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STUDIES ON THE MECHANISM OF CELL DIVISION (III). PENETRATION OF SPERMATOZOON INTO EGG INTERIOR, AND FUSION OF BOTH PRONUCLEI.

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ABSTRACT The unfertilized eggs of a sea urchin, *Hemicentrotus pulcherrimus*, were first immersed in hypotonic sea water, then, centrifugalized by an ordinary driven electric centrifuger. The eggs were remarkably stratified forming conspicuous clear hyaloplasmic layer. In the case of the sperm attachment at the site of this layer, the behavior of the spermatozoon in the process of invasion, and the course of penetration through the egg surface, were precisely observed.

To ascertain the mechanism of synkaryon (fusion of two pronuclei), the fertilized eggs in the various stages of development were exposed to sea water containing phenylurethan. In the exposure, the formation of the astral rays and spindle fibers were completely suppressed. Through the experiments, the behavior of two pronuclei in each period of the exposure, and the recovering process were observed. As the results, following conclusions were obtained.

Phenylurethan completely inhibits the formation of the astral rays which are radiating from the centrosome of the sperm head (the sperm head later swells into male pronucleus). Under these situations, the fusion of both pronuclei never took place as far as the writer's experiments were concerned. From the above facts, it seems clear, that the astral rays of the sperm pronucleus play an important role in the mechanism of the compromise and the fusion of two pronuclei.

In the eggs, exposing to phenylurethan beginning ten minutes after insemination, and transferred to sea water within 45 minutes, synkaryon took place about 30 minutes later. In this case, the mitotic apparatus was rapidly formed. Cleavage began almost at the same time in the control eggs. The rapid formation of the mitotic apparatus and synchronous cleavage of the recovering eggs are probably based upon the adaptation to the endoplasmic condition of the treated eggs, advanced regularly irrespective of the immersion of the reagent.

INTRODUCTION

Fertilization is defined ultimately to the fusion of a sperm nucleus (male pronucleus) of the

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Akita University

paternal origin with an egg nucleus (female pronucleus) of the maternal origin, to form a zygote nucleus (having double number of chromosomes) of the own species (Wilson, 1916).

Lillie found the sperm aggulutinating substances in sea water in which unfertilized eggs had been stocked, and developed his 'fertilization theory'. He was the first researcher on the establishment of mutual action between the sperm and the eggs. Since then, there have been many researchers studying on the mechanism of interactions between sperm and egg.

According to Monroy et al. (1965), the first process of fertilization involves three main physiological events: (a) the sperm-egg interactions before the establishment of any actual contact between the two gamates, (b) the interactions after the establishment of the physical contact, (c) egg reaction of the penetration of the spermatozoon. After the completion of the above processes, the second process (penetration of the spermatozoon into an egg, and the fusion of a sperm nucleus with an egg nucleus) continues successively.

The movement of the spermatozoon into the egg interior was thoroughly studies by Wilson. However, because of the invisible changes, the information available for the mechanism of synkaryon are still unsolved. In the writer's experiments, it will be worthy of special mention that the inhibition of the elongation of the astral rays by treating fertilized eggs with phenyl-urethan, affected the movement of each pronucleus. Concerning the problem, he would like to comment the behavior of the spermatozoon in the egg, arranging his works collectively.

The reviews (1993a, 1993b and 1994) consisting of three volumes, is mostly of his researches of his life on the expermental morphology, using eggs of some marine invertebrates (mainely, sea urchin eggs). To sum up his researches, it consist of six major parts: (1) Behavior of a spermatozoon at the time of fertilization-invasion and penetration-(Yamamoto, '90a and 94a): (2) Determination and formation of cleavage furrow (Yamamoto, '57, '64a, '65b, '72b, '75a, '89a, '93a and '93b): (3) Modification of meiotic spindle to mitotic spindle for inducing cell division (Yamamoto, '65c, '72a, '74a and '80): (4) X-ray and electron irradiation-60MeV-during gametogenesis (Yamamoto, '58, '60, '68a, '68b, '69b and '69c): (5) Anatomy of some duterostomia (Yamamoto, '85, '89b, '90b, '91b, '91c and '94b): (6) Notes concerning the correlationship between shape and function in the living organisms (Yamamoto, '71a, '76, '81, '91a and '92b).

When the writer set to research after graduating from the university, Harvey (1951) had established his beautiful configurations of the stratified eggs of sea urchin, *Arbacia punctulata*, to investigate the mechanism of cell division. These experiments were performed by using sufficiently high speed electric centrifuger (about 10,000 xg). This was an effective means to analyze the nature of the egg organelles.

In those days, even a high speed electric centrifuger was not equipped in the writer's laboratory, because of the scantiness coming from deficiency due to the defect of his country (World War II). At that time, he was engaging in works to ascertain the evidence of the mitotic apparatus whether it was rigid or not. The mitotic apparatus (spindle) was hardly to move by the force through an ordinary driven electric centrifuger equipped in his laboratory (about 3,500 r.p.m.). He searched for other techniques to move and to segregate major organelles present in the egg itself. After reading 'Cell biology' (Heilbrunn, 1952), he came to the technical skill to move the mitotic apparatus by centrifugation, when the fertilized eggs, previously immersed to hypotonic sea water for a short period of time, were centrifugalized. Using this method, he

has been able to carry out a series of experiments, to ascertain the mechanism of cell division. This foundation led him to be initiated to his works, described below.

Continuing previous papers (Yamamoto, 1993a and 1993b), the writer finally would like to consolidate his ideas in a review, concerning the behavior of a spermatozoon at the time of penetration, and the process of copulation (nuclear fusion) of male and female pronuclei as an initiating process of cleavage.

ACKNOWLEDGEMENT

Most of his works were performed at the Marine Biological Station of Asamushi, Tôhoku University, 1957-1994. Therefore, before going further, the writer would like to express his sincere appreciation to all the members of the Station. The writer's gratitude is also offered to all the colleages of the Akita University for their courtesy.

Upon retirement from the University, the writer would like to express his cordial thanks to his late father, mother and sisters, and Clinton M. Anderson, his brother-in-law. Their sudden passing caused him much grief and pain. He would like to extend his deepest apologies to those close to him at the time of his mourning, to whom he might have caused some annoyance.

He also expresses his sincere appreciation to existing family members, particulary to Mr. & Mrs. Takehiko Yamamoto (his brother) and Mrs. Masao Honma (Mari Akagi-his niece), for their encouragement, kind cooperation and manuscript.

The three volumes containing this report (1993a, 1993b and 1994) are didicated to his wife, Hiroko Yamamoto, in appreciation of her great support during the course of his career.

RESULTS AND DISCUSSION

1. PROCESS OF SPERM PENETRATION INTO EGG

The purpose of fertilization is ultimatery the fusion of the male and the female pronuclei to form single nucleus of new indivisual. Its process consists of three major phenomena: Recognition and attachment of a spermatozoon onto egg surface (Epel, 1980); Sperm invasion and penetration through egg surface (photographed evidently by Schatten and Mazia, 1957); Fusion of male and female pronuclei. In the following of this report, the behavior of the spermatozoon in penetration, and the mechanism of attraction between both pronuclei, will be discussed.

Owing to the difficulties of the detection of the spermatozoon inside the egg (after penetration), the writer obtained an extraordinary stratified eggs having clear transparent cytoplasmic layer, of which unfertilized eggs were centrifugalized after treatment of hypotonic sea water (Yamamoto, 1964 and 1988). After insemination, the point of the sperm attachment and the process of invasion were clearly observed. The most remarkable phenomena were the rapid breakdown of the cortical granules, taking place on the egg surface, to elevate fertilization

Akita University

membrane (Fig. 1). The role and the detailed structure of these granules for the elevation of fertilization membrane, have been elucidated up to the present (Takashima, 1960 ; Schroeder, 1979 and many researchers). Generally, the cortical granules are hardly recognizable in the living unfertilized eggs, however, in the writer's present experiment, he was able to ascertain the direct collupse of these granules, forming fertilization membrane (Fig. 1, E and F). Namely, the rapid collupse of these granules was easily recognized using writer's technique of hypotonic pretreatment. It took place from a point of the sperm attachment, propagating in all over the directions to the whole surface of the egg.

At the initiation of sperm invasion, a tiny cone-like protrusion was formed at the point of the sperm attachment.. This is the fertilization cone designated by Runströme (1963). Recently, the formation of fertilization cone is considered to establish by the cluster of the elongated microvilli on the egg surface, around the sperm attaching microvillus (Schatten, 1983 and Trimmer, 1986). At the time of the sperm entrance, the deformation of the egg contour into flat form was observed around the sperm penetrating point, however, it soon recovered into the original form. According to Hamaguchi and Hiramoto (1980), the cytoplasmic movement is carried by the contraction and expansion of the cortex which start at the point of the sperm entrance. Then, it propagates toward the opposit side of the egg. In the writer's case, the fertilization cone was clearly recognized as compared to the normally fertilized egg. Longo (1986) also suggested, that the membrane for the fertilization cone in *Albacia* eggs came from the surrounding microvilli, arranged on the surface of the egg as Schatten and others pointed out.

Within a short period of time (about 90 to 150 seconds after insemination), the head of the spermatozoon began to be swallowed toward the egg interior. The duration of the spermatozoon in penetration through egg cortex, took comparably long time about two to three minutes. This will be due to the rigidity of the egg cortex, encircling just beneath the egg surface. During the penetration, the egg cortex, at the point of sperm attachment, began to be retracted in a form of conical depression (Fig. 1, D). The writer, in the previous paper (1990a), calculated the thickness of the egg cortex as less than 4 μm or so, using the preparations of the centrifugalized eggs stained with Lillies PAS method. Concerning detailed description of this value, see next chapter. Hiramoto (1957), by using the micrurugical technique, measured that it was about 3 μm, and got thicker to about 4 μm with the progress of development. Consequently, it can be understand, that the spermatozoon at the time of penetration, takes such a long period of time to pass through the stiff cortex of gelated constitution.

According to Dawid and Blackler (1972), and Giles et al. (1980), the sperm nucleus and its centrioles first separate from the mitochondria and the flagelum of the spermatozoon. However, the writer could not ascertain its separation owing to such a small entities of the spermatozoon in the case of the living condition. The sperm head is considered to move by cytoplasmic streaming, taking place in the start of an activation, as well as the sperm tail attaching (Hamaguchi and Hiramoto, 1980). The sperm head, after its complete engulfment of the spermatozoon into the egg, was generally known to turn about 180° degree facing to the egg cortex, with the centrosome in front (Dan, 1950 ; Flemming, 1881). While the sperm head moves toward the egg nucleus, it little by little, changes into the male pronucleus. Soon the sperm head

Akita University

begins to swell into the pronucleus at the place where it complets penetration. The male pronucleus, which was easily able to discern, began to approach toward the direction to the position of the female pronucleus. According to the text book of development written by Gilbert et al. (1991), the envelope of the male pronucleus is formed through very complicated processes of the membrane alterations.

In the amphibian egg, the path of the sperm invasion is at first straight, but soon changes its course toward the direction of the position of the female pronucleus. In the text book of amphibian development (Rugh, 1951), the newly changed path was designated as copulation path. In the following chapters, the writer would like to review the mechanism of approaching and union (synkaryon) of the male pronucleus to the female one.

2. THICKNESS OF CORTICAL CYTOPLASM (SEA URCHIN EGG)

The cortical granules found in the superficial cytoplasm of sea urchin egg, have a imovable character to resist outer moving force. Consequentry, the gelated constitution of this cytoplasm (constituting outermost layer of egg) has been supposed, and this was designated as the cortical cytoplasm or the egg cortex up to the present. Marsland (1970) found out that this layer could be displaced under the treatment of strong centrifugation, when the eggs of sea urchin, *Arbacia pustrosa*, were exposed to strong hydrostatic pressure before centrifugation. This fact clearly shows that the cortical cytoplasm is constituted usually in a gel, and is capable of turning into a sol state under exposing the eggs to several conditions. Motomura (1954) established a method of maceration of the cortical granules in the living sea urchin eggs as well as the fixed materials. In this experiment, he first exposed unfertilized sea urchin eggs to an acid solution (N/10 to N/100 HCl) for 40 minutes. In this condition, instead of disintegration of the cortical cytoplasm, the granules could be observed without inflamation. As described before, the writer recognized the cortical granules, embedding in the gelated layer of the cortex in the centrifugalized unfertilized eggs of sea urchin, *Hemicentrotus pulcherrimus* (Fig. 1, C).

In order to ascertain the structure and the nature of the gelated cortical cytoplasm (cortex) during the process of development, the writer fixed the centrifugalized eggs (after hypotonic treatment), at various stages of development. He stained them with Lillies PAS staining method. As it has been well known, that the cortical granules are composed of mucopolyssacharide, they must be well stained by PAS method. The cortical cytoplasm can easily be recognized because of the coexistence of the cortical granules. Namely, the existence of the cortical cytoplasm can be distinguished as the negatively stained whitish colored layer containing numerous granules. In the staining, the sectioned preparations were previously oxidized with Periodic Acid solution (0.8 gr of $Na_2H_3IO_6$ in 100 dl of 70% HNO_3), then, transffered to Schiff's reagent. The stained preparations were immersed with Hydrochloric Acid, then rinsed. As the result, the cortical cytoplasm was confirmed as a thin sheet of cytoplasm, encircling outermost layer of the whole eggs (Fig. 1, I). Its thickness mesured was about 4 μm or so in the most stages of development with the exception of the furrowing stage. At the time of cleavage, the cortical cytoplasm became undistinguishable particulary in the region of furrowing. Marsland (1970) postulated that, at the beginning of furrowing, the cortical gel layer began to

Akita University

deform and contracted the eggs to be elongated, in the direction of an axis of spindle. Consequently, the diminution of the cortical cytoplasm may be due to qualititative change ocurring during the process of furrowing.

Generally, the cells tend to shrink considerably during the course of fixation, therefore, direct mesurement of the thickness of the cortex in the stained preparations connot show real value of cellular constituents. For this reason, the thickness of the cortical cytoplasm was calculated by using the proportion, (diameter of the fixed egg/diameter of living egg). In this manner, the proportion of the thickness (cortex) was estimated about one twenty second of egg diameter, so, the thickness was determined approximately as 4–4.5 μm. The presence of the cortical cytoplasm of eggs under hypotonic treatment, was distinguished by the strong contrast to darkly stained yolk granules full in endoplasm. However, the reason of the prominence of the cortex of above mentioned eggs comparing with the control eggs is not certain, whether it was produced by the change of cytoplasmic qualities by water absorption.

Running parallel with investigation, the behavior of the endoplasmic granules (PAS positive granules) during the course of cleavage was observed. The role of the endoplasmic granules in the period of furrowing is unfortunately obscure as far as the writer's awareness, but an assembly of these granules on the equatorial plane at telophase, must be important for the analysis of the mechnism of cell division (Fig. 1, J). The interested readers would like to reffer to the writer's previous papers (Yamamoto, 1964 and 1988).

3. ENDOPLASMIC DEVELOPMENT WITH THE ADVANCEMENT OF CELL DIVISION

The process of the cleavage is closely associated in connection with the function of mitotic apparatus. By using centrifugation accompanying with pretreatment of hypotonic sea water, the writer could easily shift the mitotic apparatus (mainly, spindle) at metaphase toward centripetal direction. In this condition, most of the mitotic apparatus were settled in parallel with the endoplasmic stratification (Fig. 2, A–C). In this condition, the egg cleft in a plane, perpendicular to an axis of the displaced spindle. When the centrifugation was applied after anaphase, the furrow was, in most cases, formed at the original position where the mitotic apparatus had settled (Fig. 2, D and E). Considering the facts, it seems clear that the mitotic apparatus is at least necessary for an early half of cell division. And, when passing through critical stage, a potentiality of contraction for furrowing is established on the superficial cytoplasm of the cortex (prospective girdle for the cleavage).

By application of the above experiment, when the eggs of a bivalve, *Caecella chinensis*, was centrifugalized before the extrusion of first polar body, the mitotic apparatus was displaced as a whole, toward the egg interior (Yamamoto, 1965). In this condition, the furrow was formed across the middle of the displaced spindle, dividing the egg into two equal blastomeres (almost the similar size of 1st cleavage) (Fig. 2, H and I). From the facts, it is certainly a reasonable hypothesis, that conversion of the minute meiotic spindle into the larger mitotic spindle for cell division, seems to be controlled by encircling endpolasmic conditions. The meiotic and the mitotic spindle may be determined in the position where it is settled on the region in egg surface or in egg interior (endoplasm).

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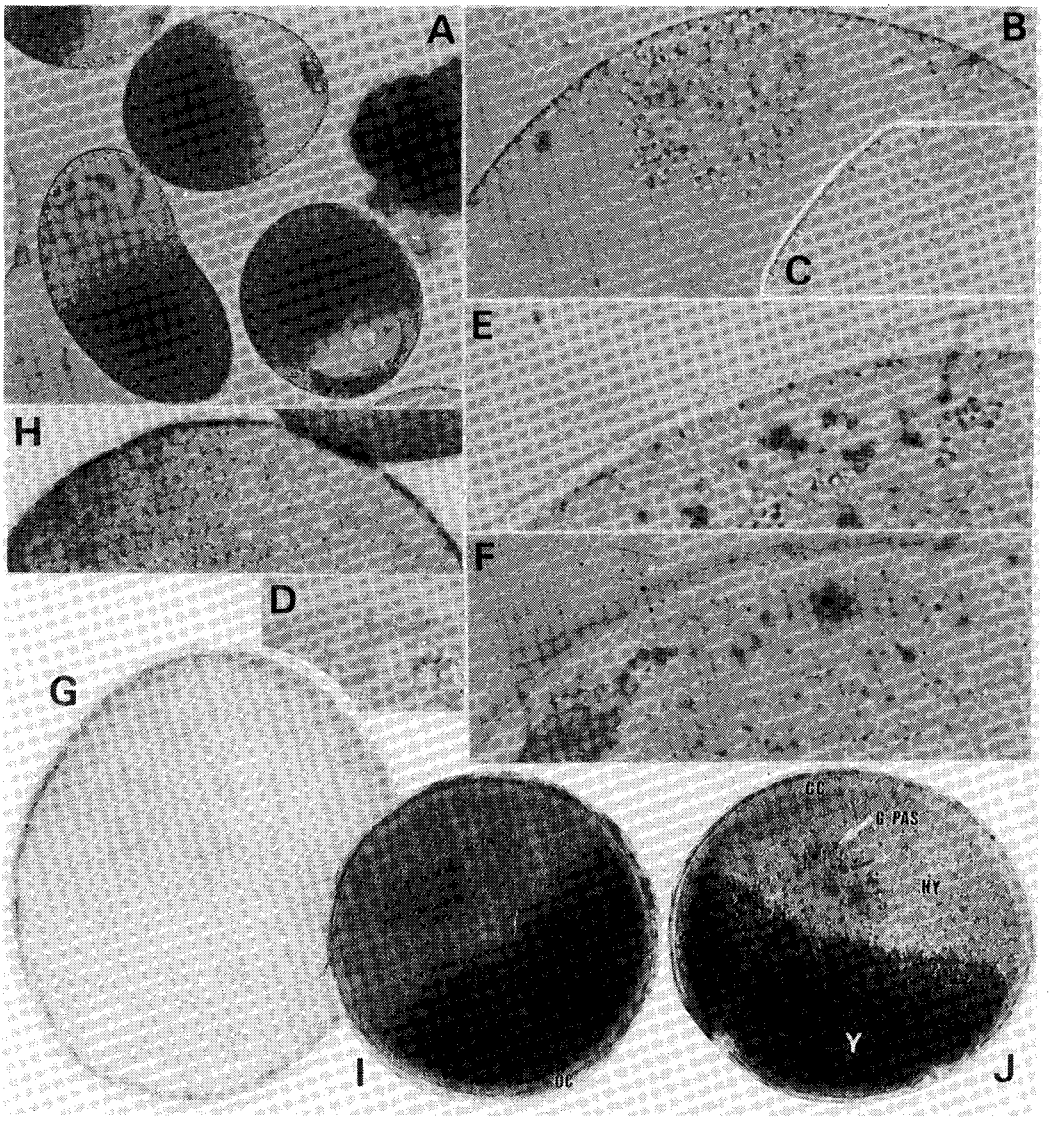

Fig. 1 Photomicrographs showing effects of centrifugal force on sea urchin eggs. The eggs were immersed to hypotonic sea water before centrifugation. A : Clearly stratified hyaloplasmic layer. B : High magnification of centripetal portion of the egg showing cortical granules. C : The same as B. Female pronucleus is clearly observable. D : Invasion of a spermatozoon into hyaloplasmic layer showing depression of cortical cytoplasmic layer, at a point of sperm invasion. E and F : Process of elevation of fertilization membrane, during breakdown and extinction of cortical granules (E and F) just after fertilization. G : Sectioned and stained preparation (Heidenhein's iron hematoxylin), showing a layer of cortical granules on egg surface. Egg was sectioned through hyaloplasmic layer in a plane in parallel with the stratification. I and J : Stained preparation of centrifugalized egg during development toward 1st cleavage. PAS stained granules are seen around mitotic apparatus. Note, cortical cytoplasm is evidently found as a thin faintly stained layer around egg periphery.

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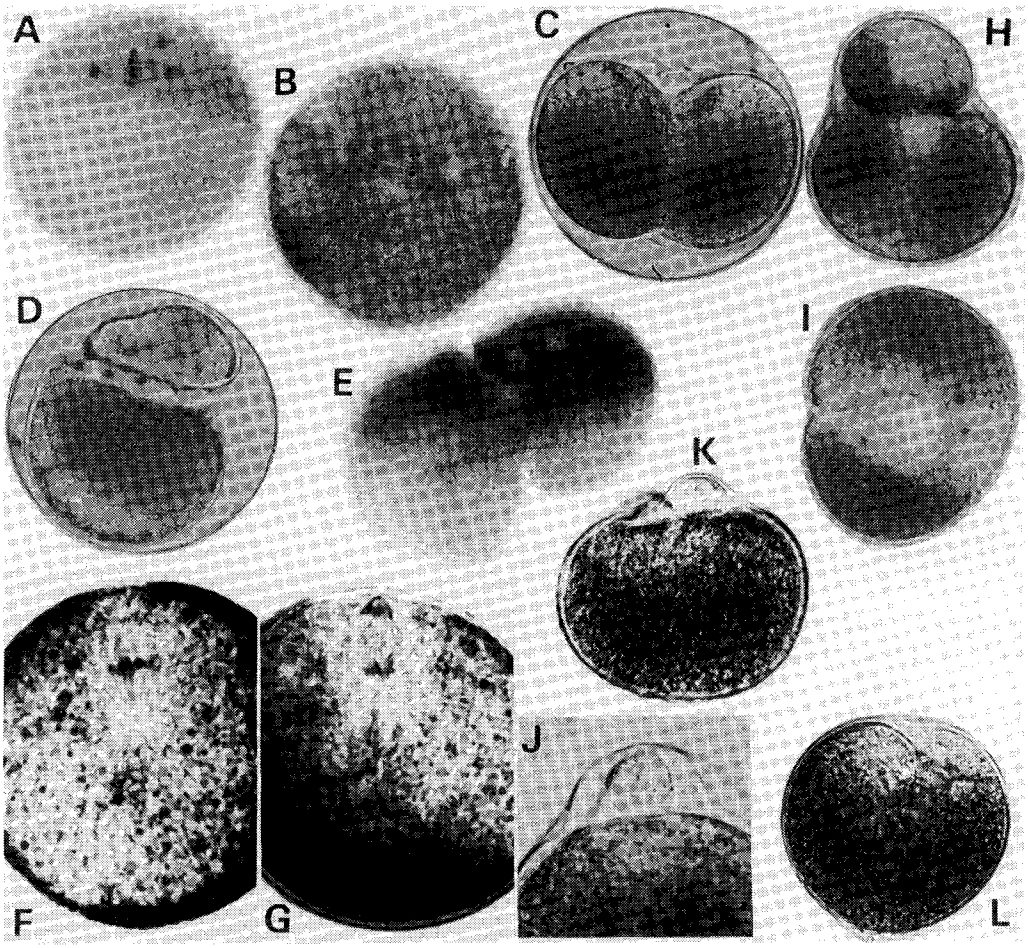

Fig. 2 Photomicrographs showing effects of centrifugal force on fertilized sea urchin eggs. The eggs were treated with hypotonic sea water before centrifugation. A–C : Successive changes of development, centrifugalized before metaphase. The mitotic apparatus was easily displaced toward hyaloplasmic layer. The axis of spindle lies in parallel with stratification. D and E : Cleavage without relation to final position of mitotic apparatus. Eggs were centrifugalized after critical stage of inducing furrow (beyond anaphase). F–I : Photomicrographs showing formation of giant polar body and induction of cleavage of the eggs of *Caecella chinensis*. F : Enlarged meiotic spindle of *Caecella* egg treated with DNP. G : Control. J–L : Giant polar body formation on above mentioned eggs. H and I : Induction of cleavage by meiotic spindle. Fertilized eggs were centrifugalized at stages just after germinal vesicle breakdown.

The endoplasmic change (qualititative), taking place during the course of development, has been postulated by many researchers. Kojima (1961 and 1962) obtained an evidence of cyclic change of the endoplasmic development by the analysis of a thickness of a hyaline layer, and a reformation of the sperm asters at every stages of development in the activated sea urchin eggs. For the establishment of the mitotic apparatus in accordance with the endoplasmic development, the writer ascertained recovering process of the mitotic apparatus (disintegrated or blocked), fixing his eyes upon the encircling circumstance of the endoplasmic conditions (mainely, aging). The drug used was phenylurethan, known to have specific action to the formation of aster by an inhibition of astral ray elongation of tubuline molecules (Yamamoto,

Akita University

1967). The fertilized eggs of a sea urchin, *Hemicentrotus pulcherrimus* and *Strongylocentrotus nudus*, were exposed to sea water containing phenylurethan (0.0001-0.00001 per cent) at various developmental stages for various periods of time, then, part of the treated eggs was transferred to ordinary sea water. In this manner, the processes of disintegration and recovery of astral rays and the mitotic apparatus were ascertained.

Continuous exposure to low concentration of phenylurethan ten minutes after fertilization was as follows: Within 20 minutes, it appeared a sperm nucleus as a pale small sphere (Fig. 5, B), then, within 60 minutes, it swelled up till almost the equal size as the female pronucleus (Fig. 5, D) (but, slightly smaller). However, the fusion of both pronuclei was never recognized. With the progress of development, each pronucleus became obscure in contour showing the entrance into metaphase (Fig. 5, E). It stood still in that condition for several minutes of time. Ultimately, one or both of the pronuclei divided into two or several small spheres (Fig. 5, F-H) (micronuclei). From sectioned preparations, it seems quite evident, that the small spheres were formed by one or few dispersed telophase chromosomes by the absence of the mitotic apparatus (karyokinesis without chromosome movement) (Fig. 7, F). Interestingly, in these eggs, a rhythmic deformation of the egg contour was recognized in the certain stage, almost at the half way of the course of each cleavage in the control eggs. The deformation restored gradually to an original spherical egg form with the advace of development, before the appearance of the furrow. Therefore, some intrinsic changes of the endoplasmic development seem to proceed with the advancement of nuclear development. The writer's present findings are, in the most parts, almost the same as Kojima's conclusion stated before. Accordingly, the writer suggested the reality of the endoplasmic change, independently to proceed during development. The appearance of the mitotic apparatus is probably controlled as being adapted to the aging or development of the endoplasmic condition.

In the next experiment, the fertilized eggs ten minutes after insemination were exposed to phenylurethan for 7.5, 15, 30, 45 and 60 minutes, then, put back to ordinary sea water. The time required for cleavage was 83, 78, 68, 57 and 60 minutes, respectively (control egg, 87 minutes). In the case of the transfer after the immersion within 45 minutes, the eggs, after accomplishment of synkaryon, continued development as shown in the figure (Fig. 6, M-N). The initiation of cleavage of the treated eggs within 60 minutes treatment, was almost the same time as the initiation of the cleavage of nontreated eggs (control egg). The time required for synkaryon was about 30 minutes in the shortest, then, the eggs entered into a course of the cleavage. As seen in the experiment, the process of the formation of the mitotic apparatus was hastend considerably despite of duration of the treatment. As shown in the figure (Fig. 6, O), the formation of the mitotic apparatus after the completion of synkaryon, evidently hastened correlating to the rhythmic cycle of an endoplasmic development. Accordingly, this fact suggests an existence of the range of the period of recovery, that is capable for an achievement of synkaryon.

In the case of the transfer once beyond the critical stage (more than 45 minutes), almost all of the eggs failed to accomplish synkaryon (Fig. 5, I-N). After raising full width of delay, they continued subsequent cleavage (irregular). When the fertilized eggs were transferred to the ordinary sea water, after the treatment for more than 80 minutes, these eggs kept still in that

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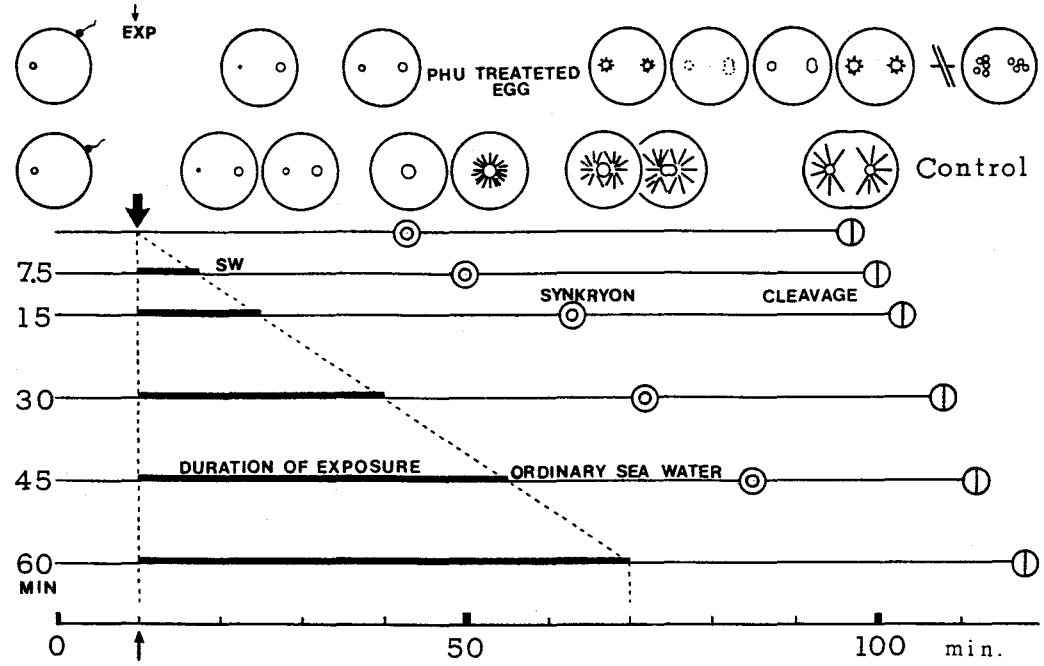

Fig. 3 Diagram showing onset of first cleavage. Eggs ten minutes after fertilization were immersed in 0.0001 per cent phenylurethan sea water for different periods of time, then returned to sea water. ◎ : Fusion of male and female pronuclei. ⦶ : Onset of cleavage. Uppermost drawings schematically illustrate the changes of eggs, continuously exposed to low concentration of the reagent.

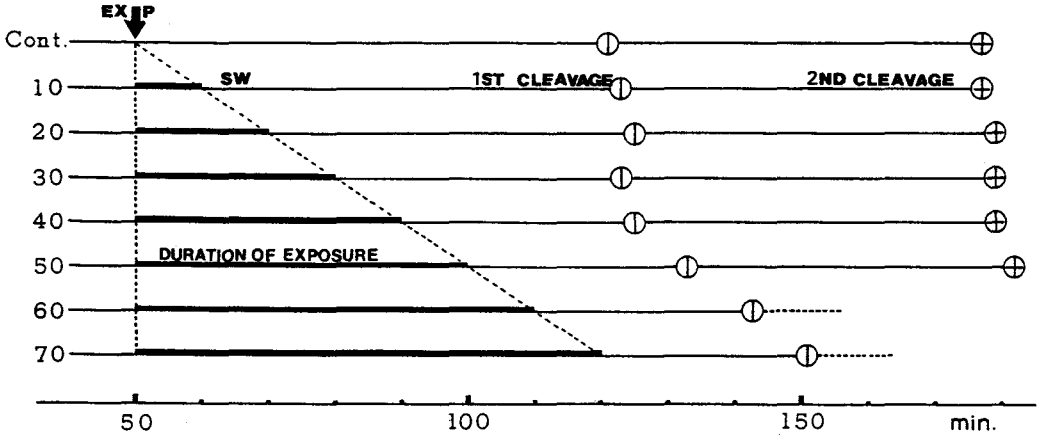

Fig. 4 Diagram showing onset of first and second cleavage. Eggs fifty minutes after fertilization were exposed to 0.0001 per cent phenylurethan sea water for different periods of time, as shown in diagram. Then, they were returned to sea water. ⦶ : Onset of first cleavage. ⊕ : Onset of second cleavage.

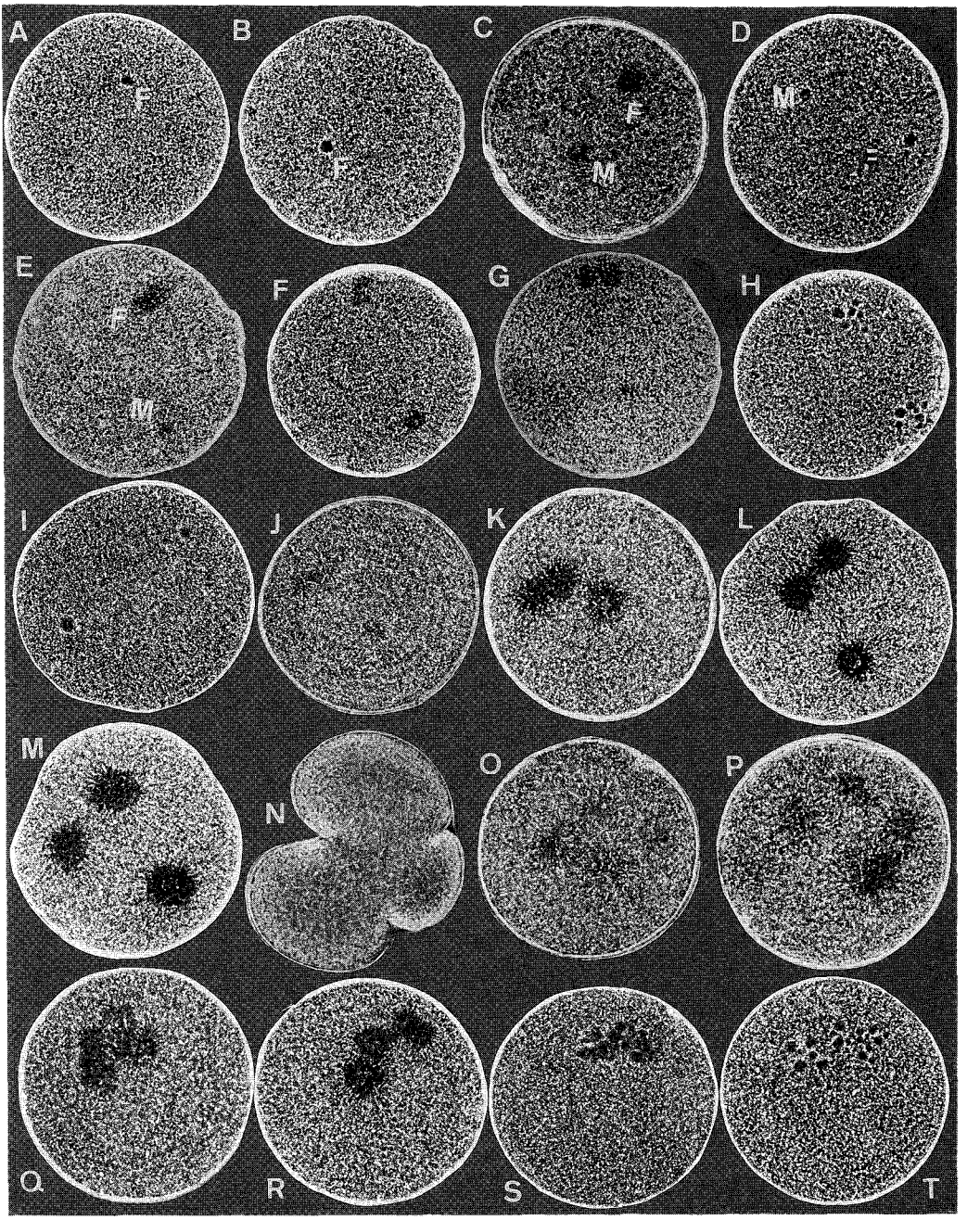

Fig. 5 Phase contrast micrographs (negative high) showing effects of phenylurethan on development of sea urchin eggs. Changes of the eggs exposed continuously to low concentration of reagent. A : Egg just before exposure. B–H : Successive changes of eggs showing inhibition of synkaryon and advancement of nuclear division, without forming definite mitotic apparatus. Periods of time since exposure was begun were : B, 15 ; C, 60 ; D, 30 ; E, 70 ; F and G, 80 ; H, 140 minutes, respectively. I–N : Recovering process of exposed eggs after transfer to sea water. Eggs ten minutes after fertilization were exposed to 0.0001 per cent phenylurethan sea water for 60 minutes, then returened to sea water. I : Egg just before transfer. J–M : Successive changes showing recovering processes of nuclear division without synkaryon. N : Multicleavage (irregular cleavage). O–T : Process of recovery with longer treatments, then transferred to the sea water. Note, nuclear division without presence of mitotic apparatus.

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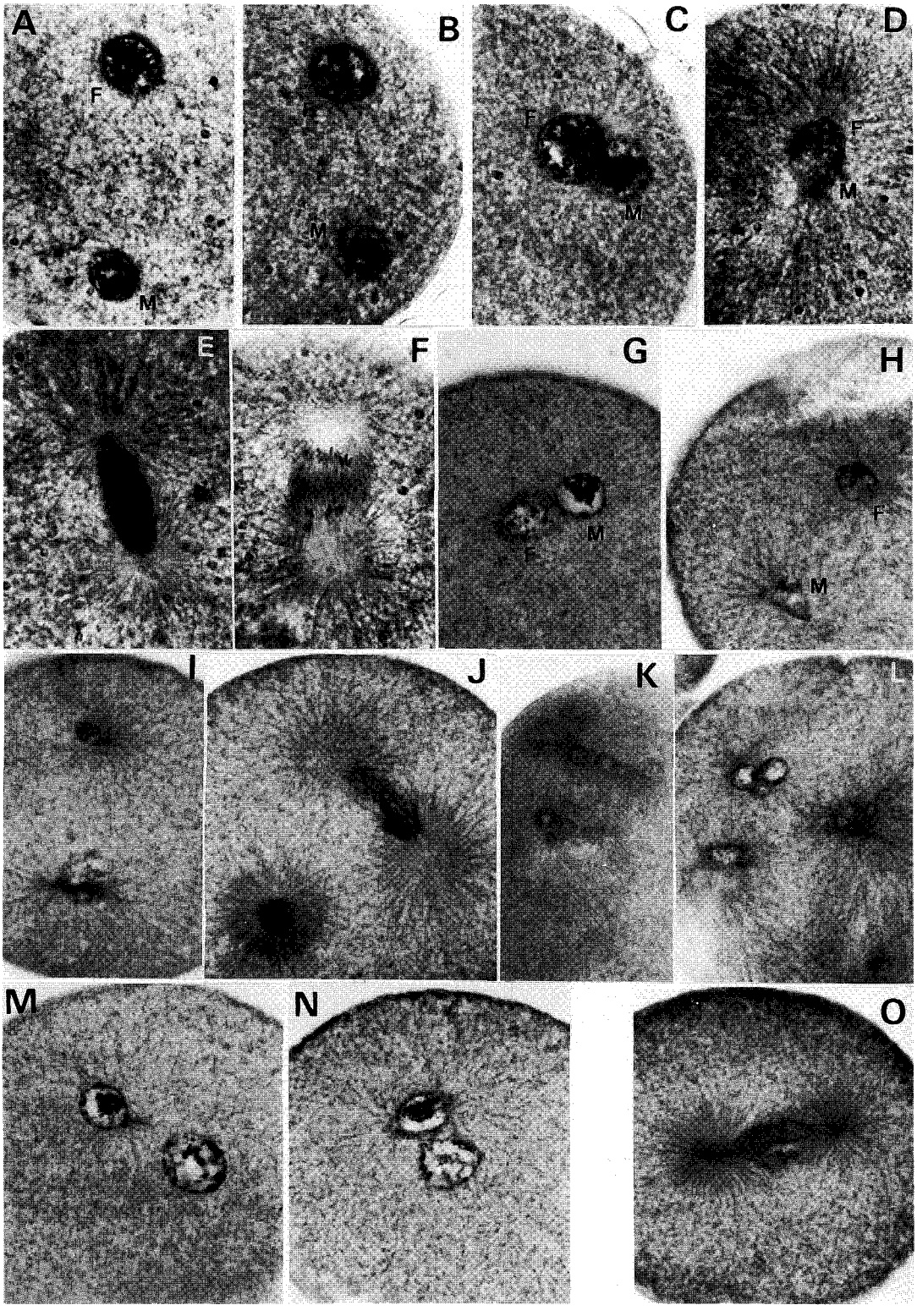

condition for about 30 minutes or so without proceeding any sign of developmental change. Subsequently, there appeared small asters around each of the male and the female pronucleus (Fig. 6, H-J). Above all, they (especially, the female pronucleus) began to divide after the completion of the small size of the mitotic apparatus (this means an occurrence of nuclear division). The initiation of cleavage, though much or less irregular in form, took place nearly at the same time as second cleavage of the control eggs (sometimes, multicleavage). In the case of exposure beginning just before 1st cleavage, neither delayed nor blocked the 1st cleavage. Continuing the exposure for more, there appeared a mass of micronuclei consisiting of several vesicular spheres, in the central part of each blastomere (Fig. 6, K and L).

In all our discussions thus far, an attention has been paid to rhythmic chang of the endoplasm, which exerts profound affection to the progress of nuclear change toward cell division (of course, involving mitotic apparatus). Osanai (1964), by an observations of partial activated eggs pretreated by acetone, pointed out that the mitotic apparatus (involving chromosomes, centrosomes, astral rays and so on), were appropriatery formed in response to the endoplasmic advance in the degree of an activation. The principal parts of his conclusion, though involving slight disacord, coincide with the writer's synopsis introduced by the before mentioned experiments. Collectively speaking, it may suggest the presence of the development of the endoplasm itself in the course of cell division. For preference, it may rather be considered that the process of the nuclear development may be introduced with response to the endoplasmic development, progressing rhythmically from start of an activation despite of the exposure to the reagent.

4. FUSION OF MALE PRONUCLEUS TO FEMALE ONE

According to Rugh (1951), the spermatozoon in the process of invasion, is at first rather straight (penetration path), indicating a certain directional force on the penetrating spermatozoon. The middle piece of the spermatozoon containing centrosome enters the egg behind attaching to the sperm head. As this head swells and loses its identity (becomming into male pronucleus), the associated centrosome divides. The separating centrosomes establish an axis at right angle to the penetrating direction, however, the sperm penetration path will not lead the sperm pronucleus, directly to the final position toward the position of the female pronucleus. This new direction is designated as 'the copulation' path by Rugh. Meanwhile, the two pronuclei meet. The sperm centrosomes are then well separated, and finally induce both centers

Fig. 6 Photomicrographs showing effects of phenylurethan on fertilized sea urchin eggs. Recovering processes of the eggs after transfer to sea water. Eggs ten minutes after fertilization were exposed to 0.0001 per cent phenylurethan sea water for 45 minutes, then returned to sea water. Sections were made ten micra in thickness and stained with Heidenhein's iron hematoxylin. A-C: Successive changes showing process of synkaryon. D-F: Advancement of nuclear division. H-L: Recovering processes of treated eggs after transfer to sea water. Eggs ten minutes after fertilization were exposed to the reagent for 90 (G-I) and 120 (J-L) minutes, respectively. M-N: Successive changes of recovery showing proximity of sperm and egg nuclei, and formation of mitotic apparatus. The fertilized eggs ten minutes after fertilization were exposed to the reagent for less than 60 minutes, then transferred to sea water.

Akita University

of division spindle for cleavage.

In the case of sea urchin egg, the spermatozoon once penetrating into egg rotates approxiately at 180°, so that the centrosomes come ahead of the spermatozoon (Dan, 1950 and 1952). The spermatozoon invades deeper into the egg interior, swelling gradually to form the male pronucleus. Meanwhile, the small aster consisting of feeble astral rays, was formed around the sperm nucleus. With the enlargement of the astral ray, one extremity of the astral ray will reach the egg surface and the other, the periphery of the egg nucleus. The rate of the extension was mesured by Hamaguchi and Hiramoto (1980) as about 0.1 μm in a second. The movement and the rotation of the sperm head result from pushing by the sperm tail, being engulfed into the egg at the time of fertilization.

The reasons why the sperm pronucleus directs and approaches toward the egg nucleus, and what sort of force acts between both pronuclei, have been obscure as far as the writer's awareness. Chambers (1939) proposed, that the motive force for the approach of the male pronucleus toward the female one was based upon the action of pushing force of elongating astral rays onto the inner wall of the cortex. Nakamura (1963) observed the behavior of the spermatozoon in the egg interior, and suggested that the fusion of both pronuclei is achieved by an attractive force acting between the two pronuclei. He assumed the force of attraction, working through certain changes on the astral rays (shrinkage). This seems to be the reasonable interpretation about the mechanism of synkaryon, based upon the assumption of stretching and shrinking force of the astral rays.

In the writer's observation, when the tips of the astral rays reached the cortex, the movement of the sperm nucleus accompanying with the growth of the astral rays, began to move toward the egg interior. In order to prove the actual interaction standing between the two pronuclei with a degree of an elongation of the astral rays, the writer designed an inhibition and a recovery of the astral rays, in which the process of synkaryon was arrested or not. Since, phenylurethan has been known to have specific inhibitory action on the formation of mitotic apparatus, the present chapter deals with the effect of this reagent on the behavior of the sperm nucleus in the process of synkaryon.

As stated previously, continuous exposure of the fertilized sea urchin eggs to phenylurethan inhibits synkaryon (Fig. 7, A-F). In the present experiment, when the fertilized sea urchin eggs were exposed, the formation of the mitotic apparatus was completely suppressed. After the exposure for ninety minutes, the sperm nucleus, initially small as a vesicular form (like a dot), gradually grew until almost an equal size to the egg nucleus (slightly smaller). Nevertheless, its movement toward the egg nucleus was still not occurred. Soon, the nuclear membranes of both pronuclei became obscure, it certainly showed that each pronucleus probably got into the course of nuclear division (without mitotic apparatus). The chromosomes of each of the pronucleus were scattered in a mass in the area of an unbounded nuclear sphere full of faintly stained nucleoplasm (Fig. 7, D) (sectioned preparations). In the meantime, the above mentioned mass of the scattered chromosomes together entered into telophase (Fig. 7, L and M), and finally formed a mass of several nuclear spheres (Fig. 7, M and N., sometimes, dumbbell shaped). Frequently, one or a few chromosomes apart from the main mass, formed one or a few small nuclear spheres (Fig. 7, F, M and N., micronuclei).

Akita University

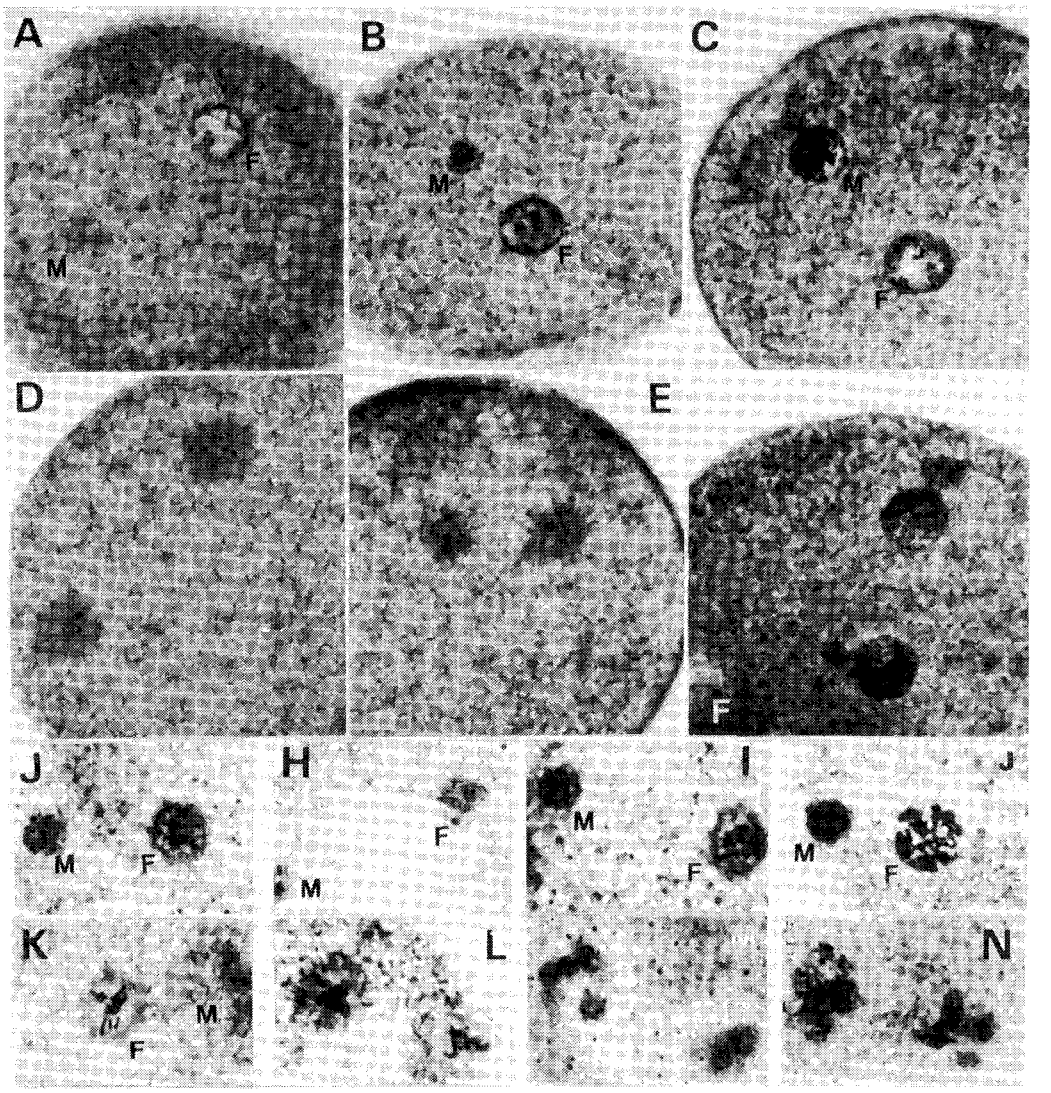

Fig. 7 Photomicrographs showing effects of phenylurethan. A-F : Continuous exposure of fertilized eggs in the reagent. Duration of exposure was : A, 30 ; B, 60 ; C, 90 ; D and E, 120 ; F, 150 minutes, respectively. J-N : Nuclear changes taking place in eggs with periods of exposure. Note, nuclear division without forming mitotic apparatus (sectioned and stained preparations).

Continuous exposure for even more than 150 minutes, the fusion of both pronuclei never occurred. From the facts, it becomes clear that the fusion of both pronuclei is led in the presence of the astral rays. This means that there must be some mutual relationship between both pronuclei, through mediation of the elongation of the astral ray.

It is generally agreed that phenylurethan inhibits the formation of mitotic apparatus, however, the nature of its action has remained unsolved (probably, an inhibition of polymerization of tubulin molecules). The change and the advance of the pronuclei in the egg interior

Akita University

(metaphase to telophase) continued still even during the exposure (Fig. 7, J-N). This means, that phenylurethan is inefficacious on the advance of nuclear development. The changes of an endoplasmic condition in the egg interior (involving endoplasmic development described before), in spite of the suppression of the mitotic apparatus, progress normally even at the case without synkaryon. The factors exerting the development of the nucleus, are probably assumed to be depend upon the advance of endoplasmic changes starting at fertilization (activation). No matter how the endoplasmic advance proceeds, the inhibition of the astral rays exerts no mutual attraction between both pronuclei.

Under continuous exposure of fertilized eggs for less than 60 minutes, then, they were transferred to ordinary sea water, soon, feeble astral rays were observed radiating their rays from each spherical formed pronucleus (Fig. 6, M and N). In the sectioned preparation, the appearance and the growth of the rays were observed around the sperm nucleus (Heidenhein's iron haematoxylin). With the visualization and the growth of the rays, the sperm and the egg nuclei came up approaching each other, then, they entered the course of synkaryon. Interestingly, the two asters were found mainly on the sperm nucleus side. After synkaryon, the egg made steady progress of nuclear division, and finally began cleavage within forty minutes. As stated before, the formation of the mitotic apparatus was considerably hastend, therefore, the duration of nuclear division was accelerated as shown in the figure (in accordance with the endoplasmic advance stated before).

From the evdence of an appearance of the minute amphiaster from the beginning of the recovery, it seems likely, that the development and duplication of the centriole has been started normally even in the exposure to phenylurethan. Namely, the appearance of amphiaster, immediately after the transfer, suggests the continuous development of the centriole, even in the exposure without affection to phenylurethan. The existence of divided centrioles in the sectioned preparations is thought, that the centriole developes automatically, depending upon the development of the endoplasmic advance.

In the case of the transfer after the exposure for more than 70 minutes, the process of synkaryon was completely suppressed. In this case, in spite of the formation of the astral rays (having doubled centrioles), why synkaryon between both pronuclei is perfectly inhibited (Fig. 6, H-I)? The analysis of this problem is needed immediate attention, so that the careful experiments will be done by the writer's colabolators as graduation theses (Ikeda A., Naka C. and K. Suda, 1994).

When the fertilized eggs were treated with phenylurethan for more than 90 minutes, and transferred to ordinary sea water, synkaryon did not occur. Both pronuclei, apart from each other, were found in the endoplasm (their appearance was quite different, as compared to the eggs which were exposed for shorter minutes). Within 15 minutes, the numerous feeble astral rays were formed around each pronucleus. Continually, the nuclear membrane of each pronucleus became obscure, showing that the egg entered into metaphase inspite of synkaryon. In this case, the perfectly assembled mitotic apparatus was never formed, accoudingly, one or two groups of the multinucleated vesicles were seen in the egg interior. They later formed micronuclei at the places where the pronuclei had been located. Soon, the cleavage took place dividing the egg into two or several blastomeres in irregular sizes. The reason for the

Akita University

incompletion of synkaryon inspite of the presence of the astral rays, was inferred that it was not the sperm aster but the more advanced amphiaster, which had the role for making the furrow, not for synkaryon. It will be based upon the inadequacy of the growth of the astral rays to act on both pronuclei, adjusting to the advanced developmental condition of the endoplasm. Accordingly, it can presume the presence of certain stage of endoplasmic development for the approach and the fusion of both pronuclei (critical stage for synkaryon, mediated by the elongation of the astral rays). Perhaps, in accordance with the endoplasmic condition, the process of synkaryon proceeds in close connection to the formation of the astral rays. Of course, the elongation of the rays are controlled by the situation of the centriole which has faculty of binding tubuline molecules. Here, the writer came to the conclusion, that the main factors of the fusion of both pronuclei are probably due to an action of the astral rays corresponding to the critical stage of endoplasmic development.

CONCLUSION

At fertilization, the spermatozoon carries centrosome, attaching on the distal end of the sperm head, into egg interior. After invasion, the astral rays are formed radiating from the centrosome toward every directions. The each ray will be thought to an array of microtubules. The centrosome later divides into two centrioles that participate the formation of the mitotic apparatus. The egg, of course containing own centrosome, have been considered that, in certain stage, the centrosome will be lost during last meiotic division.

Up to the present, there has been many researchers who have been engaged in the analysis of the process and the mechanism of fertilization (Epel, 1986; Longo, 1987 and Metz and Monroy, 1955, and others). In the writer's present experiments, he observed the invading spermatozoon, using clearly stratified sea urchin eggs by centrifugation. From the facts obtained, he discussed the mechanism of the sperm penetration, and synkaryon of both pronuclei as the mutual interaction of the astral rays. By the inhibition of the formation of sperm aster (rays), the fusion of the sperm and the egg nuclei never occurred. As the result, he came to the conclusion, that the motive force for the movement of the sperm pronucleus, and the attractive force acting between the two pronuclei, are based upon the pushing and the pulling actions of the astral rays.

Later, Hamaguchi and Hiramoto (1981), by using the differential interference microscope in the fertilization of the eggs of a heart-urchin, *Clypeaster japonicus*, indicated that the sperm pronucleus was carried to the center of the egg by the growth of the sperm asters, and that the egg nucleus was carried to the center of the aster by the filamentous structure formed between them. Morover, they showed that the curved pass of the egg nucleus in the egg interior, was interpreted as the combination of the movements of the astral center and the egg nucleus moving toward the center of the aster. These facts indicate the similar hypothesis proposed by the writer, that the behavior of both pronuclei is regulated by the force of the rigid astral rays. The inward movement of the sperm nucleus is derived from the pushing action of the astral rays when the extending tips of the rays reach the wall of egg cortex.

Akita University

The results of the continuously exposed fertilized eggs to phenylurethan commencing ten minutes after insemination was described already. After about ten minutes, the sperm nucleus was observed as a small refracting dot, around which no sign of the formation of the sperm aster was recognized. In this case, the fusion of both pronuclei never occurred. Maintaining this status consecutively, the sperm nucleus initially small and conpact in size, gradually swelled into the spherical form involving chromatin like entities, until growing to the similar size of the egg nucleus.

According to Chambers (1939) on the observation of the fertilized sea urchin eggs, the movement of the sperm aster toward the egg nucleus, is accomplished by the pushing action of the astral rays in a direction to egg cortex. Nakamura (1963), from the observation of the behavior of the astral rays which were extending with the egg interior, suggested that the motive force of the nuclear fusion was based upon the pulling action of the rays, stretching between both nuclei. In any cases, it is certain, that the growth of astral rays extending around sperm pronucleus, plays an important role in the mechanism of synkaryon. Namely, inhibition of the fusion of both pronuclei is inferred to be derived from the complete suppression of the formation of astral rays by the reagent (mitotic inhibitors, such as phenylurethan and colcemid).

After the continuous exposure to phenylurethan for more than ninety minutes, the volume of the sperm nucleus reached almost an equal size of the egg nucleus. Nevertheless, the movement of the sperm nucleus toward the egg one was still not observed. After a while, the nuclear membrane of both of the two pronuclei became obscure, showing that they got into metaphase without forming mitotic apparatus. The chromosomes were scattered into a mass of the nucleoplasm (darkly stained in blue) on each area, where pronucleus had been present. Soon, a chromosome or several numbers of them developed into a mass, consisting of the several small spheres in irregular sizes (micronuclei). This means the presence of the endoplasmic change which developes automatically in the fertilized eggs even in the exposure of this reagent. In accordance with this change, each chromosome decides it state of development without the presence of mitotic apparatus.

Moreover, the migration of the egg nucleus in the fertilization process, is examined by using time lapse cinematography (Yoshida, Nagai and Yamamoto 1986). In the analysis of the pictures, the female pronucleus is initially settled keeping still at anywhere in the egg, then, starts to move in the direction toward the center of the egg. The initiation of mevement was almost the same period of time during the inward movement of the male pronucleus. The shape of the female pronucleus at the time of migration was conical sphere, pointing its apex in the direction of the sperm nucleus. This means, that the migration of the egg nucleus inward, will be controlled by the pulling action of the astral rays.

The force exerted to pull up the egg nucleus (by astral rays), was calculated by Hamaguchi and Hiramoto (1980), comparing with the movement of an iron particle in the endoplasm of heart urchin egg under magnetic field (5 μm/sec). They showed, that this value was consistent with muscular contraction of the single muscle filament or the single microtubule during the cilliary beating. They cited the works done by Zimmerman and Zimmerman (1967) and Aronson (1973), that the migration of the egg nucleus was inhibited by an application of colcemid, and the inhibition was romoved by an irradiation of 366 μm light. This fact suggests,

Akita University

that the astral rays may be composed of microtubule system which plays an important role in the migration of the egg nucleus.

In the text book of Developmental Biology (Gilbert, 1991), Yudin and others illustrated the clear photographs of the process of fertilization in hamster egg (Yudin, 1988 ; Cherr, 1986 and Bavister, 1980). According to them, the mammalian sperm nucleus enlarges while the egg nucleus completes its second meiotic division. Soon, each pronucleus migrates toward each other. Upon meeting, the two nuclear envelopes then break down. Instead of producing common zygote nucleus, the chromatins condense into chromosomes that orient themselves upon a common mitotic spindle for cell division.

On the contrary, a true diploid nucleus in mammals is first seen, not in the zygote but at 2 cell stage. Glancing at photographs, the sperm aster could not ascertained. This is unplausible difference between mamalian and sea urchin eggs during fertilization. Therefore, in the mammalian fertilization, it must be considered an other physiological interaction on the process of the nuclear fusion, comparing with the conclusion written by the writer.

Concerning develpment of the endoplasm, starting from activation by fertilization (egg is metabolically dormant before fertilization), it has been noticed cytoplasmic segregation in the frog egg just after fertilization (gray crescent formation). Namely, the spermatozoon serves to trigger a series of the program for development on every aspacts of egg itself (unfortunately, an essential quality of activation is up to date obscure). To ascertain the phenomenon, Danilchik and Denegre (1991) examined the behavior of yolk platelets under fluorescent microscopy (the sections were previously stained by Trypan blue). They recognize the cytoplasmic migration which occurred during the middle of the cell cycle from ventral egg side toward dorsal direction. Thus, the local migration of the cytoplasm results the egg into irreversible qualititative development on endoplasm. Thus, the difference of the concentration of the egg substance, accompanying with the progress of development, is the real entities of endoplasmic development. These changes induce a series of developmental progresses important for embryonic development.

Fortunately, concerning the chemical essence of an activation itself, the initial change occurring from fertilization in the sea urchin eggs is examined to be mediated possibly by the changes in ion concentration within the egg (Whitaker et al., 1985). According to the text book of Molecular Biology (Alberts et al., 1989), it was shown that the rise in the intercellular pH in some organisms induces the later synthetic events of an egg activation. And, the low intercellular pH is thought to be responsible for keeping the unfertilized eggs metabolically inactive (Dube et al., 1985).

In conclusion, the inhibition of the astral rays (consisting of microtubules) impairs the migration of both pronuclei in the egg interior. Consequently, it is evident that the astral rays are important for the movement of the sperm and the egg nuclei in the process of synkaryon (possibly to be as motive force).

The mechanism of the facing of both pronuclei remains still unclear, however, for an

Akita University

extensive reviews on the process of the facing of each pronucleus, see Longo and Anderson (1968, 1969 and 1970).

The writer completed writing this review in early September in 1994. After having published more than one hundred various papers (References), this paper will probably be his last paper. Unfortunately, during the course of writing, the writer suffered from *chololithiasis*. Therefore, the completion of this paper was continued with much co-operation and encouragement from Miss Rio Yoshida, to whom the writer is extremely grateful.

At the conclusion of the paper, the writer, looking back on his long university career, believes that for a researcher, learining should always take priority over unavailing meetings and miscellaneous businesses. If he is a true researcher at all, he would not have the liberty of allowing idleness of other duties to interfere with his research.

The writer's present state of mind is, of course, full of heartfelt appreciation. He wuld like to express his sincerity quoting the Chinese proverb, 'Jiû de qì, xin de bû lai !'

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