal and endodermal cells, on the other hand, are rounded (Fig. 5C,D). Cytoplasmic projections extending into the hyaline membrane, which lines the archenteron lumen (*l*), are withdrawn (Fig. 5D).

Loss of cells during the procedure

It turned out that about a half of the cells, as measured by the amount of protein, are lost during the dissociation process either as undissociated bits of the material embryo (ca. 3%) or by the damage caused by pipetting (ca. 50%). Among those recovered as dissociated cells some 30% fail to reaggregate and 40% more are dropped during the formation of the external epithelium (stage 2). Reaggregates are usually very stable after this stage.

The situation is, therefore, that 13·3% of the overall material and 30% of the dissociated cells survive and are participating in the reconstruction process.

DISCUSSION

The ability of dissociated embryonic cells of the starfish to reaggregate and to reconstruct the larval form is shown. This process consists of six steps, namely reaggregation (stage 1), formation of the external epithelium (stage 2), development of the internal structures (stage 3), 'gastrulation' (stage 4), and formation of the mouth (stage 5) which renders the reaggregate the larval form (stage 6).

The morphogenetic events of reconstruction differ from the normal course of development in several respects. (1) Rudiments of three germ layers segregate directly from one another within the structureless reaggregate, instead of segregating sequentially by means of formation of the archenteron and migration of the mesenchymal cells therefrom. (2) The entire later events depend on the achievement of stage 2, as inferred from the fact that internal structures never develop until, or unless, the external epithelium is fully established. (3) 'Gastrulation' is performed in an entirely specific manner. That is, the ectoderm invaginates, touches and connects with the endoderm which is already established as epithelial vesicle(s) suspended in the blastocoel. (4) While the events of

reconstruction up to stage 4 ('gastrulation') proceed synchronously among re-aggregates of the same experimental batch, morphogenesis after this stage is size dependent. (5) In the case of 'gastrula' having only one site of invagination, the body axis is determined when that site is decided. This conclusion is deduced from the fact that the mode of swimming is switched from uncoordinated spinning or rolling of the 'blastula' to a coordinated spin-and-advance mode of the 'gastrula'. The site of invagination is always kept at the posterior end in the latter mode. (6) The body axis of the 'gastrula', which has formed more than one site of invagination, is determined at the same time as the position of the oesophagus (see below for further discussion). (7) 'Large' reaggregates tend to reconstruct multi-anal bipinnariae, in contrast to the multi-embryonic forms which result when two or more early embryos are joined (Dan-Sohkawa, 1977) (see below for further discussion).

The major factor influencing the shape of the reconstructed larva is the number of sites of invagination formed at the time of 'gastrulation'. Each of these sites will fuse with the nearest point of the internal epithelium to form a blastopore which will become the morphogenetic centre of the embryo, as in normal development. That is, the blastopore always constitutes the posterior end of the embryo, as was mentioned above, and the oesophagus, and eventually the mouth, develop from the other end of the internal epithelium. Therefore, when only one blastopore is formed, morphologically normal larva will develop (Figs 2J, 4A,B) and, when more than one blastopore is formed, the larva will become multi-anal (Fig. 4C–E).

In addition, the smaller the size of the reaggregate, the greater is the chance that it will develop into a 'normal' bipinnaria. Indeed, the great majority of bipinnariae having body lengths less than 225 μm was 'normal'. It is inferred from this fact that there is a certain range of cell population size which is more suitable for the mechanism of reconstruction. Since 'normal' reconstructed bipinnariae do not differ in morphology from experimentally induced dwarf larvae (cf. Dan-Sohkawa & Satoh, 1978), we have estimated the size of the preferred cell population for reconstruction, at the time of 'gastrulation', by referring to previous counts of the constituent cells of dwarfs of the same body lengths (Dan-Sohkawa & Satoh, 1978). In this previous experiment, 1/8 and 1/4 dwarfs, which had an average of 555 and 980 cells respectively, at the time of gastrulation, grew up to bipinnariae having body lengths around 150 and 225 μm, respectively. The preferential cell population at the time of 'gastrulation', therefore, is estimated to fall roughly between 500 to 1000 for the reconstruction process. The size of this population is in sharp contrast to 5000 for the normal gastrulating embryo (Dan-Sohkawa & Satoh, 1978). This difference points to the possibility that there is a mechanism in the normal embryo which either enables a greater distance for cell-to-cell communication or prevents cells located at a distance from the vegetal pole participating in the gastrulation events. The same mechanism is also considered responsible for the difference in the shape between 'large' reconstructed bipinnariae with many ani (Fig. 4C,D) and joined larvae with multi-embryonic forms (Dan-Sohkawa, 1977).

Reaggregates consisting of more than 1000 cells, on the other hand, are likely to have more than one internal epithelium at the time of 'gastrulation'. Blastopores will form between ectoderm and any of these internal epithelia, the latter eventually fusing with one another (Yamanaka *et al.* 1986) to form a common stomach (Fig. 4C). In rare cases, however, the internal epithelial vesicles stay separate and form isolated stomachs, as shown in Fig. 4D. In either case only one set of oesophagus and mouth is formed. It is only in extremely rare cases that more than one mouth is formed (Fig. 4E). As is inferred from the forms of such multi-anal bipinnariae, the second major morphogenetic adjustment takes place when the site of oesophagus formation is decided and the time required for this decision may constitute the main cause of size dependency of the later half of the reconstruction process.

Synchrony of the reconstruction events up to 'gastrulation' not only applies to reaggregates of the same experimental batch, as has been mentioned, but also to successful cases of cells dissociated from embryos at different stages of development. This fact implies that the degree of differentiation reached by the cells at the time of dissociation is not reflected in the process of reconstruction. This situation differs from that of the sea urchin in which reaggregates of gastrular cells reach the pluteus stage within a shorter period of time than those composed of blastular cells (Giudice, 1962; Giudice & Mutolo, 1970). The reason for this difference is not known.

Our results, so far, tell us nothing about the occurrence of sorting out events in the reconstruction process, as is known to occur in the sea urchin (Giudice, 1962; Spiegel & Spiegel, 1978).

It is important to mention that the reconstruction process of the sea urchin cells dissociated at the 16-cell stage (Spiegel & Spiegel, 1975, 1980) should not be discussed on the same basis with the present results because, when the sea urchin embryo is dissociated during the cleavage stages, descendants of each of the blastomeres are expected to stay close together and tend to form reaggregates by themselves or in collaboration with several neighbouring clones. In either case, much of the positional information carried by individual blastomeres would be introduced into the reaggregate. Support for this speculation is provided by the fact that the greater the number and/or the size of clusters that are allowed to remain in the dissociated cell population (Fig. 2B), the easier reconstruction is achieved (data not shown). Resemblance between the reconstruction process of the 16-cell-stage sea urchin blastomeres (Spiegel & Spiegel, 1980) and the developmental process of isolated blastomeres of the starfish (Dan-Sohkawa & Satoh, 1978) is thought to supply further justification for this argument.

The greatest difficulty concerning the present method is the low percentage of cells to survive the experimental procedure (see Results). Various efforts to raise the yield such as prolonged pretreatment with low-Ca^{2+} Jamarin, addition of a small amount of calcium ion into the same artificial sea water, addition of foetal bovine serum to the dissociation medium and to the initial reaggregation medium, and initial low-temperature conditions all helped to raise the yield to some extent,

but not satisfactorily. Taking into account the fact that septate junctions survive the pretreatment (Fig. 5B), we speculate that a considerable number of ectodermal cells are either ruptured or injured during the pipetting at the time of dissociation, while endodermal and mesodermal cells are relatively uninjured. We have no evidence, however, to show that reconstructed bipinnariae are populated by greater proportions than normal of both endodermal and mesodermal cells as compared to ectodermal cells. Efforts are under way to raise the rate of survival of dissociated cells.

We are grateful to the members of Tateyama Marine Laboratory of Ochanomizu Women's University and Asamushi Marine Station of Tohoku University for kindly supplying the material and for allowing us to use their facilities during this investigation. The kind assistance of Mr N. Miyata of Osaka City University in preparing the photographs is gratefully acknowledged.

REFERENCES

DAN-SOHKAWA, M. (1977). Formation of joined larvae in the starfish, *Asterina pectinifera*. *Devl., Growth and Differ.* **19**, 233–239.

DAN-SOHKAWA, M. & SATOH, N. (1978). Studies on dwarf larvae developed from isolated blastomeres of the starfish. *Asterina pectinifera*. *J. Embryol. exp. Morph.* **46**, 171–185.

GIUDICE, G. (1962). Restitution of whole larvae from disaggregated cells of sea urchin embryos. *Devl Biol.* **5**, 402–411.

GIUDICE, G. & MUTOLO, V. (1970). Reaggregation of dissociated cells of sea urchin embryos. *Adv. Morphogen.* **8**, 115–158.

HERBST, C. (1900). Uber das Auseinandergehen von Furchungs und Gewebezellen in kalkfreiem medium. *Arch. mikrosk. Anat. EntwMech.* **9**, 424–463.

HOLTFRETER, J. (1938). Differenzierungspotenzen Isolierter Teile der Urodelengastrula. *Wilhelm Roux Arch. EntwMech.* **138**, 522–656.

KANATANI, H. (1969). Induction of spawning and oocyte maturation by 1-methyladenine in starfishes. *Expl Cell Res.* **57**, 333–337.

LIN, T. P. & FLORENCE, J. (1970). Aggregation of dissociated mouse blastomeres. *Expl Cell Res.* **63**, 220–224.

LUCEY, E. C. A. & CURTIS, A. S. G. (1959). Time-lapse film study of cell reaggregation. *Med. Biol. Illus.* **9**, 86–93.

MILLONIG, G. (1975). Blastomere reaggregation. In *The Sea Urchin Embryo* (ed. G. Czihak), pp. 407–423. Berlin, New York: Springer Verlag.

MILLONIG, G. & GIUDICE, G. (1967). Electron microscopic study of the reaggregation of cells dissociated from sea urchin embryos. *Devl Biol.* **15**, 91–101.

MOSCONA, A. A. (1956). Development of heterotypic combinations of dissociated embryonic chick cells. *Proc. Soc. exp. Biol. Med.* **92**, 410–416.

PATRICOLO, E. (1967). Differentiation of aggregated embryonic cells of amphibians (*Discoglossus pictus*). *Acta Embryol. Morph. exp.* **10**, 75–100.

SPIEGEL, E. & SPIEGEL, M. (1980). The internal clock of reaggregating embryonic sea urchin cells. *J. exp. Zool.* **213**, 271–281.

SPIEGEL, M. & SPIEGEL, E. (1975). The reaggregation of dissociated embryonic sea urchin cells. *Amer. Zool.* **15**, 583–606.

SPIEGEL, M. & SPIEGEL, E. (1978). Sorting out of sea urchin embryonic cells according to cell type. *Expl Cell Res.* **117**, 269–271.

STERN, M. S. (1972). Experimental studies on the organization of the preimplantation mouse embryo. II. Reaggregation of disaggregated embryos. *J. Embryol. exp. Morph.* **28**, 255–261.

TOWNES, P. L. & HOLTFRETER, J. (1955). Directed movements and selective adhesion of embryonic amphibian cells. *J. exp. Zool.* **128**, 53–118.

TRINKAUS, J. P. (1963). The cellular basis of *Fundulus* epiboly. Adhesivity of blastula and gastrula cells in culture. *Devl Biol.* **7**, 513–532.

WEISS, P. & ANDRES, G. (1952). Experiments on the fate of embryonic cells (Chick) disseminated by the vascular route. *J. exp. Zool.* **121**, 449–487.

YAMANAKA, H., TANAKA-OHMURA, Y. & DAN-SOHKAWA, M. (1986). What do dissociated embryonic cells of the starfish, *Asterina pectinifera,* do to reconstruct bipinnaria larvae? *J. Embryol. exp. Morph.* **94**, 61–71.

YOKOYA, S. (1966). Cell dissociation and reaggregation in early stage embryo of a teleost, *Oryzias latipes*. *Sci. Rep. Tohoku Univ. Ser. IV.* **32**, 229–236.

(*Accepted 11 December 1985*)