Experimentally Induced Separation of Embryonic Area in the Eggs of the Horseshoe Crab

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There are many experimental embryological studies* in the horseshoe crab and also other members of Chelicerata, but few experiments of the morphogenetic movement have been known.

Recently the author studied the morphogenetic movement of the embryo of the horseshoe crab. In this study, the treatment of the embryo with $NaHCO_3$ and Ca^{++} free sea water was attempted, and it was found that the treated embryonic area was separated into two or three pieces.

The present paper deals with the characteristics of the malformation induced. Still more, the mechanism of the separation of embryonic area will be discussed.

MATERIALS AND METHODS

1. Materials

Embryos of the horseshoe crab, *Tachypleus tridentatus* were used for the materials.

Their parent crabs were collected at the beaches of Imari, Saga Prefecture and Kasaoka, Okayama Prefecture, and were transported to Shizuoka University, where the artificial insemination and the experiments were carried out.

2.Methods

Dissociation of cells; The cells of the embryos are dissociated by $NaHCO_3$ or Ca^{++} free sea water. The author used these reagents for cell dissociation. Ca^{++} free sea water was made by adding NaCl instead of $CaCl_2$ in Van't Hoff's artificial sea water.

Treatment with chemical reagents; In order to analyze the relation between $NaHCO_3$ and Ca^{++} free sea water on their malforming effects, the embryos were treated with sea water including 10^{-1} M NaCl, NaOH, HCl, Na_2CO_3, both $NaHCO_3$ and $CaCl_2$, or both $NaHCO_3$ and HCl, with Ca^{++} free sea water including ethyleneglycol bis (2-aminoethylether)-N,N,N′

*These studies are as follows: Patten (1896), Kautzsch (1909), Oka (1943), Holm (1952), Sekiguchi (1957, 1966), Ehn (1962, 1963, 1964), Juberthie (1968), Jucuñski (1969), Itow & Sekiguchi (1979)

N'-tetraacetic acid(EGTA), and Mg^{++} free sea water, respectively. Some inhibitors of the respiratory metabolism, such as iodoacetic acid, NaF, arsenite, maronate, KCN, NaN_3 and 2,4-dinitrophenol were also used for the same purpose.

Vital staining and histological techniques; The embryos, normal and treated with chemical reagents, were vitally stained with neutral red, and the change of morphogenesis was observed under a stereoscopic microscope. For histological studies, the normal and the treated embryos were fixed in Bouin's, Carnoy's, or FAA solutions, embedded in paraffin, and sectioned at 5 to 20 μm thick. The sections were stained with Mayer's hematoxylin and eosin.

RESULTS

1. The conditions for induction of the monster with separate embryonic areas

The obvious morphogenetic movement in the horseshoe crab embryos occurs at Stage 10 to 12 (during about two days after the completion of the germ disc). The monster with separate embryonic areas was induced by the treatment with sea water including 10^{-1} M $NaHCO_3$ or with Ca^{++} free sea water for 24 hours during the above mentioned period (Tables 1&2). This type of monster was sometimes induced by the same treatment at the stages of spreading of germ disc (Stage 7 to 9), but the rate was low. These monsters were not induced by the treatment at the other stages (Figs. 1&2).

Table 1. The frequency of formation of monsters having separate embryonic areas. The embryos were treated for 24 hours during the morphogenetic movement.

	Normal Embryos	Monster having separate embryonic areas	other Monsters	Developed Embryos
Normal sea water	14,262 (99.69)	5 (0.03)	40 (0.28)	14,307 (100.00)
$NaHCO_3$ 10^{-1} M	56 (53.8)	12 (11.5)	36 (35.6)	104 (100.0)
Ca^{++} free sea water	98 (46.7)	70 (33.3)	42 (20.0)	210 (100.0)

Number (%)

Table 2. Types and frequencies of monsters induced by the treatment with Ca^{++} free sea water and $NaHCO_3$ at the stage of morphogenetic movement.

	Monster having separate embryonic areas	Monster having no anterior area	Monster having no Chelicera	Monster having abnormal appendages
10^{-1} M $NaHCO_3$	12 (25.0)	8 (16.7)	8 (16.7)	20 (41.7)
Ca^{++} free sea water	70 (62.5)	9 (8.0)	4 (3.7)	29 (25.9)
Total	82 (51.3)	17 (10.6)	12 (7.5)	49 (30.6)

Number (%)

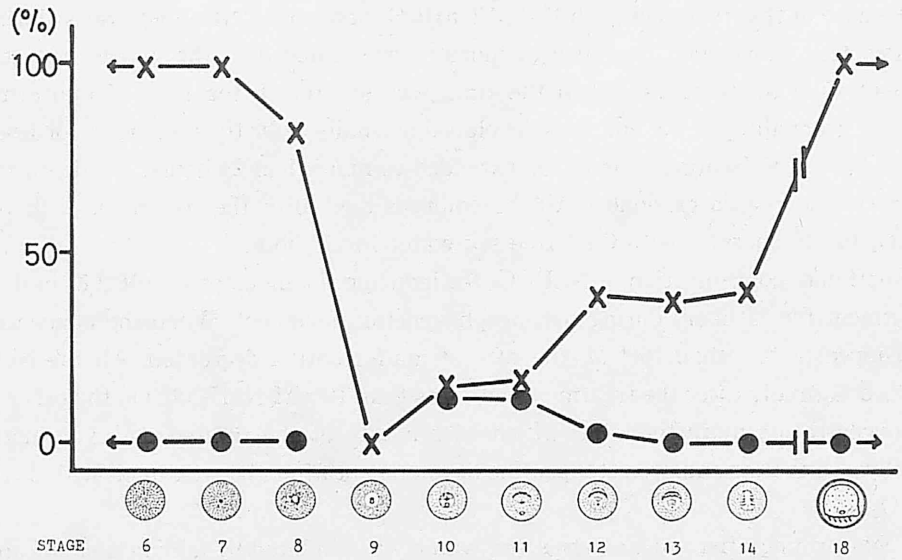


Fig. 1. Percentage of development and of the monsters having separate embryonic areas after treatment with $NaHCO_3$.

The embryos were treated for 24 hours with 10^{-1}M $NaHCO_3$ starting with the stages shown in the abscissa.

The duration of each stage is one day. ×:Percentage of developed embryos against the control. ● Percentage of the monsters among the developed embryos.

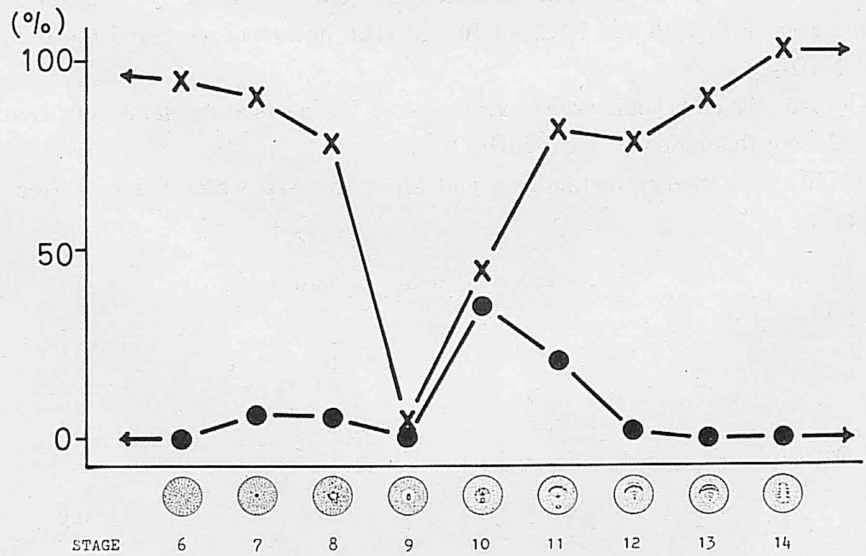


Fig. 2. Percentage of development and the monsters having separate embryonic areas after treatment with Ca^{++} free sea water starting with the stages shown in the abscissa. The duration of each stage is one day. ×: Percentage of developed embryos against the control. ●: Percentage of the monsters among the developed embryos.

In the case of the treatment with 10^{-1} M $NaHCO_3$ or with Ca^{++} free sea water during the morphogenetic movement, this type of monster was induced at the highest rate by the treatment time of 24 hours. When the time was shortened, the rate of malformation decreased gradually. All the embryos developed normally after the treatment for less than 6 hours. When the treatment time was extended to more than 24 hours, the death rate of the embryos increased gradually. All the embryos died after the treatment with 10^{-1} M $NaHCO_3$ for 48 hours or with Ca^{++} free sea water for 72 hours.

The optimum concentration of $NaHCO_3$ for inducing the monster was 10^{-1} M in the case of treatment for 24 hours during the morphogenetic movement. When the concentration was dropped to less than 10^{-1} M, the rate of malformation decreased. All the embryos developed normally after the treatment with less than 10^{-3} M $NaHCO_3$. On the other hand, in a concentration more than 10^{-1} M, the death rate of the treated embryos increased gradually. All the the embryos stopped the development after the treatment with $2x10^{-1}$ M $NaHCO_3$.

The malforming effect of Ca^{++} free sea water was inhibited by adding a slight amount of $CaCl_2$ and enhanced by EGTA in the case of treatment for 24 hours during the morphogenetic movement. After the treatment with artificial sea water including 5% $CaCl_2$ ($4.5x10^{-4}$ M Ca^{++} ion), the embryos developed normally. By treatment with Ca^{++} free sea water including 10^{-3} M EGTA, however, the degree of the malformation increased especially at the anterior part of embryos. After the addition of 10^{-2} M EGTA into Ca^{++} free sea water, the embryos did not develop (Table 3).

NaCl, NaOH or HCl in the artificial sea water did not induce the separation of the embryonic area. Either 10^{-1} M $CaCl_2$ or 10^{-1} M HCl, however, repressed the malforming effect of $NaHCO_3$.

Na_2CO_3 made the embryonic area separate into a few parts at the same conditions as the case of inducing this monster with $NaHCO_3$.

The inhibitors on energy metabolism and Mg^{++} free sea water did not effect on the malformation.

Table 3. The effect of the reagents being related to $NaHCO_3$ and Ca^{++} ion. The embryos were treated for the first half of the morphogenetic movement.

	Developed Embryos (% of developed control embryos)	Monsters having separate embryonic areas (% of developed embryos)	Monsters having no anterior area (% of developed embryos)
10^{-1} M $NaHCO_3$	13.4	11.5	7.7
10^{-1} M $NaHCO_3$ + 10^{-1} M $CaCl_2$	96.8	0.0	0.0
Ca^{++} free sea water	43.1	33.3	4.3
Mg^{++} free sea water	97.2	0.0	0.0
10^{-3} M EGTA in Ca^{++} free s.w.	4.0	0.0	50.0

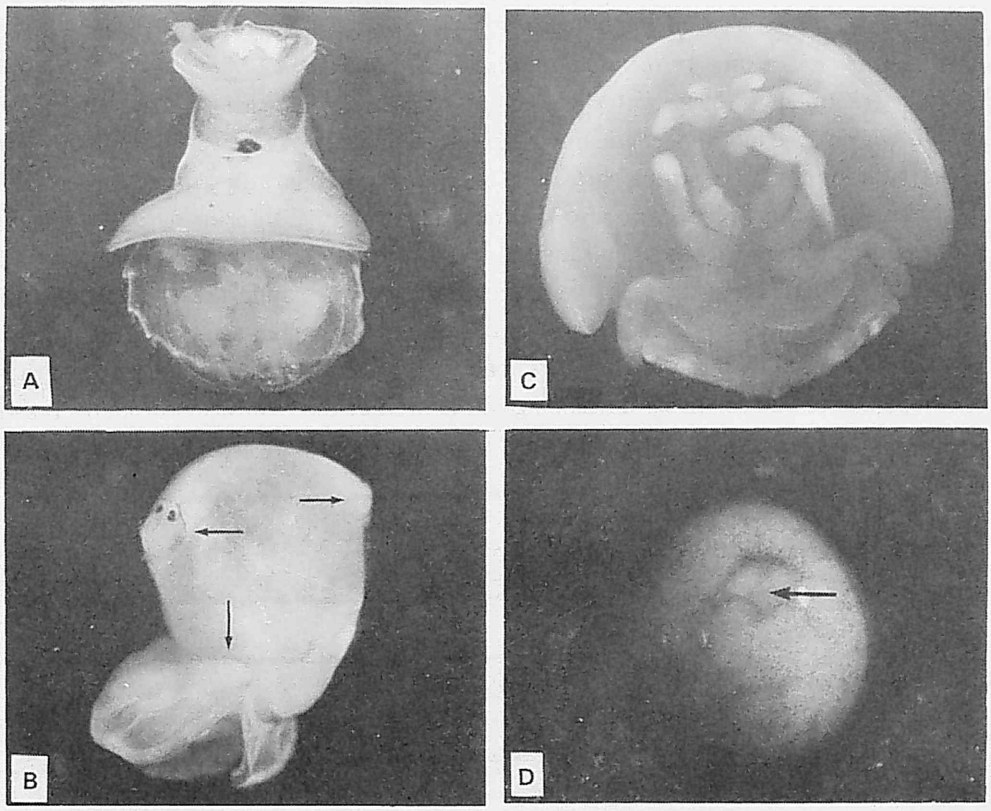


Fig. 3. Examples of monsters having separate embryonic areas.

A: The typical type of the monster, having two embryonic areas. The anterior piece consists of three prosomal segments, and the posterior one consists of three prosomal segments and the complete abdomen.

B: The monster having three separate embryonic areas. The anterior piece and the middle one have no appendages. The posterior piece comprises the 6th prosomal segment and the abdomen. The three pieces of the embryonic area are indicated by arrows.

C: The monster showing incomplete separation of the embryonic area. The anterior piece has three segments and the posterior one has three prosomal segments and the abdomen, but the separation is incomplete.

D: The embryo of one day after the treatment with Ca^{++} free sea water at the stage of morphogenetic movement. The embryonic area is separated at the prospective 3rd prosomal segment (arrow).

The malforming effect was hardly ever seen in normal natural and artificial sea water (Table 1).

2. The characteristics of the separation of embmyonic area

By the treatment with sea water adding 10^{-1} M $NaHCO_3$ or with Ca^{++} free sea water for 24 hours during the morphogenetic movement, the embryonic area was particularly injured and separated into two or three parts. The degree of this separation varied from partial to complete separation. There was also the monster losing the anterior part (Figs.3&4).

The embryonic area always separate into the anterior and the posterior part, and it never split right and left. More than 99% separated into two parts and the rest into three. In the separate embryonic areas consisting of two parts, the appendages of the last pair of the anterior part fused with those of the first pair of the posterior part at a rate of 70 to 80%. The appendages and segments developing in the area of the separation often decreased in

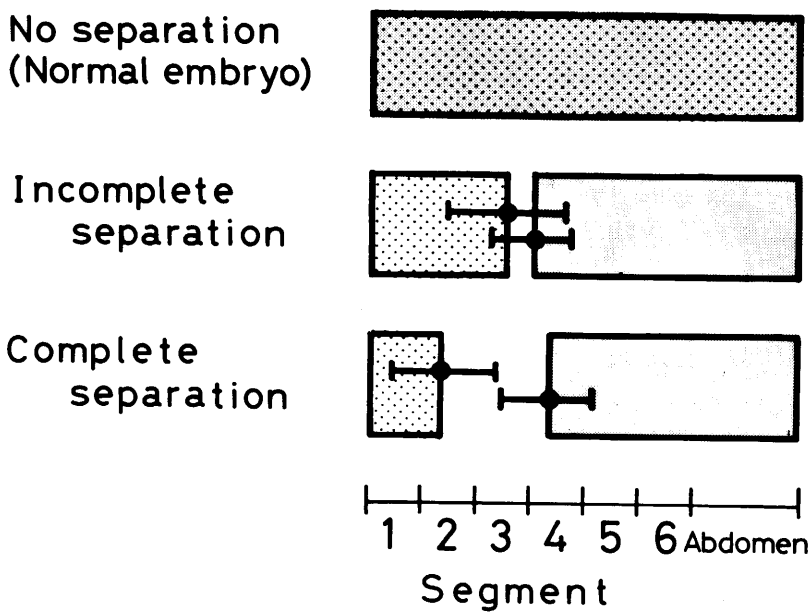


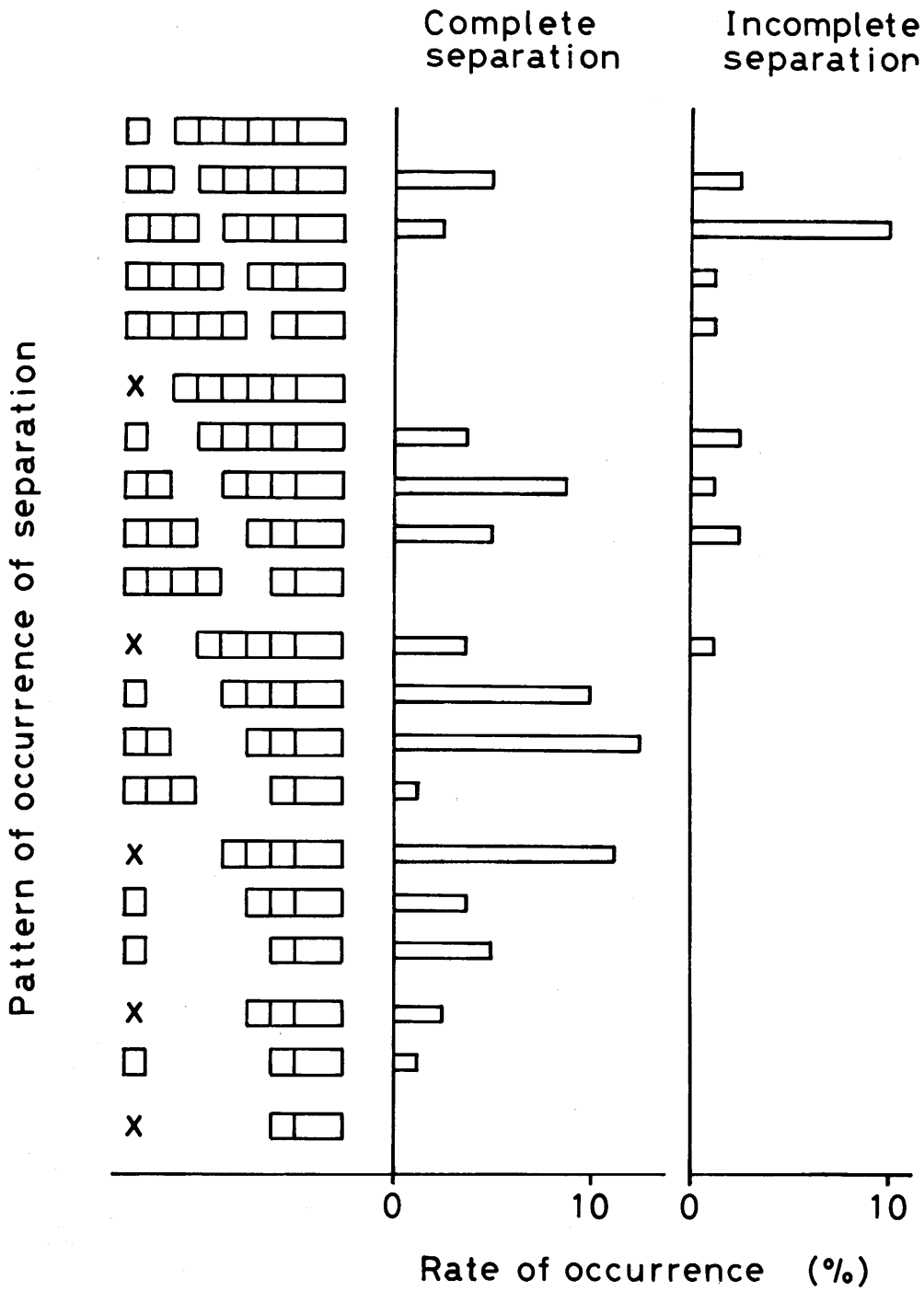
Fig. 4. The position of the separation of embryonic area.

The mean position of separation is shown in this figure. The horizontal bars with limiting marks show the range of standard deviation. The separation of embryonic area was induced by the treatment with Ca^{++} free sea water or $NaHCO_3$ for one day during the morphogenetic movement.

The anterior piece having no appendages was calculated as 0 segment.

Fig. 5. The patterns of separation of the embryonic area.

The separation of the embryonic area was induced by the treatment with Ca^{++} free sea water or $NaHCO_3$ for one day during the morphogenetic movement. □:prosomal segment having appendages. ▭: abdomen (opisthosome). ×: the separate embryonic area having no appendages.


Complete separation
Incomplete separation
Pattern of occurrence of separation
0
10
0
10
Rate of occurrence (%)

number. But they never increased in number and thir anterior to posterior sequence did not reverse. The histological features of this monster did not differ from those of normal embryos.

Fig.5 shows the position of the separation, the number of segments of anterior and posterior part, and the rate of each type of this monster. The embryonic area separated within the prosome, not within the opisthosome (or the abdomen). The separation did not occur at the border between the prosome and the abdomen.It occurred mainly at the 3rd prosomal segment (Fig. 4).

By the same treatment, other types of monsters were also induced; monsters devoid of the anterior embryonic areas and monsters with some abnormalities in the prosomal appendages. Among them, however, there were no double embryos and no monsters losing the posterior embryonic area (Table 2).

DISUSSION

It has been well known that the cells of emryos of the horseshoe crab (Itow & Sekiguchi, 1979) and various animals such as the sponge, the hydra, the sea urchin and the chick (Herbst, 1900; Giudice, 1962; Gierer et al., 1972) are dissociated by the removal of Ca^{++} ion.

When the embryos of the horseshoe crab, at the stage of active morphogenetic movement, were treated with Ca^{++} free sea water or $NaHCO_3$-added sea water, their embryonic areas were often separated.

The effective component of $NaHCO_3$ on the separation seems to be CO_3^{--}, because the monsters were induced by only CO_3^{--}, not the other ions such as Na^+, H^+ and OH^-.

It has been known that CO_3^{--} inhibits the respiratory metabolism, but the inhibitors of the metabolism used by the author did not induce the monster. This means that the inhibition of respiratory metabolism by CO_3^{--} is not the direct cause of leading the monster. As is well known, the CO_3^{--} ion combines with Ca^{++} ion, therefore the effect of $NaHCO_3$ to the formation of monster is thought to be completely repressed by means of the addition of $CaCl_2$. On the other hand, on the formation of monster, Ca^{++} free sea water was more effective than $NaHCO_3$-added sea water. In addition, on the characteristics of the monster, no appreciable difference between $NaHCO_3$-added sea water and Ca^{++} free sea water was recognized. Hereupon, we can estimate that the effect of $NaHCO_3$ on the formation of the monster is not more than depriving the embryos of Ca^{++} ions which connect the cells. The fact that the effect of $NaHCO_3$ to the formation of monster is repressed by means of the addition of HCl may be concerned with the inhibition of the combination of CO_3^{--} and Ca^{++} caused by H^+ ion.

In the author's study, the embryonic areas were always separated into two parts, anterior and posterior half, not right and left half. This fact may be concerned with the pattern of the morphogenetic movement that the cells at the periphery of the germ disc migrate into anterior end of the germ disc and then they move forward and backward along the medial body line. As the region where the cells at the periphery of the germ disc migrate becomes

the 3rd prosomal segment, the embryonic area of this monster seems to be separated nearly at the 3rd prosomal segment.

The posterior region behind the 5th or 6th prosomal segment may be formed after the stage of active morphogenetic movement. If the region was already formed at the time of the treatment by Ca^{++} free sea water or $NaHCO_3$-added sea water, the embryonic area should be separated even at the posterior region. But, the posterior regions of the treated embryos were always normal.

In the embroys of the horseshoe crab and the other members of Arthoropoda, the posterior region of the embryonic area is formed from the growth zone which exists at the posterior end of the embrvonic area. The growth zone of the embryo of the horseshoe crab may not be affected by the cell dissociation at the stage of active morphogenetic movement, because the monster devoid the posterior region was not produced by the teatment to induce the separation of embryonic area.

The embryonic area was separated by the cell dissociation at the stage of active morphogenetic movement, and in this malformation, the excess and the duplication of the body segments were not observed even at the faced margins of each separated piece. This fact means that the regeneration did not occur at these faced regions. Besides, the arrangement of segments of this monster was completely normal.On the other hand, the duplication of embryo has been induced by the cell dissociation at the stage of appearance of the germ disc (Itow & Sekiguchi, 1979). From these facts, the segment number and the characteristics of the lst to 4th or to 5th prosomal segments are thought to be determined at the period from the appearance of the germ disc to the stage of the active morphogenetic movement.

SUMMARY

For the analysis of the morphogenetic movement in the horseshoe crab embryos, the modification of morphogenesis was tried by treatment of the embryo at the stage of the active morphogenetic movement with Ca^{++} free sea water or $NaHCO_3$-added sea water.

The embryonic areas of the treated embryos were separated into a few pieces, and the characteristics of the malformation were examined. From the result, the direction of migration of cells during the morphogenetic movement and the determination of segments were discussed.

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