On the Mitotic Activity of the Nuclei in the Centrifuged Sea Urchin Eggs, with Special Reference to the Formation of the Giant Nucleus*

KENZI OSANAI

Department of Biology, University of Iwate, Ueda, Morioka, Japan.
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I. INTRODUCTION

It was previously reported that cell division and differentiation depend on the cytoplasmic factors segregating prior to cleavage in the eggs of the polychaete, *Tylorrhynchus heterochaetus* (OSANAI 1967a, b). The cells divided and differentiated only in the regions containing those factors. In the polyspermic eggs induced by being inseminated after the pre-trearment with Ca and Mg-free solution, the degree of the mitotic change of the sperm nuclei seems to correlate positively with the concentration of PAS (periodic acid-SCHIFF reaction) positive substance and inversely with that of toluidine blue-positive substance (OSANAI 1966). These observations suggest that the mitotic activity of the nuclei depends generally on cytoplasmic factors.

Methods, with which the egg cytoplasm are artificially replaced, are useful to dicide the cytoplasmic factor. When the unfertilized eggs of the sea urchin are centrifuged strongly, they are cut into several egg fragments and each of them consisits of different cytoplasmic components. The centrifuging method is considered to be ultilizable to study the nucleo-cytoplasmic relation.

In the American sea urchin, *Arbacia punctulata*, HARVEY (1932, 1940, 1946, 1956) reported on the development of the egg fragments cut with the centrifuging method. The results obtained in the *Arbacia* eggs, however, are applied not directly to the other sea urchins, because the fragmentation pattern of the unfertilized eggs by centrifuging differes in species.

When the fertilized eggs were strongly centrifuged, the cleavage proceeded rapidly in the centrifugal blastomeres, whereas it delayed or stopped in the centripetal blastomeres. Consequently, the giant cells remained in the centripetal half (PEASE 1939, MOTOMURA 1946, 1949). Studing the effect of centrifugal force on the determination of the egg axis, MOTOMURA (1946, 1949) asserted that the embryonic axis was altered in accordance with the centripetal shifting of the cortical cytoplasm. According to him, the centripetal pole became the vegetal pole in the strongly centrifuged eggs and the giant cells invaginate into the blastocoel, differentiating to the

* This was preliminary reported at the first meeting of Japanese Society of Developmental Biologist (OSANAI 1968).

mesenchyme cells.

On the other hand, KOJIMA (1959) reported that the giant cells appeared also in the fertilized *Temnopleurus* eggs centrifuged by a weak force prior to the first cleavage, though this weak centrifuging caused cytoplasmic stratification without egg streching. Therefore, he concluded that the cleavage activity of the blastomeres depended on the presence of a endoplasmic factor, the vitally stainable granules. When the unfertilized egg was cut into the two fragments by centrifuging and then fertilized, these granules were contained only in the centrifugal half which cleaved normally, while the centripetal half, which lacked these granules, failed to cleave.

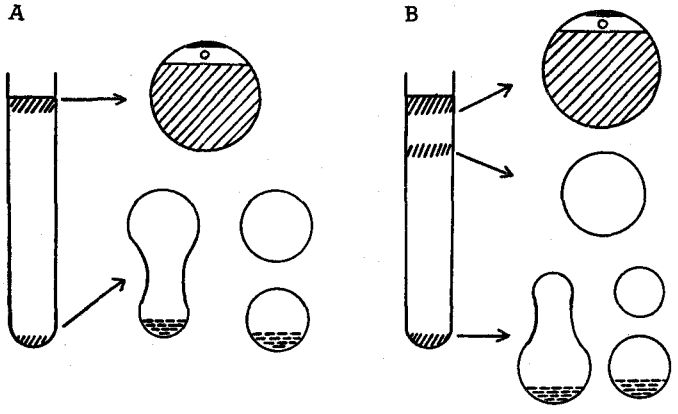
The giant nucleus was observed in the giant cell of the centrifuged embryo (PEASE 1939, MOTOMURA 1946, 1949). The mechanism causing the nuclear enlargement has been obscure. Thus, problems on this mechanism are as follows; (1) whether the enlarging of the nucleus dues to simple swelling by absorbing water or to increasing nuclear substances: (2) how it relates with the inhibition of the mitosis: (3) what cytoplasmic factors induce nuclear enlargement and whether this relates with cell differentiation, such as vegetalization or mesodermalization.

In order to analyse these problems the centrifuging experiments were carried out in the eggs of the sea urchin, *Hemicentrotus pulcherrimus*.

II. CENTRIFUGING OF THE UNFERTILIZED EGGS AND THEIR FRAGMENTATION

The materials used were the gamates of the sea urchin, *Hemicentrotus pulcherrimus*, collected at Asamushi, Aomori City. HERBST'S artificial sea water modified by MOTOMURA (1938) were used as physiological salt solution.

To stratify the cytoplasmic components in the whole eggs the unfertilized eggs were suspended in sea water which was placed on 1 M sucrose solution in the centrifuge tube and then centrifuged at 10500–12000×g for 20–25 minutes with the high-speed



Text-fig. 1. Fragmentation pattern of the unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus* when they were centrifuged in isotonic sucrose-sea water.

Table 1 Relative volume of the centrifuged fragments of the *Hemicentrotus pulcherrimus* eggs in per cent.

Fragment	A*		B*	
Centripetal	70.4		63.7	
Middle	18.2	29.6	20.2	
Centrifugal	11.7		6.0	16.1
			10.1	

* A and B correspond those in Fig. 1.

centrifuge (Marusan, Type 30–1). To produce the egg fragments, the unfertilized eggs were centrifuged in isotonic sucorse-sea water solution (mixture of 6 parts of 1 M sucrose solution and 4 parts of sea water). By centrifuging in this medium the egg were cut into two, three or four fragments (Text-fig. 1). When they were cut into three separate fractions in the centrifuge tube, the centripetal fraction, the lightest fraction, consisted of the centripetal orange frament, which contained the oil drops, the hyaloplasm, the female pronucleus and the yolk granules. The heavy centrifugal fragment, which contained the clear cytoplasm and the large granules, were sedimented at the bottom of the centrifuge tube. The middle fraction consisited of the clear egg fragments. The relative volume of each fragments calculated by measuring their diameter on the photographs are shown in Table 1. To observe the development, the centrifuged whole eggs and the egg fragments were returned to normal sea water and then fertilized after washing.

III. DVELOPMENT OF THE CENTRIFUGED EGGS AND FRAGMENTS

1. Fertilizabilities and cleavage activities of the egg fragments

The egg frament were inseminated in normal sea water, in which they were allowed to develop. The cleavage activities were conspicuously lower in the centrifugal and middle fragments than in the centripetal ones (Table 2). The developing

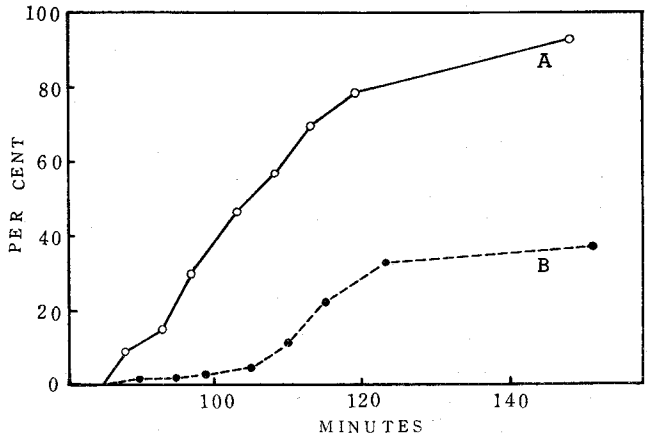
Table 2 Fertilizability and cleavage of the centrifuged egg fragments (*Hemicentrotus pulcherrimus*).

	Centripetal fragment	Centrifugal fragment
Formation of fertilization membrane* (%)	98	92
Cleavage** (%)	93	38

The unfertilized eggs were brokne into the fragments by centrifugal force (20 minutes at 10500 × g) in sucrose-sea water, and then inseminated in sea water.

* Observed 30 minutes after insemination.

** Observed 3 hours after insemination.



Text-fig. 2. Cleavage of the centripetal (A) and centrifugal (B) fragments which were cut by centrifuging in isotonic sucrose-sea water at 11000×g for 20 minutes and then inseminated in normal sea water. The ordinate shows the number of the cloven fragments in per cent and the abscissa the time in minute after insemination.

fragments were fixed with BAKER's formalin-calcium chloride solution after various intervals following insemination and the numbers of the cleaving fragments at each time were counted (Text-fig. 2).

The centrifugal fragments not only delayed in the first cleavage, but also low in the precentage of cleavage. The cleavage activities in the centrifugal and middle fragments are not attributed to decrease in fertilization, because the most of these fragments formed the fertilization membrane after insemination. The female pronucleus was contained only in the centripetal fragment. Therefore, the fertilized centrifugal or middle fragment contained a haploid nucleus derived from the spermatozoon. The low mitotic activity, however, can not be due to the haploidy, because the non-nuclear fragments cut with the glass pieces or needle were known to cleave normally after fertilization (cf. HARVY 1956).

2. Development of the centrifugal and middle fragments

The centrifugal and middle fragments were very low in developmental ability. The most of them could not divide, or stopped to divide after a few division even if they began to cleave. The cleavage blocking was more remarkable in the centrifugal than in the middle fragments. The fertilized fragments were fixed with BOUIN's solution 4 hours after insemination, sectioned with the usual paraffin method and then stained with HEIDENHAIN's hematoxylin. In the undivided centrifugal fragments the nuclei derived from the sperm nuclei were yet condensed and stained deeply with hematoxylin. The small asters developed around the nuclei, but the astral rays were short and small in number. In the fragments derived from the centrifugal end the nucleus was difficult to distinguish because it contained the basophilic minute cytoplasm. Frequently, the clear sphere lacking the basophila was observed in the central position of the fragment.

This sphere seems to correspond with the astral sphere in the other fragments and the normal egg.

3. Development of the centripetal fragments and the centrifuged whole eggs

The developmental pattern of the centripetal fragments was similar to that of the strongly centrifuged whole eggs. The first cleavage was nearly normal. The cleavage delay or blocking began to appear at the second or the third cleavage. When the first cleavage furrow was formed prependicularly to the centrifugal direction, the centripetal blastomere failed or delayed to cleave. If the first cleavage plane was formed in parallel with the centrifugal direction, the cleavage blocking began to appear at the third cleavage in the centripetal blastomeres. Thus, the centrifugal blastomeres divided into the many small cells, but the centripetal remained relatively large-sized (Plate I, Figs. 4–9). When the centrifugal force adopted was within an optimum range, the giant cells originated from the centripetal blastomere invaginated into the blastocoel, differentiating to the large mesenchyme cells (Plate I, Fig. 8; Cf. MOTOMURA 1946, 1949). But, it is obscure whether or not these cells are the same in function as the normal mesenchyme cells.

IV. GIANT NUCLEUS

1. Nuclear volume in the centrifuged embryo cells

The nucleus contained in the giant cells was also remarkably large in size. The normal gastrulae, 47 hours after insemination, and the centrifuged embryos, 24 and 44 hours after insemination, were fixed and sectioned. The cell nuclei were sketched with the ABBE's drawing apparatus. Nuclear size was measured on the sketches and the volume was calculated by regarding the nucleus as a sphere or an ellipsoid (Table 3).

The nuclei of the giant cells were remarkably larger than those of the other cells of the centrifuged embryos and the cells of the normal gastrulae. In the normal embryos, the nuclei in the cells of apical regions of the ectoderm and the archenteron wall and in the invaginated mesenchyme cells were considerably larger than in the other cells. These results suggest the presence of some relationship between cell differentiation and nuclear volume.

2. Cytochemistry of the giant nuclei

The giant nuclei contained a large quantity of the hematoxylin-stained substances. This shows that the enlargement of the nucleus is not due to simple swelling by absorbing water. To detect intra-nuclear substances, the FEULGEN method for DNA and the alkaline bromophenol blue staining for basic proteins were adopted.

The developed centripetal fragments and the centrifuged embryos were fixed with BOUIN'S solution or CARNOY'S alcohol-chloroform-acetic acid solution and sectioned with the praffin method. They were stained with the FEULGEN method after deparaffinization. The giant nuclei reacted as positively as the normal nuclei (Plate II. Figs. 13–15). The optical density of the FEULGEN-reacted nuclei was measured with the microphotometer which was constitued with the microscope and the photomicrographic

Table 3 Volume of nuclei in the sea urchin embyros, normal and centrifuged.

Embyro	Tissue	Nuclear volume (μ^3)
Normal gastrula I	Ectoderm, apical	14.1
	Ectoderm, lateral	7.2
	Endoderm, apical	20.4
	Endoderm, lateral	11.5
	Mesenchyme	14.1
Normal gastrula II	Ectoderm, apical	29.3
	Ectoderm, near-apcial	9.2
	Ectoderm, lateral	9.2
	Ectoderm, basical	11.5
	Endoderm, apical	38.8
	Endoderm, apical	14.1
	Endoderm, lateral	14.1
	Mesenchyme	24.3
Centrifuged embryo*	Giant cell	143.7
	Giant cell, mesenchymal	81.4
	Giant cell, mesenchymal	65.4
	Giant cell, mesenchymal	59.5
	Ectoderm	19.9
	Ectoderm	11.5
	Ectoderm	9.2

* The unfertilized eggs were centrifuged in isotonic sucrose-sea water for 20 minutes at 12000 $\times$g, and then inseminated and allowed to develop in normal sea water for 24 hours.

apparatus (Olympus PM III) combined with the multiplier photometer (Tokyo Photoelectric Laboratory) (Cf. OSANAI 1966). The relative content of nuclear DNA was shown with product of the optical density per one micron of thickness and the nuclear volume, $d/\mu \times v$ (Table 4). From this result it is clear that DNA content of the giant nucleus is remarkably more than that of the normal nuclei. This shows that in the giant cells the duplication of nuclear DNA proceeded normally though their cytokinesis was inhibited. Therefore, the enlargement of the nucleus in the giant cells is concluded to be attributed to the failure of separation or of segregation of chromosomal DNA, which seems to be synthesize normally.

As to the abnormal increase in DNA content the two explanations are possible: the endomitosis and the re-fusion of the incomplately separated nuclei. The multinuclear cells or the irregular-shaped nuclei, which were often observed in the giant cells, supports the later possibility (Plate II, Fig. 13). The former, however, is not completely given up because the giant cells contained the single spherical or ellipsoidal nucleus in many cases.

Generally, nuclear basic proteins increase in parallel with nuclear DNA multiplication. To determine whether this is the case in the giant nucleus or not, the centrifuged embryos, which were fixed with BOUIN's solution and sectioned with the

Table 4 Relative content of DNA (FEULGEN-positive substance) in the centrifuged sea urchin embryo nuclei

Nucleus	Optical density (d)	d/μ	Nuclear volume (v)	Relative content (vd/μ)
Giant	.168	.020	310 μ^3	3.20
Giant	.081	.014	176	2.46
Ectodermal	.051	.013	34	0.44
Ectodermal	.056	.013	40	0.52
Mesodermal	.046	.011	45	0.49
Mesodermal	.066	.021	26	0.53
Mesodermal	.092	.033	11	0.36

The centrifuged embryos were fixed with CARNOY's solution 44 hours after insemination.

paraffin method, were stained with 0.01 per cent bromophenol blue-0.035 M borate buffer solution (pH 8.3) after the pre-treatment with 5 per cent trichloroacetic acid solution at 95°C for 15 minutes (cf. RINGERTZ and ZETTERBERG 1966). The normal nuclei were clearly positive in the reaction, while the giant nuclei were negative or slightly positive. (Plate III, Figs. 18 and 19). This idicates that basic proteins in the giant nuclei were not multiplied in corresponding with DNA duplication, and that nuclear basic protein seems to regulate nuclear activity correlating with DNA synthesis.

3. Cytoplasmic factor inducing the enlargement of the nucleus

The enlargement of the nucleus in the giant cells seems to be induced by a cytoplasmic factor. To detect cytoplasmic differences among the cell, the sectioned embryos were stained with the periodic acid-SCHIFF reaction (PAS) method for polysaccharides and with toluidine blue for basophilic substances. The yolk granules were stained deep-red with the PAS method, but no differnce in their distribution was observed. On the other hand, the giant cells contained the coarse granules stainable with toluidine blue, while the centrifugal cells lacked these granules.

As reported previously, the mitotic changes of the sperm nuclei in the polyspermic eggs of the polychaete, were correlated inversely with the gradient of the toluidine blue-positive substances (OSANAI 1966). AGRELL (1958) pointed out that the sea urchin micromeres, which are low in mitotic activity, are highly active in RNA metabolism. These reports support the estimation that the toluidine blue-positive granules contained in the giant cells influence repressively on the mitotic activity. Therefore, it can be concluded that some cytoplasmic components, such as the toluidine blue-stainable granules, which block the separation of the nuclear substance and the cytokinesis, were distributed unequally by centrifuging, resulting the nuclear enlargement.

V. SUMMARY

1. To observe nucleo-cytoplasmic interactions concerning cell division and cell differentiation, the centrifuge experiments were carried out in the eggs of the sea

urchin, *Hemicentrotus pulcherrimus*.

2. When the unfertilized eggs were centrifuged in isotonic sucrose-sea water (6:4), they were cut into two, three or four fragments. The centrifugal and middle fragments which were fertilized in sea water were remarkably lower in cleavage activity than the centripetal fragments.

3. The centripetal fragments and the centrifuged whole eggs proceeded their cleavage. But, the division stopped or delayed in the centripetal blastomeres, which remained as the giant cells.

4. The nucleus was enlarged also in the giant cells. The nuclear enlargement is attributed to blocking the separation of nuclear DNA which multiplied normally.

5. The results obtained show that the nuclear and cytoplasmic divisions are depended on some cytoplasmic factors, which can be replaced with the centrifugal force.

REFERENCES

AGRELL, I. 1958 A cytoplasmic production of ribonucleic acid during the cell cycle of the micromeres in the sea urchin embryos. Arkiv Zool., **11**: 435–440.

HARVEY, E.B. 1932 The development of half and quater eggs of *Arbacia punctulata* and of strongly centrifuged whole eggs. Biol. Bull., **62**: 155–167.

———— 1940 A comparision of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. Ibid., **79**: 166–187.

———— 1946 Structure and development of the clear quater of the *Arbacia punctulata* egg. J. exp. Zool., **162**: 253–275.

———— 1956 The American Arbacia and Other Sea Urchin. Princeton Univ. Press., Princeton.

KOJIMA, M.K. 1959 Relation between the vitally stained granules and cleavage-activity in the sea urchin egg. Embryologia, **4**: 191–209.

MOTOMURA, I. 1938 Effect of some salt solution on the parthenogenetic membrane formation of sea urchin eggs. Sci. Rep. Tohoku Univ. Ser. IV (Biol.), **13**: 85–88.

———— 1946 Modification of the position of the embryonic axis in the ultra-centrifuged eggs of sea urchin. Seibutu **1**: 193–200. (In Japanese witn English résumé)

———— 1949 Artificial alternation of the embryonic axis in the centrifuged eggs of sea urchins. Sci. Rep. Tohoku Univ. Ser. IV (Biol.), **18**: 117–125.

OSANAI, K. 1966 Relation between the mitotic change of the sperm nuclei and ooplasmic distribution at maturation division in the eggs of the Japanese Palolo. Ibid., **32**: 219–228.

———— 1967a Promotion of development with periodic acid in the Japanese Palolo. Ibid., **33**: 121–128.

———— 1967b On the development-inhibitor in the eggs of the Japanese Palolo. Ibid., **33**: 163–174.

———— 1968 On the giant nucleus in the centrifuged embryo of the sea urchin. Jap. J. Dev. Biol., **22**: 37–38. (In Japanese)

PEASE, D.C. 1939 An analysis of the factors of bilateral determination in centrifuged echinoderm embryos. J. exp. Zool., **80**: 225–247.

EXPLANATION OF PLATES

Plate I

Figs. 1–3: Fertilization membrane formation of the centrifuged egg fragments (*Hemicentrotus pulcherrimus*). The unfertilized eggs were cut into the three frements by being centrifuged in isotonic sucrose-sea water (6:4) at 12000×g for 20 minutes and then inseminated in normal sea water. 30 minutes after insemination. Fig. 1, centripetal frements; Fig. 2, middle fragments; Fig. 3, centrifugal fragments. (×Ca. 100)

Figs. 4–9: Development of the centripetal fragments. The unfertilized eggs were centrifuged at 10000×g for 20 minutes in sucrose-sea water and then fertilized in normal sea water. Figs. 4 and 5, 4 hours 10 minutes after insemination. Figs. 6 and 7, 23 hours after insemination. Figs. 8 and 9, 48 hours after insemination. (×Ca. 400)

Plates II and III

Cytochemical reactions in the centrifuged embryos of *Hemicentrotus pulcherrimus*.

Plate II. The unfertilized eggs were centrifuged in sucrose-sea water at 11000×g for 20 minutes and the centripetal orange fragments were inseminated in normal sea water. They were fixed with BOUIN's solution 24 hours after insemination and sectioned with the paraffin method. (×Ca. 600)

Figs. 10–12: Stained with HEIDENHAIN's hematoxylin.

Figs. 13–15: Stained with the FEULGEN method for DNA (Fig. 14, fixed with CARNOY's solution).

Plate III. The unfertilized eggs were centrifuged in sucrose-sea water. The centripetal fragments were inseminated in normal sea water. They were fixed with BOUIN's solution 19 hours after insemination. (×Ca. 900)

Figs. 16 and 17: Stained with toluidine blue.

Figs. 18 and 19: Stained with alkaline bromophenol blue (pH 8.3) after pre-treatment with trichloroacetic acid.

Plate I

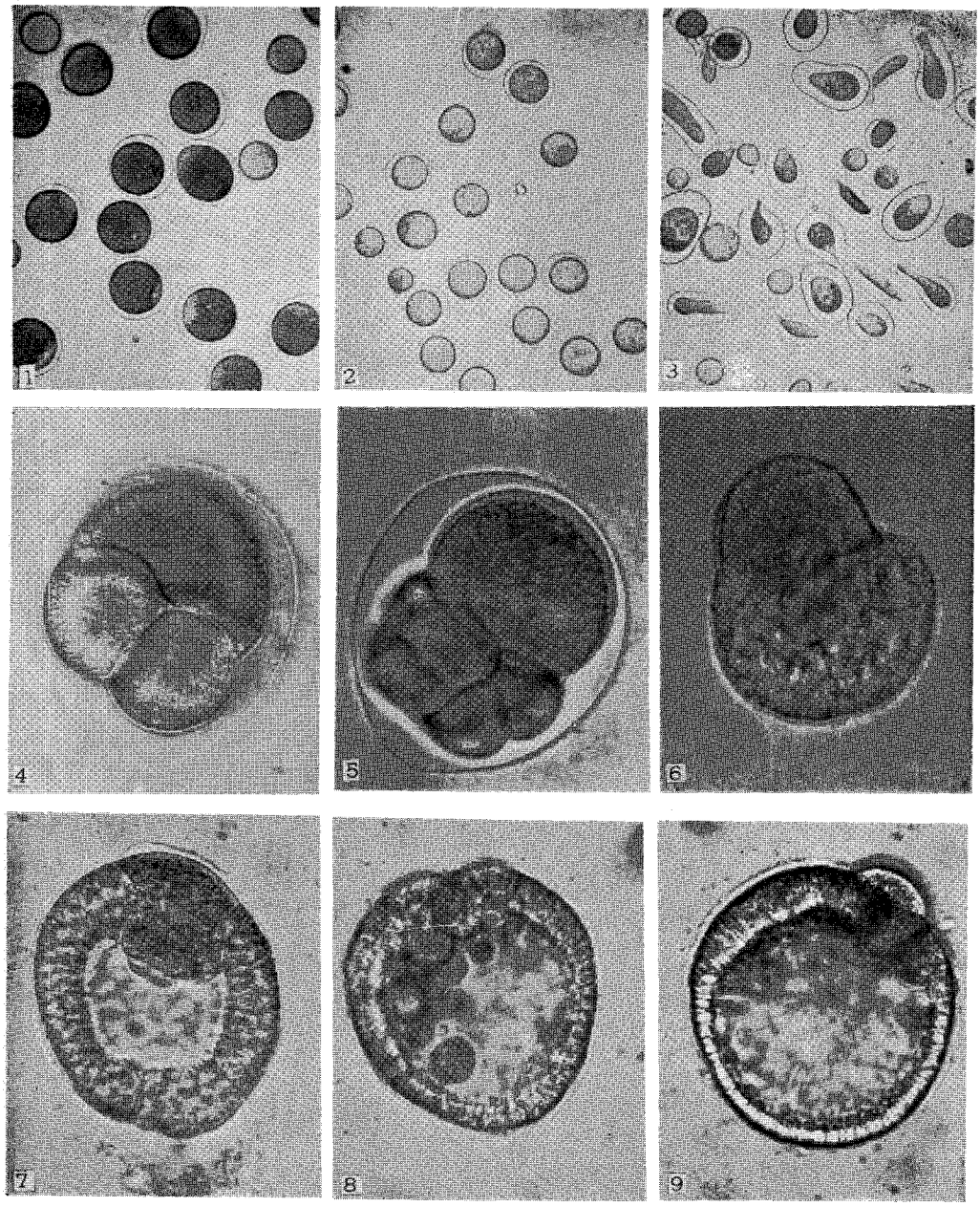
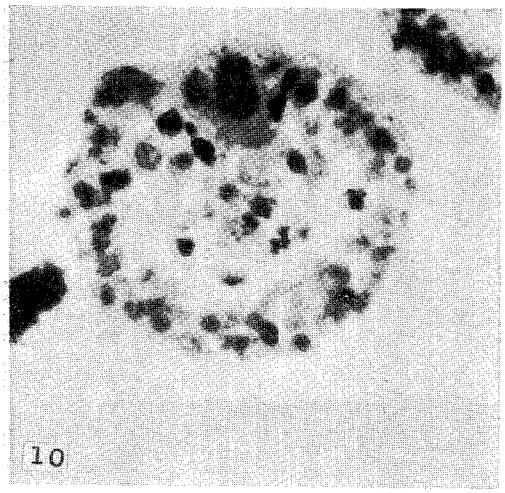
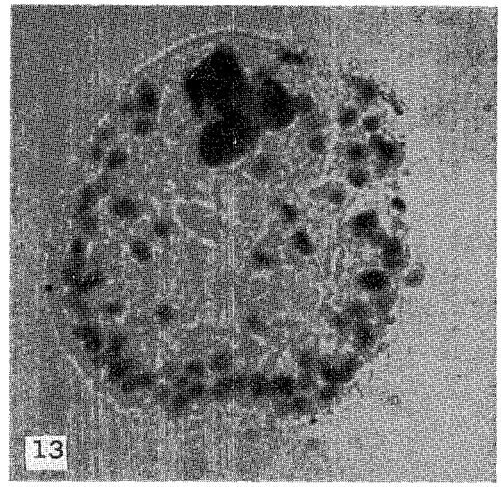
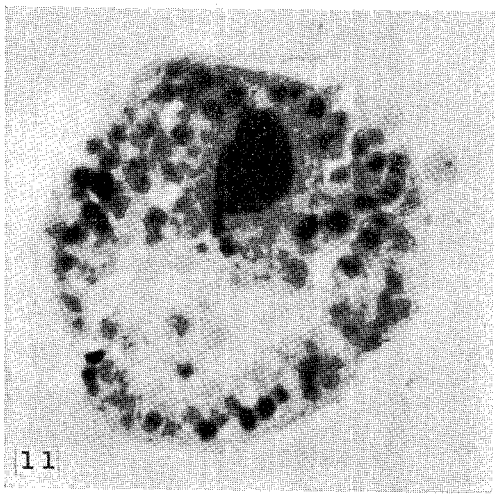
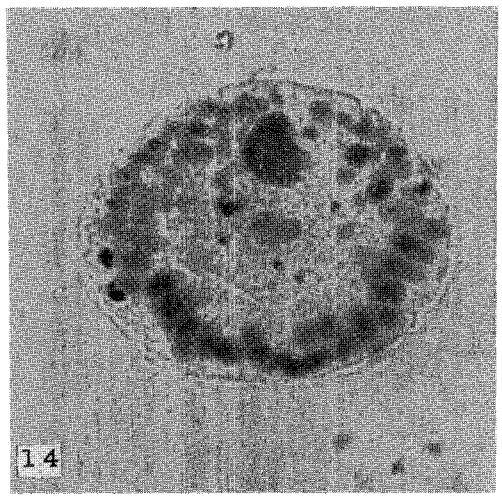


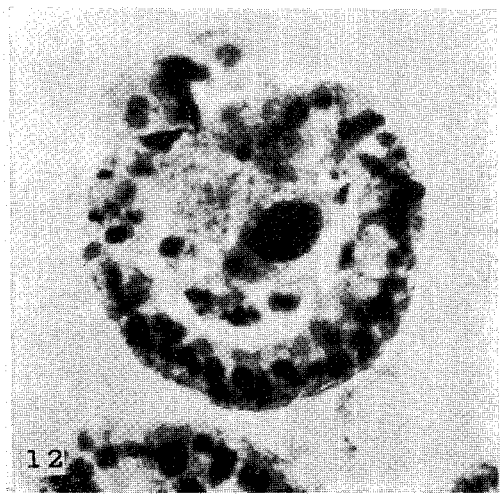
Plate II











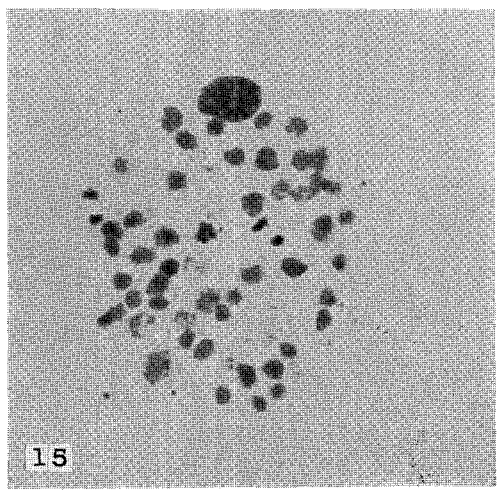


Plate III