Temporal Correlation between Sperm Aster Development and Cytoplasmic Cycle in Starfish Oocytes

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Enucleated fragments of maturing oocytes of starfish (*Asterina pectinifera*) still show cyclic changes in cortical tension with a temporal pattern similar to that exhibited by intact oocytes during meiotic division (Yamamoto and Yoneda, 1983). This autonomous cyclic activity (cytoplasmic cycle) may be involved in driving cell cycle. We also found that each peak in the tension of the enucleated fragments is always found to occur earlier (about ten min) than that in the nucleated partner. This "phase advance" of the cytoplasmic cycle seems to imply some relation with the acceleration of sperm aster development in enucleated and then fertilized fragments of starfish (*Asterias forbesi*) oocytes, which reported by Chambers and Chambers (1949). Therefore, temporal correlation between these two events was examined in detail in *Asterina pectinifera* oocytes: While sperm aster developed about ten min earlier in fragments enucleated 5-15 min after the germinal vesicle breakdown (GVBD) (Early Bisection) than in nucleated partner, this acceleration of sperm aster development was not found in those enucleated 25-40 min after GVBD (Late Bisection). On the other hand, the phase advance of the cytoplasmic cycle was not found in the enucleated fragment obtained by Late Bisection. This close temporal correlation suggests that cytoplasmic activities associated with the cytoplasmic cycle are involved in controlling sperm aster development.

INTRODUCTION

Starfish oocytes are known to show remarkable changes in cortical tension after they were induced to mature by the treatment with the natural maturation-inducing hormone, 1-methyladenine (1-MeAde) (7, 17) : After decreasing rapidly from an initial high level to a low level (8), the tension of the oocyte fluctuates twice concurrently with the two successive meiotic divisions. These dynamic changes in the cortex seem to be involved in the process of cytokinesis to produce the polar body (PB) (3). On the other hand, enucleated fragments of maturing oocytes, which never form polar bodies, were found to exhibit cyclic changes in the tension with a temporal pattern similar to that of the changes accompanying meiotic divisions of normal oocytes (17). This result indicates that the cytoplasm of the maturing oocyte has an autonomous cyclic activity ("cytoplasmic cycle") which is not dependent on the presence of a nucleus or a mitotic apparatus. In animal oocytes or eggs of various species, similar autonomous cyclic activities were reported not only in meiotic divisions (12) but also in mitotic divisions (4, 9, 11, 14, 19). The cytoplasmic cycle may be involved in progression of both meiotic and mitotic cell cycle. In amphibian eggs, there are two reports which support this

hypothesis (10, 13).

In our previous paper (17), we also found that each peak in the tension of the enucleated fragments always appeared earlier than that in the nucleated partner by about ten minutes. This "phase advance" of the cytoplasmic cycle reminds us of the early observations by Chambers and Chambers (2): They reported that, on oocytes of starfish, *Asterias forbesi*, if the oocyte is bisected fairly before the first PB formation and then fertilized, the sperm aster appears earlier in the fragment lacking the oocyte nucleus than in the oocyte-nucleated partner. This apparent coincidence of these two phenomena in enucleated oocytes seems to imply that there is some causal relation between them. In order to clarify this possible relation, temporal correlation between these two events was examined in detail using oocytes of starfish, *Asterina pectinifera*. First, the results of Chambers and Chambers (2) were re-examined with Nomarski differential interference optics: Timing of the sperm aster development was studied in enucleated and fertilized fragment of maturing oocytes. Enucleation (= bisection) was undertaken either just after the germinal vesicle breakdown (GVBD) (Early Bisection) or just before the first PB formation (Late Bisection). In the enucleated fragments obtained by Late Bisection, Chambers and Chambers (2) did not found the "acceleration" of the sperm aster appearance. Second, changes in the cortical tension of enucleated fragments were studied: It was examined whether or not the phase advance of the cytoplasmic cycle is found in the enucleated fragments obtained by Early Bisection and Late Bisection. The results obtained by the experiments indicate that there is a close temporal correlation between the phase of the cytoplasmic cycle and the timing of the sperm aster development.

MATERIALS AND METHODS

Materials

Starfishes (*Asterina pectinifera*) were collected during their breeding seasons; in May at Tokyo Bay and in September at Wakasa Bay. They were stored in aquaria supplied with cold sea water(15-18℃).

Solutions

Artificial sea water (ASW) was used throughout (Jamarin U, Jamarin Laboratory, Osaka). Ca^{2+}-free sea water (CaFSW), KCl-rich sea water, and 1-methyladenine (1-MeAde) solution were prepared as previously described (17).

Preparation of Gametes and Enucleated Oocyte Fragments

Follicle-free immature oocytes were obtained by treating isolated ovaries with CaFSW for about 40 min and subsequent transfer to KCl-rich sea water. Maturation of the oocytes was induced by treating them with ASW containing 2 μM 1-MeAde. In the first experiments in which timing of the sperm aster development was examined, intact maturing oocytes were bisected manually into nucleated and enucleated halves with a fine glass needle as previously described (6, 17). As Chambers (1) reported, resultant two fragments were held together by the vitelline coat. On the other hand, in the second experiments in which cortical tension of the

enucleated oocyte fragments was measured, oocytes deprived of the vitelline coat were used (6, 17). The bisection was undertaken in the two stages of maturing oocyte; (1) 5-15 min after GVBD (25-35 min after 1-MeAde application) and (2) 25-40 min after GVBD (45-60 min after 1-MeAde application). In this paper, the former bisection is termed "Early Bisection" and the latter is termed "Late Bisection".

The sperm suspension was obtained by diluting semen, which is taken from an isolated testis, with ASW. The insemination of the bisected pairs was done at 40-50 and 55-65 min after 1-MeAde application in Early Bisection and Late Bisection, respectively. Once fertilized, the enucleated fragment is no longer "enucleated". For convenience sake, however, whether fertilized or not, the fragment derived from an enucleated fragment of the oocyte is called "enucleated fragment "and that derived from a nucleated one is called "nucleated fragment" in this paper. The pairs in which difference in the timing of the fertilization-envelope elevation was less than 3 min were exclusively used for the observation. A inseminated pair was mounted in a rectangular microchamber which was made on a glass slide with a piece of Mending Tape (Scotch), in order to prevent an excess compression of the fragments and to minimize an evaporation of water. Observation was performed with Nomarski differential interference optics.

Measurement of Cortical Tension of Oocyte Fragments

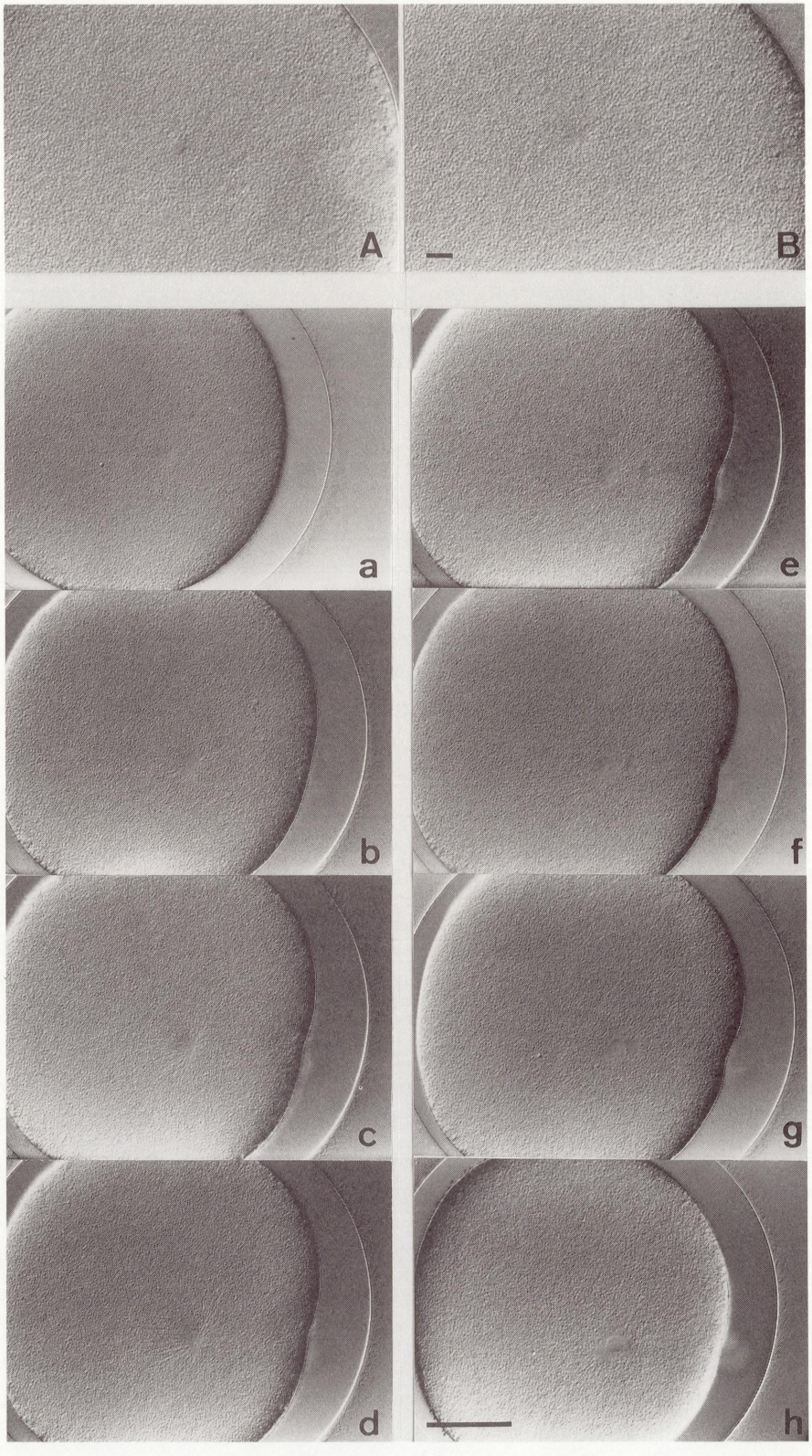
Changes in mechanical properties of oocyte cortex was examined by the compression method (17, 18). Cortical tension of the oocyte fragment (T) was calculated by measuring the thickness (Z) of the oocyte when compressed under a constant force (F), using F, Z, and the initial diameter (Z_0) as parameters.

RESULTS

Sperm Aster in Normal Maturing Oocyte

Behavior of a sperm aster in normal, fertilized oocytes was observed first. Maturing oocytes, inseminated about 40 min after the application of 1-MeAde, were kept in an incubator as a stock (20℃). Then, from this stock, several oocytes were mounted on a glass slide every 15-20 min.

A small sperm aster, which had been formed ten to fifteen min after sperm entry (5), disappeared about 10 min after the formation of the first PB and a small granule-free spot which seemed to represent a sperm nucleus appeared (Fig. 1A). About ten min later (5-10 min before the second PB formation), a small sperm aster appeared again (Fig. 1B). This small aster (Fig. 1a, b) enlarged rapidly about 5 min after the second PB formation (Fig. 1c, d). Hereafter this rapid enlargement of the sperm aster is called "sperm aster development" and timing of this event in oocyte fragments was examined in the following first experiment. Three to five min after the sperm aster development, a cluster of karyomere-like structures appeared in the center of the aster (Fig. 1e). Within ten min after the sperm aster development they fused to form a sperm pronucleus (Fig. 1f). The sperm aster disappeared during this process. Simultaneously with the formation of the sperm pronucleus, a female pronucleus was also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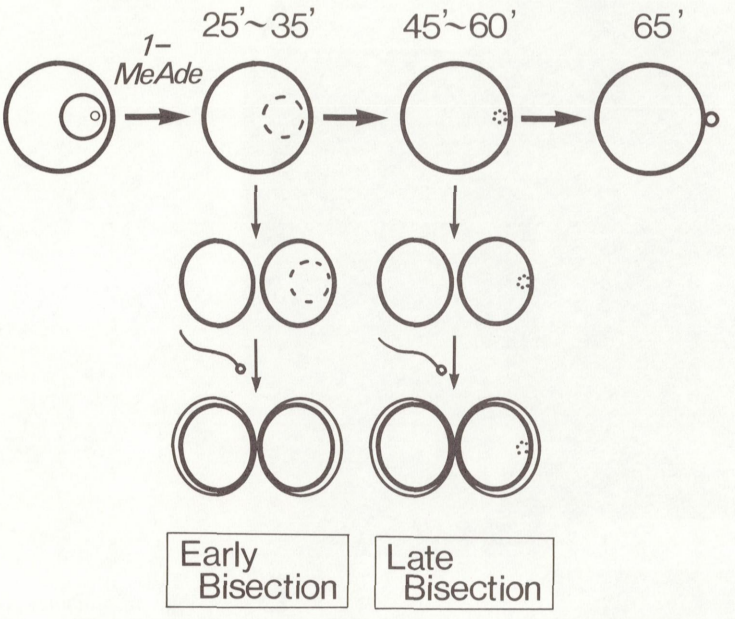

Fig. 2. Diagram of the procedure of the first experiment.

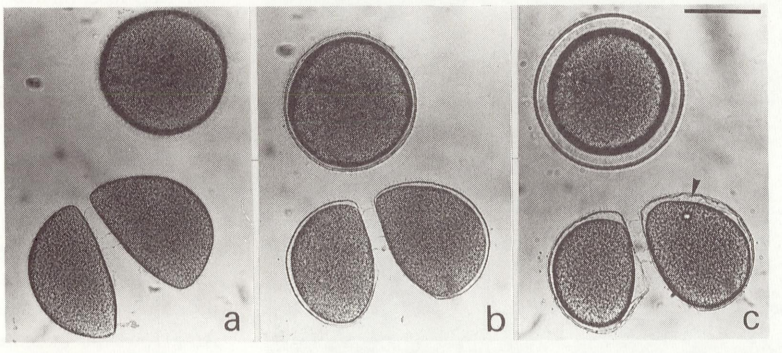

Fig. 3. Light photomicrographs of a pair of oocyte fragments (lower) obtained by Early Bisection and a normal oocyte (upper). 26 (a), 36 (b), 55 (c) min after 1-MeAde application. They were inseminated 33 min after 1-MeAde application. An arrowhead (c) indicates the first PB in the nucleated half. Scale bar : 100 μm.

Fig. 1. Nomarski photomicrographs of normal, fertilized oocytes. (A, B) and (a-h) show two sets of serial micrographs. Inseminated 35 min after 1-MeAde application. 71 (A), 75 (B) min after 1-MeAde application. 82 (a), 88 (b), 90 (c), 91 (d), 94 (e), 97 (f), 99 (g), and 110 (h) min after 1-MeAde application. The second polar body (PB) was formed 86 min after 1-MeAde application in the latter oocyte (PBs are out of focus in (b)-(h)). 20-20°C. Scale bars : 10 μm (A, B), 50 μm (a-h).

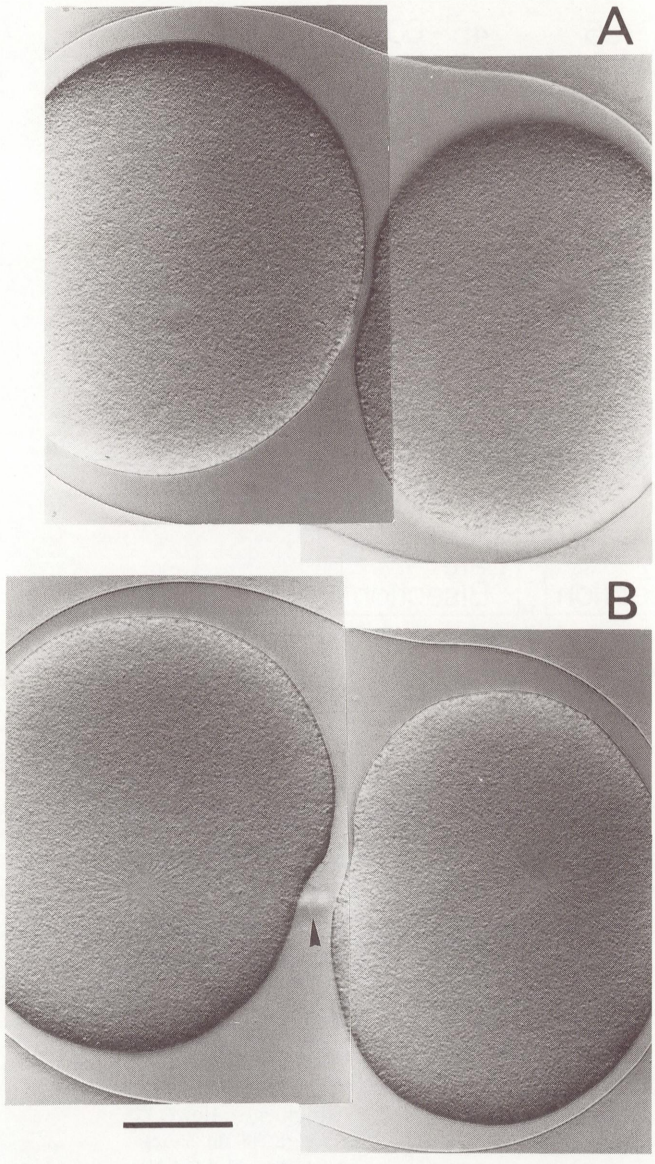

Fig. 4. Nomarski photomicrographs of a pair of oocyte fragments obtained by Early Bisection. Bisected 30-36 min after 1-MeAde application and inseminated 40 min after 1-MeAde application. 20°C. (A) 93 mim after 1-MeAde application. While a small sperm aster is seen in the nucleated fragment (left), sperm aster has already enlarged in the enucleated fragment (right). (B) 100 min after 1-MeAde application. The sperm aster has developed in the nucleated one. In the enucleated one, formation of a sperm pronucleus has started. An arrowhead indicates PBs emitted by the left fragment. Scale bar : 50 μm.

formed in the cytoplasm beneath polar bodies. After a female pronucleus moved towards the sperm pronucleus (Fig. 1f, g), both pronuclei began to fuse to form a zygote nucleus (Fig. 1h). Though Hirai *et al.* (5) reported that the sperm pronucleus moved towards the female pronucleus, the movement of the female pronucleus was always more remarkable in this study. The cause of the discrepancy is unknown. Except for this point, observations obtained here were consistent with the results already reported by Hirai *et al.* (5).

Sperm Aster Development in Early Bisection

According to the procedure shown in Fig. 2, a maturing oocyte was bisected 25-35 min after 1-MeAde application and then, the resultant enucleated and nucleated pair was inseminated 40-50 min after 1-MeAde application (Fig. 3). After the first PB was formed in the nucleated

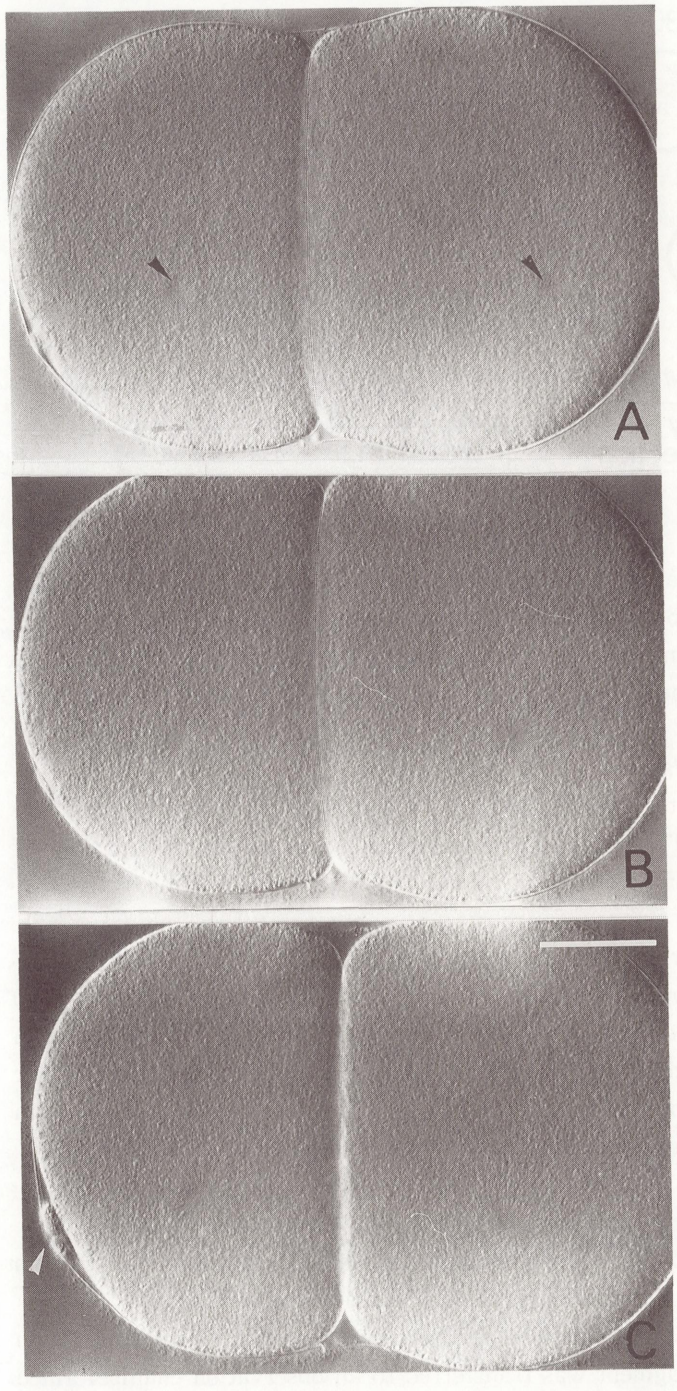

Fig. 5. Nomarski photomicrographs of a pair of oocyte fragments obtained by Late Bisection. Bisected 51-59 min after 1-MeAde application and inseminated 62 min after 1-MeAde application. 20°C. 93 (A), 100 (D), 104 (C) min after 1-MeAde application. The small sperm asters (arrowheads) are seen in both fragments (A). The sperm asters begin to develop in both fragments (B) and they have developed fully (C). By the presence of PBs (an arrowhead), one can recognize the left fragment as the nucleated fragment. Scale bar : 50 μm.

fragment, the pair was mounted. Only the pairs in which the nucleated one was recognizable by the presence of the first PB were used for the observation.

Seven (at about 23℃) and eighteen (at about 20℃) pairs were examined. As shown in Fig. 4, the sperm aster developed earlier in the enucleated fragment than in the nucleated partner in all cases. Differences between the timings of the sperm aster development in most pairs were

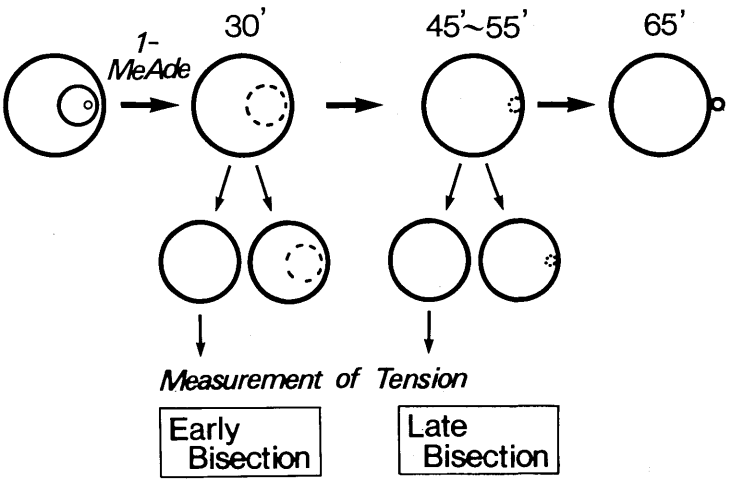

Fig. 6. Diagram of the procedure of the second experiment.

10-15 min at 20℃. Subsequent formation of a sperm pronucleus and disappearance of the sperm aster in the enucleated fragment also occurred 10-15 min earlier in most pairs. Thus, the timing of a series of events from the sperm aster development to the sperm pronucleus formation is accelerated in the enucleated fragment.

Sperm Aster Development in Late Bisection

Next, timing of the sperm aster development in the fragment pair obtained by Late Bisection was examined (Fig. 2). The bisection of a maturing oocyte and the insemination of the bisected pair were undertaken 45-60 and 55-65 min after 1-MeAde application, respectively.

In contrast to the results in Early Bisection, the sperm aster developed almost simultaneously (95-100 min after 1-MeAde application) in both fragments in eighteen out of twenty-two cases examined (Fig. 5). Differences between the timings of the sperm aster development in these eighteen pairs were less than 1 min. In the rest of the four pairs, the sperm aster developed 3-10 min earlier in the enucleated fragment than in the nucleated one. The sperm pronuclei also appeared simultaneously in most fragment pairs.

Cytoplasmic Cycle in Early Bisection

As the second experiment, the phase of the cytoplasmic cycle in the enucleated fragments was compared with the timing of PB formation in the nucleated partner.

In Early Bisection, a maturing oocyte was bisected about 30 min after 1-MeAde application (Fig. 6). A resultant enucleated fragment was transferred to an observation chamber filled with ASW and then, changes in cortical tension of the fragment were monitored continuously by the compression method. Simultaneously, the timing of PB formation in the nucleated partner was also examined. The time of PB formation was recorded when a protrusion appeared at the surface of the fragment. The temperature of ASW in Petri dish in which the nucleated partner was incubated was kept equivalent carefully to that of ASW in the observa-

tion chamber.

As previously reported (17), each peak in cortical tension of the enucleated fragment always appeared earlier than each PB formation in the nucleated partner (Fig. 7). The average of differences between them for six cases was 9.8 min with a standard deviation of 1.6 min for 1st peak - 1st PB, and 7.1 min with a standard deviation of 2.0 min for 2nd peak - 2nd PB (Table 1).

Cytoplasmic Cycle in Late Bisection

In Late Bisection, the enucleated fragments were obtained from the maturing oocytes which were 45-55 min after 1-MeAde application (Fig. 6). Since a mitotic apparatus was seen beneath the animal pole as a granule -free clear spot in a maturing oocyte of this stage, the bisection was undertaken using this spot as a marker of the animal pole; the fragment having this spot was detected as a nucleated one. Changes in the tension of the enucleated fragment and the timing of PB formation in the nucleated one were examined in the same way as in Early Bisection.

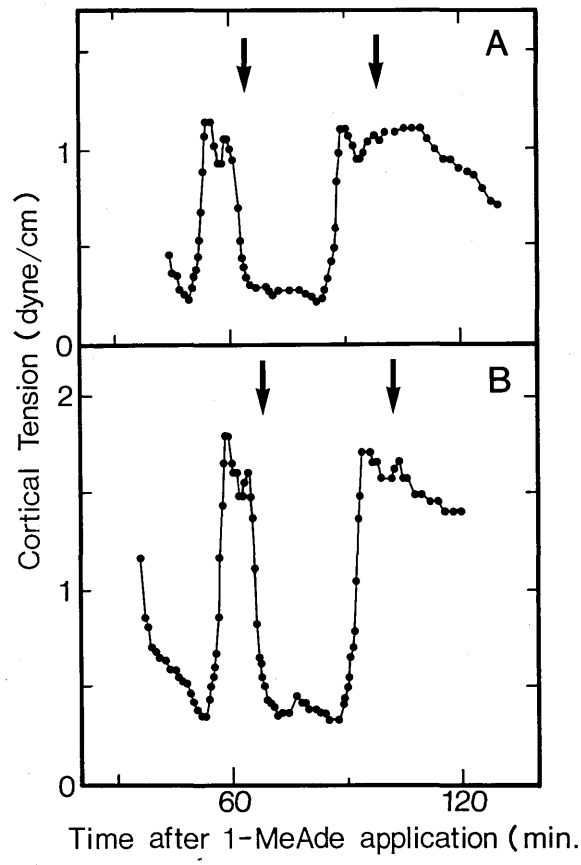

Fig. 7. Cortical tension of the enucleated oocyte fragments obtained by Early Bisection. Arrows indicate the times of PB formation in the nucleated partners. 20°C. (A) Bisected 28 min after 1-MeAde application. $Z_0 = 122$ μm. (B) Bisected 30 min after 1-MeAde application. $Z_0 = 123$ μm. The applied force (F) was 1.23×10^{-2} dyne.

Table 1. Timing of peaks in tension of enucleated fragments obtained by Early Bisection compared with the timing of PB formation in the nucleated partners at about 20°C.

	Enucleated		Nucleated	
bisection	1st peak	2nd peak	1st PB	2nd PB
28	54	89	64	99
31	60.5	97	73	106
30	58.5	94	68	102
28	55	89.5	62	95
28	57	99	67	104
29	59.5	97	69	102

Times in minutes of bisection, each peak, and PB formation are measured from 1-MeAde application. GVBD occurred 18-21 min after 1-MeAde application in these oocytes. Data for enucleated and nucleated pairs derived from a single oocyte are shown on the same line.

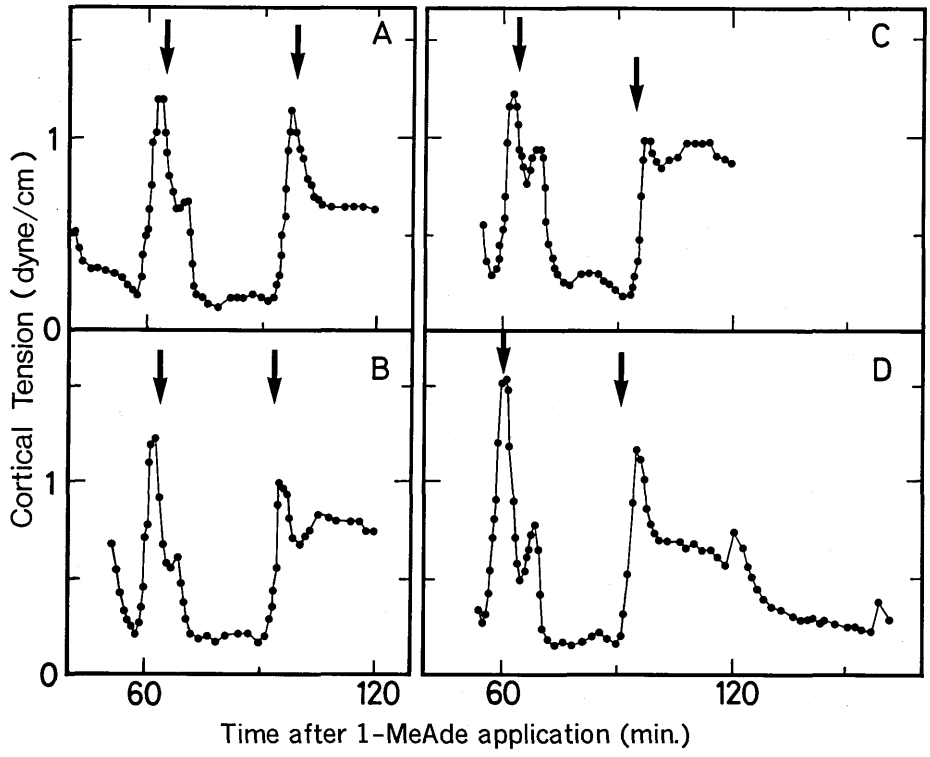

Fig. 8. Cortical tension of the enucleated oocyte fragments obtained by Late Bisection. Arrows indicate the times of PB formation in the nucleated partners. 20°C. Bisected 37 (A), 45 (B), 47 (C), 47 (D) min after 1-MeAde application. $Z_0 = 138$ (A), 123 (B), 128 (C), 122 (D) μm. The applied force (F) was 1.23×10^{-2} dyne.

Table. 2. Timing of peaks in tension of enucleated fragments obtained by Late Bisection compared with the timing of PB formation in the nucleated partners at about 20°C.

	Enucleated		Nucleated	
bisection	1st peak	2nd peak	1st PB	2nd PB
49	60	92	60	93
55	63	97	64	91
47	60	95	60	91
37	63	98	65	99
45	63	95.5	64	94.5
47	62.5	97.5	64	99
52	62	96.5	64	—

Times in minutes of bisection, each peak, and PB formation are measured from 1-MeAde application. GVBD occurred 18-21 min after 1-MeAde application in these oocytes. Data for enucleated and nucleated pairs derived from a single oocyte are shown on the same line.

As shown in Fig. 8, each peak in the tension of the enucleated fragment was found almost simultaneously with the formation of each PB in the nucleated partner. The average of differences between them for seven cases was 1.1 min with a standard deviation of 0.8 min for 1st peak - 1st PB, and 1.3 min with a standard deviation of 2.8 min for 2nd peak - 2nd PB (Table 2).

DISCUSSION

The results of the first experiment of this study confirmed the observation of Chambers and Chambers (2): When a oocyte-fragment pair obtained by Early Bisection was fertilized, the sperm aster developed 10-15 min (20℃) earlier in the enucleated fragment than in the nucleated partner. In the pair obtained by Late Bisection, on the other hand, such an acceleration of the sperm aster development in the enucleated fragment was no longer found; the sperm aster developed almost simultaneously in both fragments. In most pairs, the following formation of a sperm pronucleus and disappearance of the sperm aster in the enucleated fragment were also accelerated in Early Bisection but occurred simultaneously in Late Bisection. Thus, these results indicate not only that the behaivor of the sperm aster and nucleus observed in the enucleated fragment are identical to those changes in normal fertilized oocytes, but also that the timing of these changes is accelerated in the enucleated fragment obtained by Early Bisection.

Interestingly, the second experiment has revealed that the phase of the cytoplasmic cycle in the enucleated fragment behaves in the same manner as the time of the sperm aster development: Each peak in cortical tension of the enucleated fragment obtained by Early Bisection was found about ten min earlier than each PB formation in the nucleated partner. The average of time differences (9.8 min) between the first peak appearance and the first PB formation is comparable to that (9.2 min) obtained in our previous paper (17). On the other hand, such a phase advance in the cytoplasmic cycle was not found in the enucleated fragment obtained by Late Bisection. Thus, a close correlation was found between the time of the sperm aster development and the phase of the cytoplasmic cycle. These results appear to imply that after two rounds of cyclic change is completed, a cytoplasmic condition shifts to promoting sperm aster development and subsequent sperm pronucleus formation. In other words, the cytoplasmic activities associated with the cytoplasmic cycle may control the timing of the sperm aster development and the sperm pronucleus formation. Though the evidence obtained in this study is indirect, the fact that neither the cytoplasmic cycle (17) nor sperm pronucleus formation (5) is detected in the enucleated fragment obtained from an immature oocyte, supports the above hypothesis. Indispensability of the contents of the germinal vesicle (GV) has been demonstrated for these two events (15, 16). In order to verify the hypothesis, further studies will be necessary; for example, whether or not the phase of the cyclic changes in the fertilized, enucleated fragment behaves in the same way as that in the non-fertilized enucleated fragment remains to be elucidated.

Hirai *et al.* (5) reported that a sperm aster was formed even in the enucleated fragment of an immature oocyte. Apparently this observation does not support the above hypothesis.

However, such a sperm aster formation is not followed by the sperm pronucleus formation (5) and the formed aster does not disappear for several hours (Yamamoto, unpublished observations). Therefore, it seems that such sperm aster formation is not identical to that in the enucleated fragment of maturing oocyte and the normal maturing oocyte.

Yamamoto (16) has shown that the contents of GV are required for inducing the cytoplasm of starfish oocytes to initiate the cytoplasmic cycle. To what extent GV contents are intermingled with the cytoplasm may be involved in determining the phase of the cytoplasmic cycle, since in Early Bisection a considerable part of the contents still remains undiffused, while they are diffused fully in Late Bisection.

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