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Allocation of mesendodermal cells during early embryogenesis in the starfish, *Asterina pectinifera*

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SUMMARY

The volume of archenteron tissue (mesendoderm) in the early-to-middle gastrula of the starfish *Asterina pectinifera* was nearly one-quarter of whole embryo. Treatment with LiCl during 7–10 h increased this volume ratio by about 30 %, whereas the total volume and the total number of cells of whole embryo remained unchanged. Such a relative increase in mesendodermal part and simultaneous reduction in ectodermal part by LiCl treatment was confirmed by counting the number of constituent cells of these parts at early bipinnaria stage. Pulse treatment with LiCl revealed that the effective period of the treatment is limited from 7 to 10 h of development, when tightly packed blastulae are formed through increase in adhesiveness of blastomeres. These results indicate that a fraction of presumptive ectodermal cells can change its fate to mesendoderm during 7–10 h of development. Cellular interactions during a specific stage of development are suggested to be involved in the determination of mesendodermal tissue.

INTRODUCTION

Echinoderm embryos are known to exhibit highly regulative properties when they are dissociated into single blastomeres. Especially in starfish embryos, such capacity for regulation is very distinctly shown; each of the blastomeres derived from an embryo isolated even at the 8-cell stage is still able to give rise to dwarf but morphologically normal larva (Dan-Sohkawa & Satoh, 1978). In this case, descendent cells derived from one of four blastomeres at the animal hemisphere reconstruct axial organization and, by altering the fates of a certain population of cells, yield constituents of endodermal archenteron, though the descendants are normally destined to make ectodermal cell exclusively. This evidence of regulative development by isolated blastomeres suggests the existence of interactions between cells, not only in experimental conditions but also in the normal circumstances.

Only some reports, however, are concerned with the nature of cellular interactions in normal development of starfish embryos. In *Asterias* embryos, electrical couplings are shown to appear from the 32-cell or the 64-cell stage onward (Tupper & Saunders, 1972). In the starfish *Asterina pectinifera* it is noteworthy that blastomeres contact each other so loosely during early cleavage stages that cleaved blastomeres spread in a sheet on non-adhesive

substrata if the fertilization membrane is stripped off experimentally, yet this sheet 'curls up' into a hollow blastula after 8th cleavage (256-cell stage), suggesting an increase in cell surface adhesiveness (Dan-Sohkawa, 1976). In the tightly packed blastulae, which are formed after the 'curling up', junctional septate desmosomes are observed at the close apposition sites near the distal end of cells (Dan-Sohkawa & Fujisawa, 1980).

Little is known about the kind of intercellular interaction or the normal developmental stage at which it occurs. The present study is aimed at demonstrating interactions among blastomeres during cleavage stages, with respect to the determination of presumptive mesendodermal cells in whole embryos of starfish. Experiments were designed to change the number of cells which participate in the formation of the mesendoderm by treating the embryos with solutions of LiCl. It will also be shown that treatment with this reagent is effective during a rather short period of development, thus pinpointing the developmental stage during which interactions are taking place.

MATERIALS AND METHODS

Animals

The starfishes *Asterina pectinifera* were collected and kept in aquaria supplied with circulating sea water at 20°C. Animals were fed occasionally until their oocytes became full grown. Six batches of eggs (denoted by A–F) were used in this study.

Fertilization

Oocyte maturation was induced in the standard way, using 1-methyladenine (Kanatani, Shirai, Nakanishi & Kurokawa, 1969). Oocytes were fertilized as previously described (Kominami & Satoh, 1980). Embryos were cultured in artificial sea water (A.S.W., Jamarin U, Jamarin Lab., Osaka) at 20 ± 0·5°C.

Treatment with lithium chloride

Isotonic LiCl in distilled water was diluted with A.S.W. to a concentration of 0·03 M before use. Treatment was initiated by immersing the embryos in this diluted solution and was stopped by washing the embryos twice with A.S.W.

Measurement of embryo volume

Early gastrulae with radial symmetry around the animal–vegetal axis were fixed with 10 % formalin in sea water. Photographs were taken of embryos lying with their animal–vegetal axis horizontal to the slide glass. At the same time, the length of the embryo and the height of archenteron (along the animal–vegetal axis) were also measured. Owing to the radial symmetry of the embryos, both the ectodermal layer and the archenteron can be regarded as

shells of revolution. The volume of these shells, and thus the volume of the ectoderm and mesendoderm were approximated by regarding each as derived from a stack of short hollow cylinders 5 μm in height with the diameters and thickness derived from photographic prints.

An examination was made for possible error in such optical measurements which could arise from differences in refractive indices between cytoplasm and the surrounding medium: mature eggs of *Asterina pectinifera* deprived of jelly coat was compressed between cover and slide glass, and the refractivity of the egg cytoplasm was determined by measuring the shift of focal plane of the glass surface viewed through the egg, as done by Hiramoto (1957). The refractive index of the living egg was found to be comparable to or smaller than that of sea urchin eggs (1·40, Hiramoto, 1957). Rough calculation indicates that the error in optical measurement of wall thickness (hence the volume) of ectoderm and archenteron of gastrula whose refractive index is 1·40 would be no more than 4 %. The error seems to be still lower when the fixed embryos are observed, since using mature eggs after fixation with 10 % formalin in sea water, such a shift of focal plane was scarcely observed. Virtually no correction will be necessary in the present study.

Cell numbers

Embryos at the early-to-middle-gastrula stage (about 24 h of development) were fixed with Carnoy's fixative and the number of constituent cells was examined on squash preparations (Takahashi & Okazaki, 1979, Kominami & Satoh, 1980). Early bipinnariae (about 48 h) were fixed with 10 % formalin in sea water, dehydrated with an alcohol series and embedded in paraffin. Specimens were serially sectioned at 6 μm thick, and stained with haematoxylin. Numbers of cells in epithelium, digestive tract, coelomic sacs and mesenchyme were scored by serially examining all of the sections by Nomarski interference optics (interference apparatus NT, Nikon, Tokyo, Japan). In cases where a cell was dividing, one mitotic figure was counted as two nuclei.

RESULTS

General aspects of development

Early development of this starfish has been reported in several papers (Komatsu, 1972, Dan-Sohkawa & Satoh, 1978, Teshirogi & Ishida, 1978, Kominami & Satoh, 1980). In the present batches, gastrulation was initiated about 16 h after the start of 1-methyladenine treatment at 20°C. Release of mesenchyme cells from the archenteron tip into the blastocoel took place after another 8 h. By that time of development, the invagination of archenteron appeared to be complete (see fig. 5 and 8 in Kominami, 1983). These early-to-

middle gastrulae are still radially symmetrical around the animal–vegetal axis (Fig. 1B and its inset). Thereafter the ventral–anterior ectoderm becomes swollen and the embryos gradually lose their radial symmetry, exhibiting the dorsoventral polarity. Most parts of the embryo become fully differentiated into larval tissues at the early bipinnaria stage (Fig. 1C and Fig. 2A–C).

Volume of mesendoderm

Owing to their simple shape, the volumes of whole embryos or of the mesendodermal tissues in the early-to-middle gastrulae could be obtained by the procedures described above. Volumes of ectoderm, mesendoderm and whole embryos in batch A are summarized in Table 1. Total volume of

Table 1. *Volume of ectoderm and mesendoderm in the early-to-middle gastrulae (batch A)*

No. of embryos	Ectoderm (pl)	Mesendoderm (pl)	Whole (pl)	Diameter* (μm)	$\frac{\text{Mesendoderm}}{\text{Whole}} \times 100$ (%)
1	1980	475	2450	167	19·4
2	2280	561	2840	176	19·8
3	1950	488	2440	167	20·0
4	2220	556	2780	174	20·0
5	1920	550	2470	168	22·3
6	1770	523	2290	164	22·9
7	1750	532	2290	163	23·3
8	1950	594	2550	169	23·3
9	1940	599	2540	169	23·6
10	1780	574	2350	165	24·5
Average	1950	550	2500	168	21·9
S.D.	(178)	(41)	(187)	(4·1)	(1·9)

*Calculated diameter of a sphere equal in volume to the whole embryo.

embryos was 2200 to 2800 pl (average: 2500 pl). These values of embryo volume are equivalent to the volume of spheres with the diameters of 163 to 176 μm, as shown in "Diameter" in Table 1. Since the diameter of unfertilized eggs of this batch was 165–175 μm, we can say that the cellular volume of embryos proper apparently does not change up to the early-to-middle gastrula stage. The volume of the mesendoderm was 480 to 600 pl, or 19·4 to 24·5 % (average: 21·9 %) of the total volume of the embryo of this batch. No obvious correlation was observed between the total volume of individual embryo and the volume of its mesendodermal tissue.

The ratio of mesendodermal volume to the whole embryo volume measured in several batches of larvae (B–E) is shown in Table 2. In five batches so far examined, the average ratio ranged from 21·7 to 25·2 %. In the majority of embryos the ratio was not more than 25 %, although in 10 out of 50 embryos the ratio exceeded it, the most extreme being 27·6 %.

Table 2. *Volume ratio of mesendoderm to the whole embryos at the early-to-middle gastrula stage*

Batches of embryos	Mesendoderm/Whole × 100 (%)					Average (S.D.)
B	21·9	23·9	23·9	24·0	24·0	24·6
	24·6	25·6	25·8	26·1	26·5	(1·3)
C	19·4	20·4	20·5	20·8	22·0	21·7
	22·6	22·6	22·8	22·8	23·5	(1·3)
D	22·5	23·2	23·9	24·8	25·1	25·2
	25·9	26·1	26·4	26·4	27·6	(1·5)
E	21·2	21·3	21·8	22·9	22·9	23·2
	23·2	24·5	24·6	24·9	24·9	(1·4)

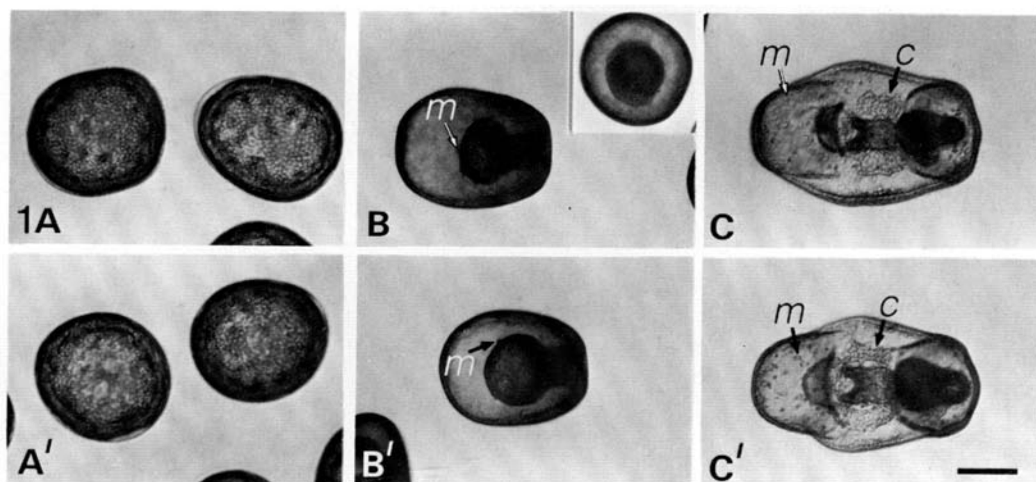

Fig. 1. Effects of LiCl-treatment on the formation of the archenteron or the digestive tract. Embryos treated with 0·03 M from 7 to 10 h of development (A′–C′) are compared with the normal embryos (A–C) at corresponding stages. (A,A′) 10 h of development. (B,B′) early-to-middle gastrulae (24 h). (B (inset)) anterior view of the gastrula, showing the radial symmetry around the animal–vegetal axis. (C,C′) early bipinnariae (48 h). Note the enlargement of the archenteron (B′ *versus* B) or the digestive tract (C′ *vs.* C) in the LiCl-treated embryos. Mesenchyme release from archenteron tip (B′ *vs.* B) or formation of coelomic sacs (C′ *vs.* C) was not delayed in experimentals. *m*, mesenchyme cells; *c*, coelomic sacs. Scale bar is 100 μm.

Embryos treated with LiCl

It is well known that the Li^+ ion causes various morphological changes in sea urchin embryos. In starfish embryos also, some morphological modifications were induced by LiCl treatment (Fig. 1). The embryos shown in Fig. 1A′–C′ have been treated with 0·03 M-LiCl during the period from 7 to 10

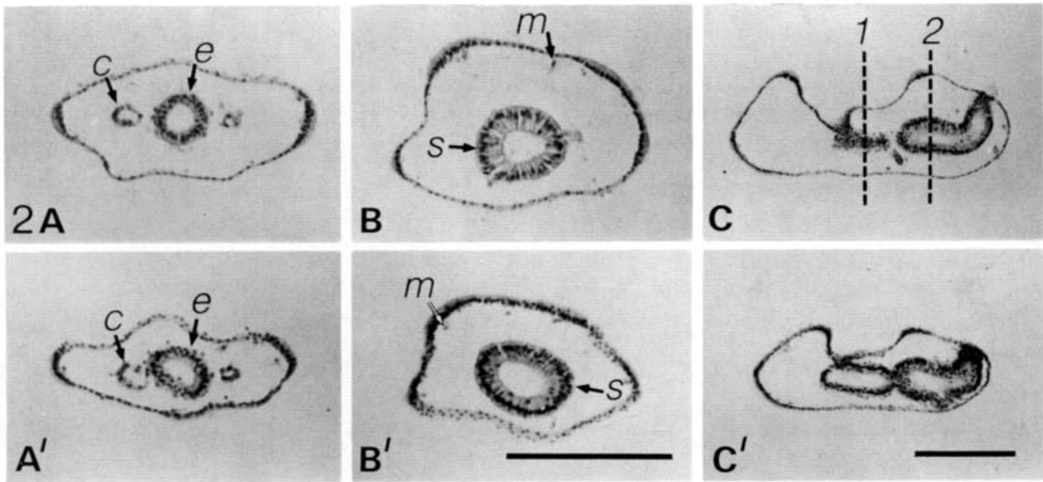

Fig. 2. Sections of the early bipinnariae. Embryos (A′–C′) were treated with 0·03 M-LiCl from 7 to 10 h of development. A–C; control. Levels of sections (*1*,*2*) are indicated in Fig. 2c. (A,A′) transverse sections at the oesophagus region (level *1*). (B,B′) sections at the stomach region (level *2*). (C,C′) meridional sections. Note the larger diameters of oesophagus (A′) and the stomach (B′) in LiCl-treated embryos compared with respective diameters (A and B) in control embryos. *m*: mesenchyme cells, *c*, coelomic sacs; *e*, oesophagus; *s*, stomach. Scar bar is 100 μm.

h of development. No immediate effect of LiCl treatment was apparent at the end of treatment (Fig. 1A, A′), but at the early-to-middle-gastrula stage, treated embryos formed archenterons that were longer and wider. The length of the whole embryo along the animal–vegetal axis became slightly shorter. Other developmental processes in treated embryos seem to be unaffected; the release of mesenchyme from the archenteron tip, the opening of oral region, and the formation of coelomic sacs were not delayed compared with the control embryos (Fig. 1B, C, B′, C′ and Fig. 2). In consequence of the enlargement of the archenteron at the early-to-middle-gastrula stage, treated embryos differentiated larger digestive tracts as shown in Fig. 2. Both in the oesophagus region (Fig. 2A, A′) and in the anterior stomach region (Fig. 2B, B′), digestive tracts of the treated larvae were larger in diameter than the controls. As for the ectodermal tissue, control larvae in turn showed larger dimensions (Fig. 2C, C′).

At the early-to-middle-gastrula stage, the relative enlargement of mesendodermal tissue in the LiCl-treated embryos to controls was substantiated by measurements of the volume of ectoderm and the mesendoderm (Table 3). In three batches of LiCl-treated embryos (A–C), the volume of the mesendoderm was 28–31 % of the total volume. This corresponds to a 30 % expansion of the volume of the mesendodermal tissue after LiCl treatment. The total volume of

Table 3. *Volume ratio of mesendoderm to the whole embryos in early-to-middle gastrulae treated with 0·03 M-LiCl in A.S.W. from 7 to 10 h after the initiation of development*

Batches of embryos	Mesendoderm / Whole × 100 (%)					Average (S.D)	Diameter (S.D.)
A	24·7	25·1	25·9	26·7	28·0	27·7	168
	28·5	28·6	28·8	29·9	30·4	(2·0)	(9·0)
B	27·8	28·1	29·3	30·5	31·2	31·2	172
	31·5	32·2	32·3	34·0	34·6	(2·3)	(4·3)
C	25·7	26·2	27·7	28·8	28·9	29·6	172
	29·8	29·8	30·9	31·8	36·3	(3·0)	(5·5)

Table 4. *Number of constituent cells in the control and LiCl-treated embryos at the early-to-middle gastrula stage*

Embryos	Number of constituent cells					Average (S.D.)
Control	4514	4687	4990	5230	5249	5196
	5283	5434	5471	5487	5513	(344)
LiCl-treated*	4701	4772	4841	4886	5061	5116
	5183	5275	5323	5375	5795	(343)

*Embryos were treated with 0·03 M-LiCl in A.S.W. from 7 to 10 h after the initiation of development.

the whole embryos (expressed as "Diameter" of the equivalent sphere in Table 3) was not significantly altered by LiCl treatment.

The number of cells in control or LiCl-treated embryos at the early-to-middle-gastrula stage are shown in Table 4. The number of constituent cells in LiCl-treated embryos (5116 ± 343 cells) was indistinguishable from that of controls (5196 ± 344 cells). These values for the early-to-middle gastrulae coincide with those reported in an earlier paper (Kominami & Satoh, 1980).

The relative increase in volume of archenteron (Table 3) at the gastrula stage, resulting in formation of larger mesendodermal tissue (Fig. 2) of bipinnariae may be due to 1) an increase in the number of cells allocated to the future mesendodermal tissues or 2) an increase in the cytoplasmic volume of a fixed number of archenteron cells, with simultaneous reduction in size of ectodermal cells.

Numbers of cells constituting ectodermal and mesendodermal tissues were therefore examined by counting the nuclei on serial sections of early bipinnariae (shown in Fig. 2). The reason for choice of such a late stage for cell counting was to know directly the cell numbers in ultimately differentiated tissues.

Table 5. *Number of nuclei of the control and LiCl-treated embryos at the early bipinnaria stage counted on serial sections*

		Ectoderm	Digestive tract	Coelomic sacs	Mesenchyme cells	Total
Control	1	7479(77·4)*	1844(19·1)	202(2·1)	140(1·4)	9665
	2	7719(78·5)	1682(17·1)	221(2·1)	199(2·0)	9827
	3	8199(76·3)	2118(19·7)	224(2·2)	208(1·9)	10749
	4	9683(78·5)	2349(19·0)	174(1·4)	133(1·1)	12339
	5	10033(73·6)	3266(23·9)	203(1·5)	139(1·0)	13641
	6	10701(76·0)	2924(20·8)	281(2·0)	172(1·2)	14078
Average		8969(76·5)	2364(20·2)	218(1·9)	165(1·4)	11717
LiCl-treated	1	7140(70·1)	2589(25·4)	276(2·7)	176(1·7)	10181
	2	7543(70·1)	2631(24·7)	296(2·8)	177(1·7)	10647
	3	8266(73·0)	2591(22·9)	282(2·5)	186(1·6)	11325
	4	8031(71·3)	3002(26·1)	333(2·9)	155(1·3)	11521
	5	8592(69·2)	3381(27·3)	285(2·3)	144(1·2)	12402
	6	9416(67·4)	3959(28·3)	398(2·8)	197(1·4)	13907
Average		8165(69·9)	3020(26·7)	312(2·7)	173(1·5)	11674

*Percentage of cells in respective tissue to the whole.

The number of cells in digestive tract derived from the archenteron dramatically increased in the LiCl-treated embryos (3020 cells), compared with that of control embryos (2360 cells), whereas the total number of nuclei were just the same in control and in LiCl-treated embryos. Digestive tract cells thus constituted 26·7 % and 20·2 % of the whole embryos in LiCl-treated and control embryo, respectively. The degree of increase in the number of nuclei of digestive tract studied at this stage of development accords well with that of the expansion of the archenteron volume measured at the early-to-middle-gastrula stage. Results obtained above favours the first possibility, i.e., increase in the number of cells which are allocated to archenteron, rather than mere swelling of archenteron.

As also shown in Table 5, the numbers of nuclei of the coelomic sacs also increased in LiCl-treated embryos, while the number of mesenchyme cells remained relatively unchanged.

Close correlation of the volume ratio with the length ratio

As stated above, the volume ratio of the archenteron served as a quantitative indicator of the number ratio of the mesendodermal cells. Measuring of the volume ratio or counting the number of constituent cells, however, are tedious procedures. When the measured volume ratio for each embryo is plotted against the length ratio (archenteron length relative to the whole embryo length. Fig. 3A, inset), there is a positive correlation in all three batches (A–C, Fig. 3), the correlation coefficients being 0·79, 0·84, and 0·89.

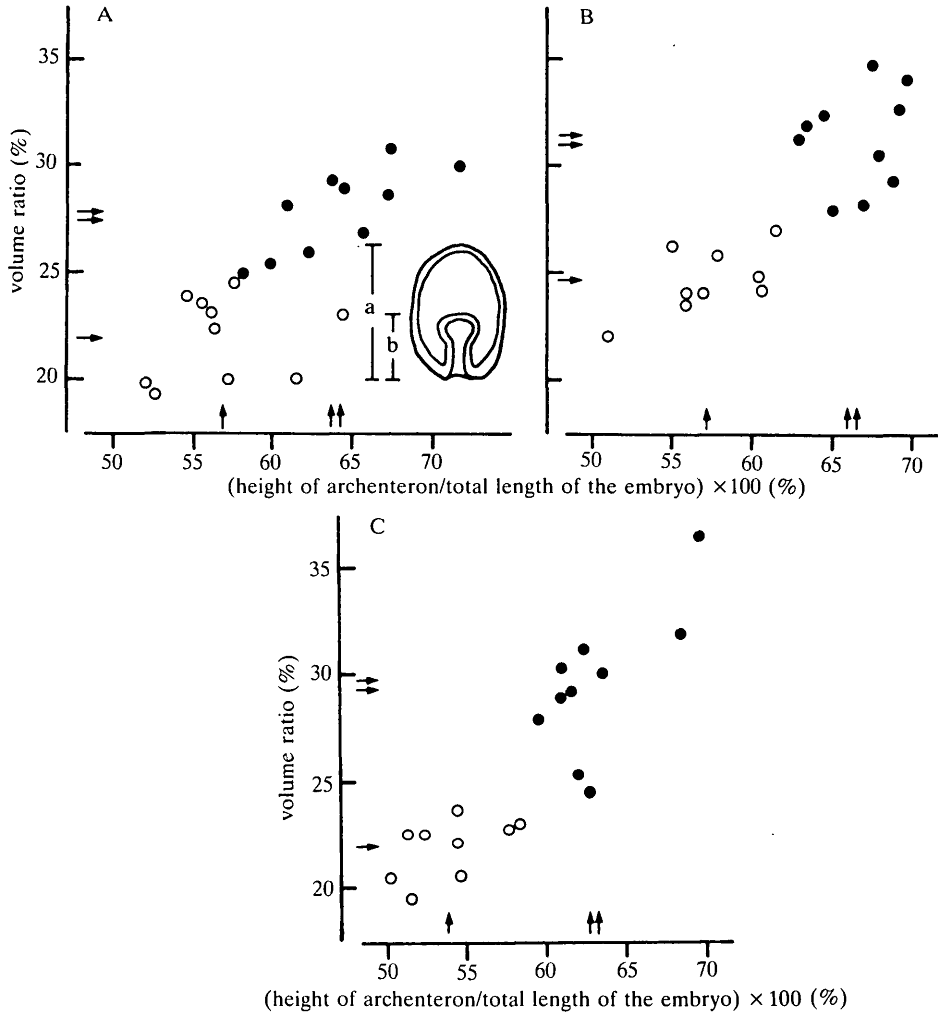

Fig. 3. Correlation between the volume ratio and the length ratio (b/a) of the archenteron to the whole embryo. The volume ratio of the archenteron in percentage (abscissa) is plotted versus the length ratio (see inset) in percentage. Results for three batches of eggs (A, B, C) are shown. Arrows indicate the average values for normal embryos (hollow circles) and double arrows for LiCl-treated embryos (solid circles).

Instead of measuring the volume ratio or number of cells, therefore, the length ratio can be used as an equivalent index of the number of the mesendodetmal cells in the experiments that follow.

LiCl-sensitive period during early embryogenesis

Embryos were treated with 0·03 M-LiCl during various stages of development (1–15 h) and the length ratios were determined at the early-to-middle gastrula stage.

Treatment with LiCl during 1 to 4 h, 3 to 6 h or during 12 to 15 h had no effect on the length ratio, but treatment during 6 to 9 h or during 9 to 12 h prominently increased the length ratio, indicating relatively longer archenterons (Table 6). The embryos thus have a short period of sensitivity to LiCl.

Table 6. *Effects of LiCl treatment on archenteron length during various 3 h periods from 1 to 15 h of development*

Time of LiCl treatment	Length ratio* (% ± S.D.)	Change in length ratio	t-test**
0 (control)	58·4 ± 2·0		
1–4	57·7 ± 3·1	−0·7	>0·30
3–6	58·6 ± 2·5	0·2	>0·70
6–9	69·4 ± 3·4	11·0	<0·001
9–12	63·9 ± 2·2	5·5	<0·001
12–15	58·6 ± 2·1	0·2	>0·70

*Ratio of the length of the archenteron to the length of the whole embryo. Averaged value of 30 embryos.

**Possibility of error by Student's t-test in the statement that the difference between control and experimentals is significant.

To confirm the nature of this sensitivity, embryos were pulse treated with 0·03 M-LiCl for one hour at various stages of development and the length ratios were measured in five batches of eggs (A–E, Fig. 4). Although the pattern of change in the length ratio was somewhat variable among batches, in all cases the one-hour treatment was consistently effective during the period of 7 to 10 h of development. In some batches (B, D) the effective period extended beyond of this range. Finally, embryos were treated continuously with 0·03 M-LiCl from 4 h after the initiation of development, and then returned to A.S.W. at various times up to 15 h (just before hatching, Fig. 5A). The length ratios of experimentals were nearly the same as controls up to 6 h and then gradually increased to a plateau at 10 h. Next, embryos were treated with LiCl at various times and returned to normal sea water at 15 h (Fig. 5B). In these cases the length ratios showed a plateau up to 10 h and then decreased to the control level. These results show clearly that the effect of LiCl was limited to the period from 7 to 10 h of development.

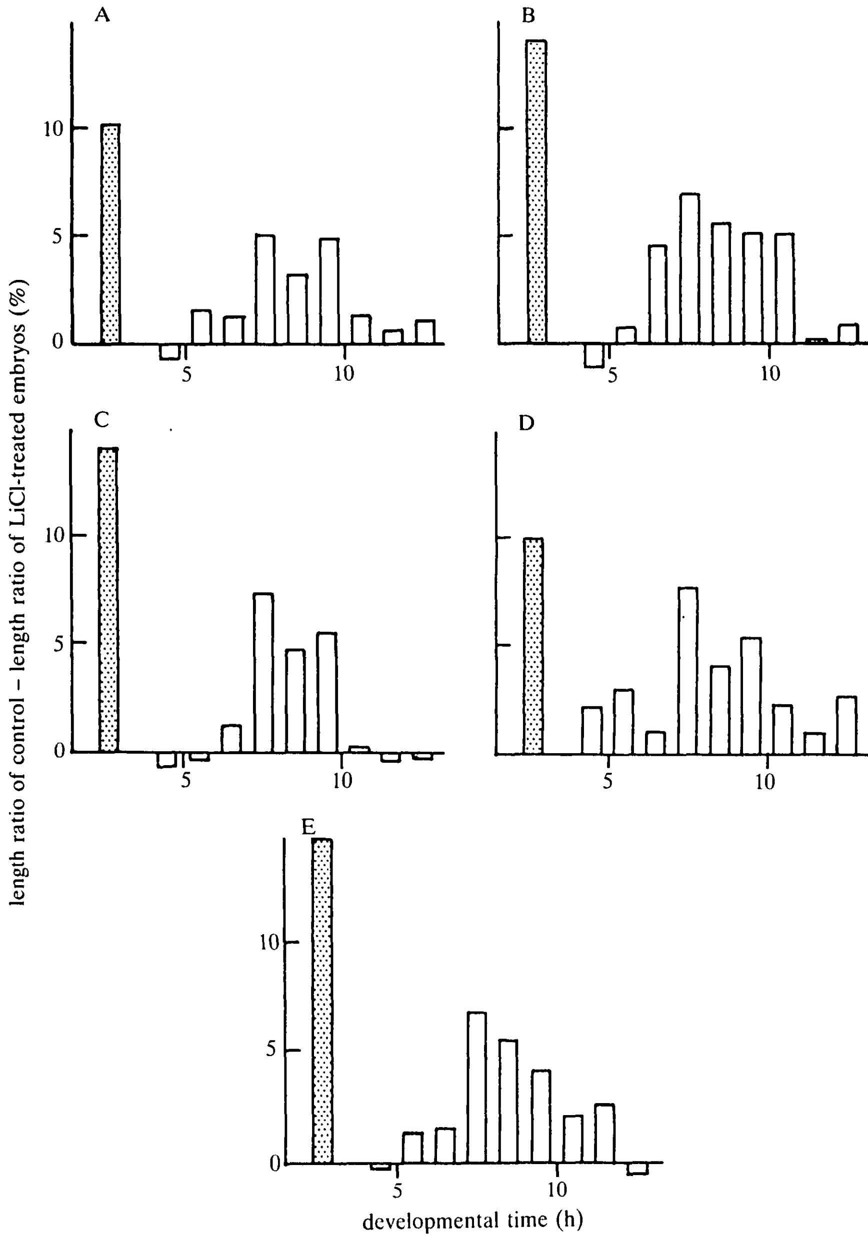

Fig. 4. Length ratios in the embryos pulse-treated (for 1 h) with 0·03 M-LiCl at indicated time of development (A–E). Dotted bars show the length ratios in the embryos treated with 0·03 M-LiCl during 7–10 h (for 3 h).

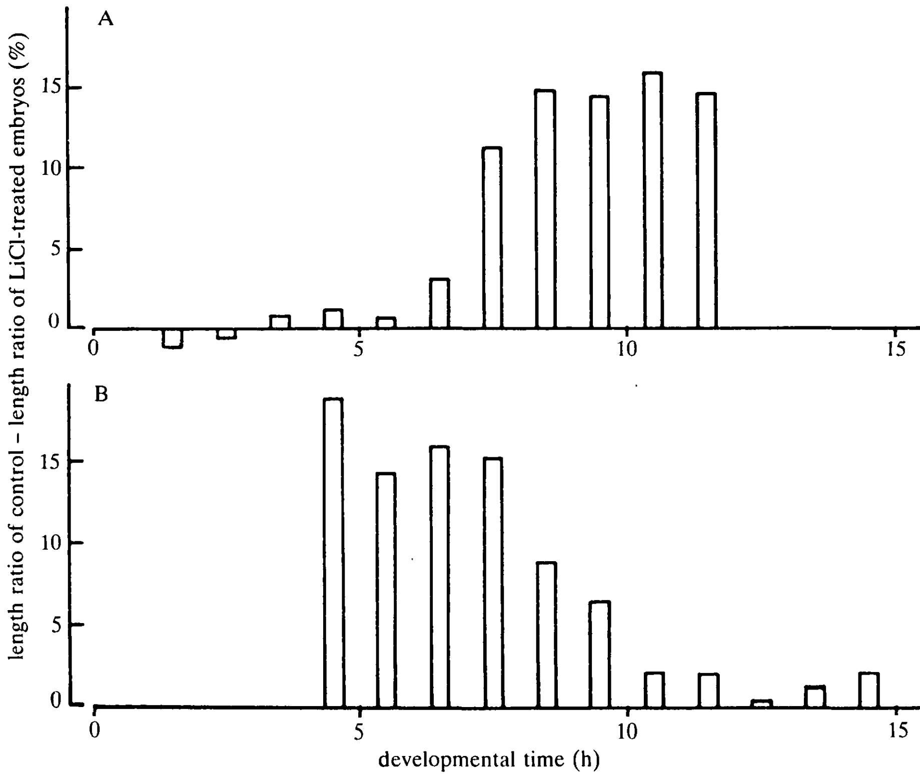

Fig. 5. Length ratios measured for the embryos of batch B treated with 0·03 M-LiCl during various periods of time. (A) treatment with LiCl initiated at 1 h of development and terminated at indicated times ranging from 2 to 12 h. (B) treatment initiated at indicated times (from 4 to 14 h) and terminated at 15 h.

DISCUSSION

Pulse treatment with LiCl at an early stage was found to increase the number of cells constituting the mesendoderm, the increase in the number (30 %, Table 5) coinciding with the increase in archenteron volume at the gastrula stage (Table 1, 2, 3). This effect of LiCl can be seen as a result of an increase in the number of cells allocated to form archenteron on gastrulation, which eventually gives rise to an enlarged digestive tract in the bipinnaria (Figs. 1, 2). Alternative explanations would involve a complicated action of LiCl, i.e., selective swelling of archenteron cells at the gastrula stage and subsequent enhancement of mitotic activity in mesendodermal tissue, while keeping the total volume and the total cell number of whole embryo unchanged. Such an intricate mechanism is inconceivable, if not impossible.

This study also demonstrates that the effective period of LiCl treatment is rather brief. It is clear that the continuous or pulse treatment of embryos with 0·03 M-LiCl is effective from 7 to 10 h of development (Fig. 4, 5 and Table 6). This period of time corresponds well with the time when blastomeres are known to come into close contact with each other: from this stage onward, blastomeres change their shape from globular to columnar and polygonal at the outer surface, resulting in tightly packed blastulae. Dan-Sohkawa (1976) reported that a sheet of blastomeres at this stage 'curls up' in the absence of the fertilization membrane. This good correlation between the effective period of LiCl treatment and the stage when blastomeres come in close contact suggests that LiCl should affect the process of increase in cellular adhesiveness, and that mesendodermal tissue may be determined through such newly formed cellular contact. Moreover, brevity of the effective period of LiCl treatment imply that the cellular interactions that make blastomeres the presumptive mesendodermal cells should occur during this stage of development.

In this connection, a classical experiment by Hörstadius on sea urchin embryos is worth noting: when the vegetal half of the embryo was removed at the 16- or 32-cell stage and replaced by micromeres implanted a few hours later, the experimental composite developed to a morphologically normal larva, but if the implantation is made after another few hours, the embryos failed to develop normally, and exhibit stronger animal properties (Hörstadius, 1936, 1973). His observation should indicate the brevity of the period during which certain interactions may take place between micromeres and the blastomeres of animal half to enable the whole system to reconstruct the animal–vegetal axis and to develop normally. It is interesting to note that this effect of micromere implantation can be mimicked by treating isolated animal hemispheres with LiCl (Hörstadius, 1936). A limitation in the effective period of LiCl treatment found in this study might correspond to such situations in sea urchin embryos. Both experiments indicate the presence of cellular interactions which take place during a rather short period of development.

The nature of LiCl actions is still not clear. In a preliminary observation I noticed that intensive treatment with higher concentration of LiCl (e.g., 0·1 M), induced such embryos to join together, resulting in monstrous aggregates (data not shown), so it is likely that treatment with LiCl temporarily enhances the adhesiveness of blastomeres at the even lower concentrations as used in the present study. Moreover, treatment with Concanavalin A, which has been shown to block the contact between blastomeres in *Asterina* embryos (Kyoizumi & Kominami, 1980), induced diminution in the size of the archenteron (unpublished data). It is, therefore, tempting to speculate that at this stage the embryos normally regulate the number of endodermal cells through newly formed cell-to-cell interactions. Li^+ ions probably make the blastomeres more adhesive and facilitate the formation of cellular contacts. Thus a larger population of blastomeres is destined to become the presumptive mesendo-

dermal cells through enhanced cell-to-cell interactions during the period of 7 to 10 h of development.

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