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Genetic Consequences of Blocking Polar Body I with Cytochalasin B in Fertilized Eggs of the Pacific Oyster, *Crassostrea gigas:* II. Segregation of Chromosomes

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Abstract. The effect of blocking polar body I (PB1) with cytochalasin B (CB) on subsequent chromosome segregation was studied in fertilized eggs of the Pacific oyster, *Crassostrea gigas.* To block the release of PB1, fertilized eggs were treated with CB (1.0 μg/ml) for 15 min beginning at 5 min post-fertilization at 25°C. Chromosome segregation in both control and CB-treated eggs was analyzed with an acetic orcein stain.

In untreated eggs, ten maternal tetrad chromosomes went through meiosis I and II, and released two polar bodies, reaching a haploid number of 10 chromatids. In CB-treated eggs, meiosis I proceeded normally and produced two groups of dyads, ten in each group. However, blocking PB1 dramatically changed chromosome segregation in meiosis II. In the majority of the treated eggs (68%), the two groups of dyads from meiosis I entered meiosis II through a "tripolar segregation", although two other types of segregation, namely "united bipolar" (7%) and "separated bipolar" (12%) were also observed. After anaphase II, chromatids at the peripheral pole were released as polar body II (PB2). The release of two sets of chromatids as PB2 through either a united bipolar or a separated bipolar segregation resulted in the formation of meiosis I triploids (14%). The release of one set of chromatids as PB2 from an unmixed tripolar or a separated bipolar segregation formed meiosis I tetraploids (20%). Aneuploids (56%) were produced, primarily when the two groups of dyads from meiosis I united or overlapped before entering the tripolar segregation.

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Introduction

It has been reported that blocking the release of polar body I (PB1) in the Pacific oyster, *Crassostrea gigas,* and other mollusks may result in triploidy (Quillet and Panelay, 1986; Arai *et al.,* 1986), tetraploidy (Stephens and Downing, 1988), or both (Stanley *et al.,* 1981; Yamamoto and Sugawara, 1988). In addition to the triploids and tetraploids reported, a previous study also revealed the production of high levels of aneuploids, which distributed unexpectedly around two peaks (Guo *et al.,* 1992). The production of triploids, tetraploids and aneuploids in specific distributions suggests that blocking PB1 may have complicated subsequent chromosome segregation.

The production of triploids and tetraploids from blocking PB1 may be explained by results from two studies. In the surf clam *Spisula solidissima,* Longo (1972) used electron microscopy to study the effects of CB on the formation of polar bodies. When CB blocked the formation of both polar bodies, he observed four groups of maternally derived chromatids, suggesting that two groups of dyad chromosomes from meiosis I might have entered meiosis II separately. If one or two of the four chromatid groups were released as PB2, tetraploids or triploids were produced. In the Japanese pearl oyster, *Pinctada fucata martensii,* blocking PB1 produced 3 or 4 maternally derived groups of chromatids; triploids or tetraploids were produced by releasing two or one set of chromatids (Komaru *et al.,* 1990).

Despite the findings that production of triploids from blocking PB1 is possible, the question still remains

whether triploids produced from blocking PB1 are genetically different from triploids produced from blocking PB2. It has been shown that meiosis I triploids have a higher level of heterozygosity and a higher growth rate than meiosis II triploids in the American oyster, *Crassostrea virginica* (Stanley *et al.*, 1984). Improved growth rate was also observed for meiosis I triploids in the Pacific oyster (Yamamoto *et al.*, 1988), in blue mussel, *Mytilus edulis* (Beaumont and Kelly, 1989), and in the pearl oyster, *Pinctada martensii* (Jiang *et al.*, 1991). Treatments applied at different times showed two windows for triploid induction, corresponding to the timing of meiosis I and II, respectively (Quillet and Panelay, 1986; Arai *et al.*, 1986; Yamamoto and Sugawara, 1988). However, cytological evidence of meiosis I triploid formation has never been reported. That tetraploids and aneuploids resulted from blocking PB1 adds further confusion to how meiosis I triploids are produced.

The mechanism by which aneuploids were produced from blocking PB1 is unknown. Overlapping of chromosome segregation during meiosis II observed by Longo (1972) may produce some aneuploids; however, specific distributions of aneuploids suggest that they were not created randomly (Guo *et al.*, 1992). Instead, the aneuploids may be created through specific patterns of chromosome segregation.

It was the objective of this study to investigate how chromosomes segregate and what is released as PB2, following inhibition of PB1 in fertilized eggs of the Pacific oyster.

Materials and Methods

Treatment and sampling

Gametes of the Pacific oyster were prepared according to methods described in Guo *et al.* (1992). Gamete handling and treatment were all conducted at 25°C. Two experimental groups were formed. In the first group, fertilized eggs were treated with 0.1% dimethyl sulfoxide (DMSO) only and allowed to develop as diploid controls. In the second group, fertilized eggs were treated with 1.0 μg/ml CB in 0.1% DMSO for 15 min beginning at 5 min post-fertilization. The experiment was repeated in a second cross using a different pair of oysters.

To study chromosome segregation, samples of developing zygotes from both groups were taken every 5 min until 80 min post-fertilization and fixed in 1:3 (v/v) glacial acetic acid and absolute methanol. Ploidy level of 8-h-old embryos was determined with karyological analysis according to methods in Guo *et al.* (1992). Twenty-five metaphase plates were counted for each group.

Staining method

Initially, two acetic orcein stains were tested: 1% orcein stain in 45% acetic acid (in distilled water) and a lactic-acetic orcein stain (Darlington and La Cours, 1962; Lu, 1986). Both resulted in dark staining of the cytoplasm and, therefore, the contrast between chromosomes and cytoplasm was poor.

Subsequently, 0.5% orcein in 40%, 50%, 60%, 70% and 80% acetic acid was tested. Cytoplasmic staining became lighter with increasing concentrations of acetic acid. But with 80% acetic acid, the staining of chromosomes was slow, taking at least 24 h to obtain good contrast. Stain with 0.5% orcein in 60% acetic acid gave satisfactory contrast with 5–10 min of staining.

For staining, drops of fixed samples were spread on slides, and the excess fixative was allowed to run off the slides. Two drops of the modified acetic orcein stain were added, and the material was covered with a cover glass. After staining for 5 min, excess stain was removed by absorption onto filter paper. The cover glass was gently pressed and sealed with fingernail polish.

Slides were examined with a Zeiss compound microscope. Photographs were taken with Kodak TMX 400 black and white film.

Results

The mature oocyte of the Pacific oyster has a large germinal vesicle which is visible under the microscope without staining. Germinal vesicle breakdown (GVBD) usually occurs after the egg has been in the seawater for 20–40 min.

Pacific oysters have a diploid number of 20 chromosomes (Ahmed and Sparks, 1967; Ahmed, 1973). After GVBD, 10 tetrad (synapsed) chromosomes were scattered in the eggs. Shortly after fertilization (within 5 min), the 10 tetrads gathered closely and were apparently ready for meiosis I (Fig. 1A).

In both the control and the CB-treated groups, development of eggs was not completely synchronized. A small proportion (<5%) of the eggs developed very slowly. These slowly developing eggs reached metaphase I when the majority of the eggs had released the second polar body.

Chromosome segregation in the control groups

In the control groups, the 10 tetrads started to segregate at 5 min post-fertilization. Anaphase I was reached in the majority of the eggs at 10 min post-fertilization with 10 dyad chromosomes moving toward each division pole (Fig. 1B). At telophase I (around 15 min post-fertilization), the 10 dyads at the peripheral pole became more condensed and compacted (Fig. 1C). PB1 was released in the majority of the eggs by 20 min post-fertilization. The ten remaining dyads continued through meiosis II, and metaphase II was reached around 25 min post-fertilization.

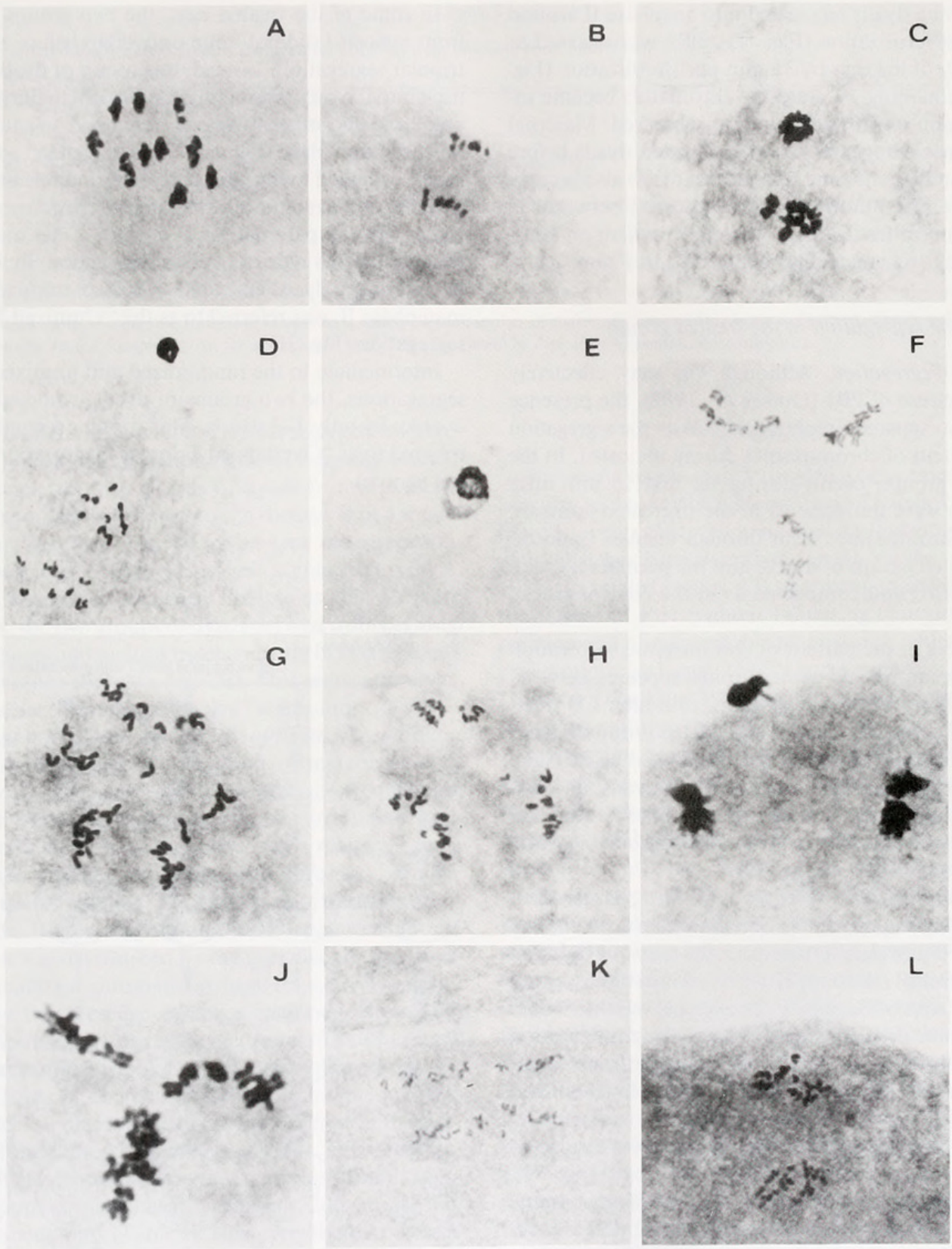

Figure 1. Segregation patterns observed in fertilized eggs of the Pacific oyster following normal fertilization (A–E) and blocking polar body I with cytochalasin B (F–L): A–E, meiosis in normal eggs; F–I, randomized tripolar segregation; J, unmixed tripolar segregation; K, united bipolar segregation; and L, separated bipolar segregation.

The remaining dyads segregated into anaphase II around 30 min post-fertilization (Fig. 1D). PB2 was released in the majority of the eggs by 35 min post-fertilization (Fig. 1E). The remaining 10 maternal chromatids became invisible. Fusion of pronuclei was not observed. Maternal chromosomes reappeared as 10 duplicated dyads before mitosis I. At the same time, 10 paternal dyads also became visible. The first mitotic division occurred between 55 and 65 min post-fertilization. The development of a normal diploid is schematically represented in Figure 2A.

Chromosome segregation in the treated groups

Tripolar segregation. Although CB may effectively block the release of PB1 (Guo *et al.*, 1992), the presence of CB had no apparent inhibitory effects on the segregation and movement of chromosomes during meiosis I. In the CB-treated groups, events during the first 15 min after fertilization were the same as in the untreated controls. Maternal chromosomes went through meiosis I and divided into two groups of dyads, and the peripheral group also condensed and compacted as in the control groups (Fig. 1C).

Differences in the pattern of chromosome segregation between the control and treated groups appeared between 15 and 20 min post-fertilization (10 min after CB treatment). In the treated eggs, the compacted group of dyads (ten) at the peripheral pole was not released as PB1 and started to dissociate, become individual dyads, and migrate back toward the remaining group of dyads. No polar body was released at 20 min post-fertilization when eggs in the control groups released PB1.

In the majority of treated eggs at 20 min post-fertilization, the two groups of dyads were united in a single cluster. At 25–30 min post-fertilization, the twenty dyads (ten from each group) randomly partitioned into three groups; each group (with 6–7 dyads on the average) was distributed on one of three division planes in a tripolar configuration (Fig. 1F). Between 30 and 35 min post-fertilization, the three groups of dyads at the three division planes entered anaphase II (Fig. 1G). During telophase II, each pole received chromatids from two adjacent groups of dyads and ended up with an average of 13–14 chromatids (Fig. 1H). After telophase II, all three groups of chromatids became condensed, and the group at the peripheral pole was released as PB2 by 40 min post-fertilization (Fig. 1I). This PB2 contained, on the average, 13–14 chromatids; its release could leave the egg as an aneuploid (Fig. 2C). Since the dyads from meiosis I appeared to group and segregate randomly, this tripolar segregation was referred to as "Randomized Tripolar" segregation (Fig. 2C). Although rare, eggs with two PB2s were also observed. The first mitosis in the CB-treated groups occurred between 60 and 75 min post-fertilization.

In some of the treated eggs, the two groups of dyads from meiosis I did not unite or overlap before entering a tripolar segregation. Instead, one group of dyads divided itself into the two division planes adjacent to the peripheral pole, and the other 10 dyads remained together at the inner division plane (Fig. 1J). Thus, a set of ten chromatids might be pulled to the peripheral pole and released as PB2 (Fig. 2D). The remaining 30 maternal chromosomes may unite with the paternal set of chromosomes and form a tetraploid. This type of tripolar segregation, in which the two groups of dyads appeared to be kept unmixed during metaphase II, was referred to as the "Unmixed Tripolar" segregation (Fig. 2D).

Intermediate to the randomized and unmixed tripolar segregations, the two groups of dyads would sometimes overlap by only 1–3 dyads. This tripolar segregation was referred to as "Overlapped Tripolar" segregation (picture not shown).

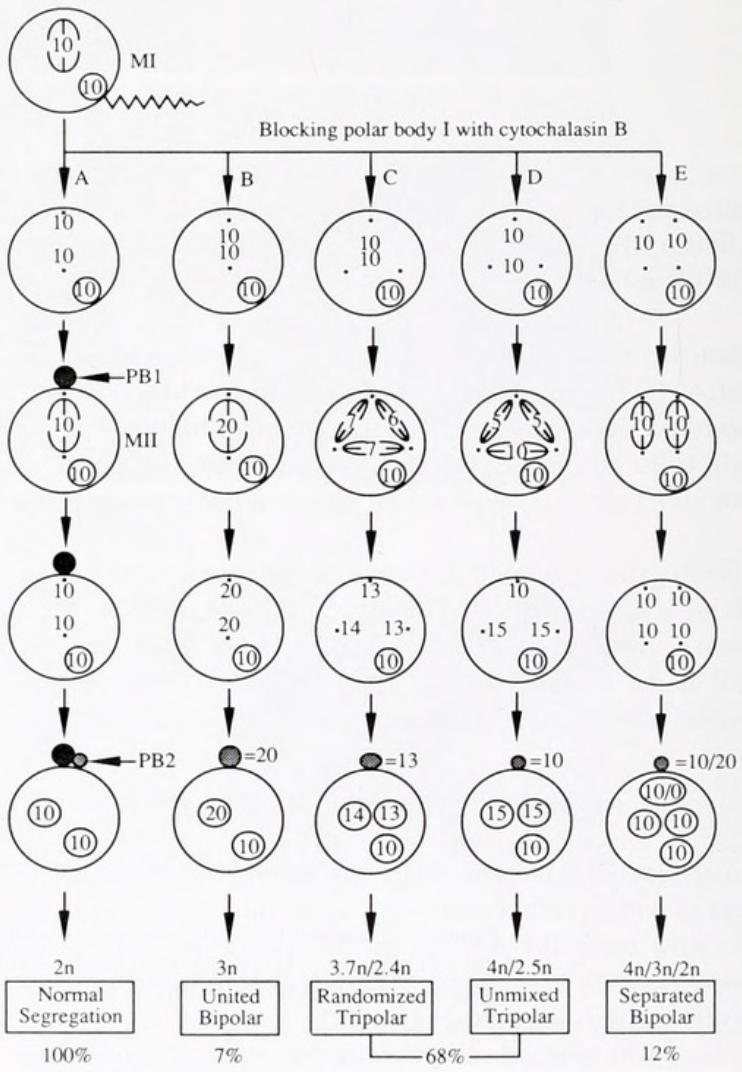

Figure 2. Schematic summary of segregation patterns observed in fertilized eggs of the Pacific oyster following normal fertilization (A) and blocking polar body I with cytochalasin B (B–E). Spindles and centrioles are hypothetical.

Table I

Percentage of different segregation patterns and ploidies observed from blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster

	Segregation patterns (n = 50)				Ploidy levels (n = 25)			
Cross	Separated bipolar	United bipolar	Tripolar all	Unclassified patterns	3n	4n	An	Other
1	12	10	64	14	12	8	68	12
2	12	4	72	12	16	32	44	8
Average	12	7	68	13	14	20	56	10

All segregation patterns were determined at telophase II based on the number of maternal chromosome groups observed. Ploidy was abbreviated as 3n for triploids, 4n for tetraploids, An for aneuploids, and Other for haploids, diploids and pentaploids combined.

Other patterns of segregation. Tripolar segregations were common, but other segregation patterns were also found. In some cases, the two groups of dyads from meiosis I completely united and went through meiosis II in a single bipolar configuration (Fig. 1K). This type of segregation was referred to as "United Bipolar" segregation, which led to the formation of meiosis I triploids (Fig. 2B). In other cases, the two groups of dyads derived from meiosis I entered the second meiosis independently in two separate bipolar divisions (Fig. 1L; Fig. 2E). This segregation was referred as the "Separated Bipolar" segregation.

Segregation patterns and ploidy of resultant embryos. Due to the relatively short duration of metaphase and anaphase, it was difficult to estimate precisely frequencies of different tripolar segregations. Between anaphase II and the release of PB2, however, there was a relatively long period when the number of maternal chromatid groups could be reliably counted. The frequency of united bipolar segregation, tripolar segregation and separated bipolar segregation was determined by the presence of two, three and four maternal chromatid groups, respectively. As indicated by the presence of three maternal chromatid groups, the majority of the treated eggs in both crosses (68%) went through tripolar segregations following blocking PB1 (Table I). The two crosses had the same number of separated bipolar segregations, but differed in the percentage of the united bipolar segregations.

Overall, ploidies of the resultant embryos from the CB-treated groups consisted of 14% triploids, 20% tetraploids and 56% aneuploids (Table I). However, the two crosses differed considerably in the frequencies of tetraploids and aneuploids. CB treatment in the second cross produced more tetraploids (32%) but fewer aneuploids (44%) than that in the first cross (8% and 68%, respectively). About two-thirds of the aneuploids fell in the range of 23–26 and 34–38 chromosomes. In some aneuploid embryos where two cells were counted, chromosome numbers sometimes varied. In the control groups, over 90% of the cells counted could be classified as diploids.

Discussion

The acetic orcein stain provided a convenient and satisfactory tool for cytological analysis of Pacific oyster eggs. Meiotic events observed in normal diploid embryos agree with what has been described for the Pacific oyster (Lu, 1986). In treated groups, CB treatment effectively blocked the release of PB1 without inhibiting chromosome segregation and movement during meiosis I, which confirms observations by Longo (1972) and Komaru *et al.* (1990). However, blocking PB1 greatly changed chromosome segregation during the second meiotic division.

Segregation patterns following inhibition of PB1 observed in this study differ from that observed in the surf clam, *Spisula solidissima* by Longo (1972). When the formation of both polar bodies was blocked in fertilized eggs of the surf clam, four groups of maternally derived chromatids were most often produced, suggesting that the two groups of dyads from meiosis I might go through meiosis II through a separated bipolar segregation. In this study, the separated bipolar segregation was observed in only a small proportion of treated eggs (12%). The majority of the treated eggs (68%) went through a tripolar segregation to produce three groups of maternally derived chromatids (Table I). Although tripolar segregation was not described in his study, Longo (1972) did observe that sometimes three maternal pronuclei of unequal ploidy were present.

In the Japanese pearl oyster, *Pinctada fucata martensii,* Komaru *et al.* (1990) reported that three (20.6%) or four (17.6%) groups of maternally derived chromosomes were produced from blocking PB1. While the authors did not discuss how dyads segregated during meiosis II, it is likely that the four groups of maternal chromatids were formed through the separated bipolar segregation. However, there are two possible explanations why three groups of maternal chromosomes were observed by Komaru *et al.* (1990). One possibility is that the three groups of maternal chromosomes were produced through the tripolar segregations,

as described in this study. The other possibility is that one group of dyads from meiosis I did not divide during meiosis II, and the other group did. Evidence of the latter possibility was not found in this study, and in fact, dyads from meiosis I always entered meiosis II synchronously. It should be mentioned that in the Komaru *et al.* 1990 study, blocking PB1 produced 1.9% triploids and 98.1% diploids, suggesting that their CB treatment was not very effective.

Mechanisms for the formation of triploids, tetraploids and aneuploids are indicated by the segregation patterns we observed (Fig. 2). Triploids could be formed through either a united bipolar segregation (Fig. 2B), or a separated bipolar segregation (Fig. 2E). Triploids produced from a united bipolar segregation would undoubtedly be meiosis I triploids, which should be genetically different from meiosis II triploids. Triploids produced from a separated bipolar segregation were probably also meiosis I triploids, because the two chromatid sets released were most likely from two different divisions rather than from the same one. The formation of meiosis I triploids has been previously suggested by the fact that (1) treatments applied corresponding to meiosis I and II showed two windows of triploid induction (Arai *et al.*, 1986; Quillet and Panelay, 1986; Yamamoto and Sugawara, 1988) and (2) the triploids produced from treatment applied during meiosis I contained a higher level of genomic heterozygosity than triploids produced at meiosis II (Stanley *et al.*, 1984). However, this study does not rule out the possibility that blocking PB1 might overlap with blocking PB2 and produce meiosis II triploids as well (Downing and Allen, 1987).

Having shown that meiosis I triploids are genetically different from meiosis II triploids, this study does not necessarily imply that meiosis I triploids always have higher levels of heterozygosity than meiosis II triploids. Heterozygosity of meiosis I and II triploids is determined by the recombination rate (r). Considering only the maternal chromosome sets, the heterozygosity is predicted by "$1 - r/2$" in meiosis I triploids, and "r" in meiosis II triploids. Thus, meiosis I triploids would be more heterozygous than meiosis II triploids only if the recombination rate is lower than $\frac{2}{3}$. In the Pacific oyster, a preliminary study estimated that the average recombination rate was close to $\frac{2}{3}$ over seven allozyme coding loci (Guo and Gaffney, in prep.).

Following inhibition of PB1, the release of one set of chromosomes as PB2 through either a separated bipolar or an unmixed tripolar segregation will lead to the formation of a meiosis I tetraploid (Fig. 2D, E). Such tetraploids contain three maternal sets and one paternal set of chromosomes. Compared with tetraploids produced from blocking mitosis I, meiosis I tetraploids should have higher levels of heterozygosity. However, meiosis I tetraploids had very limited viability (Stephens and Downing, 1988; Stephens, 1989; Guo, 1991).

Aneuploids were apparently created as a consequence of the tripolar segregations. If the twenty dyads (ten from each group) derived from blocking PB1 enter the tripolar division randomly, the release of one of the three groups of chromatids in meiosis II left the egg, on average, with 26–27 maternally derived chromatids (Fig. 2C). The 26–27 maternally derived chromosomes plus the 10 paternally derived chromosomes would form an aneuploid with 36–37 chromosomes. In our previous paper (Guo *et al.*, 1992), karyological analysis has shown that induced aneuploids formed a peak around 35–37 chromosomes.

However, the question still remains as to how aneuploids with 23–25 chromosomes were formed. Besides creating aneuploids with 36–37 chromosomes, randomized tripolar segregation may suggest two other possibilities. First, two of the three groups of chromatids (13–14 per group) might be released as PB2s, leaving the egg with 13–14 maternal chromatids, which, combined with 10 paternal chromatids, form aneuploids with 23–24 chromosomes. The second possibility is that one group of chromatids was released as PB2, but another group failed to incorporate into the first mitosis or was incorporated into only one of the daughter cells, forming aneuploids with 23–24 chromosomes or mosaic aneuploids with 23–24/36–37 chromosomes (Fig. 2C). Evidence for both possibilities exists. In this study and in that of Komaru *et al.*, 1990, some eggs were observed to release two polar bodies after blocking PB1. Komaru *et al.* (1990) also found evidence that one group of maternal chromatids might be neither released as PB2 nor incorporated into the first mitosis. Similarly, aneuploids with, on the average, 25 chromosomes could also be produced from an unmixed tripolar segregation (Fig. 2D).

Difficulties in discriminating between the various types of tripolar segregations prevented direct correlation between segregation patterns and ploidies of resultant embryos. Increased incidence of aneuploids observed in the first cross was probably caused by a higher proportion of randomized tripolar segregations. It is possible that the two dyad groups from meiosis I united more frequently in eggs of the first cross than in eggs of the second cross, producing more randomized tripolar segregations in the first (increased aneuploidy) and more unmixed tripolar segregations in the second (increased tetraploidy).

The various tripolar segregations may also be responsible for the difference in ploidies reported from different studies on blocking PB1 (Stanley *et al.*, 1981; Quillet and Panelay, 1986; Stephens, 1989; Guo *et al.*, 1992). Results of this study indicate that formation of triploids, tetraploids or aneuploids depends on the configuration of the dyads when they enter the tripolar segregation. Since the movement of the two groups of dyads can be influenced

by many factors, such as temperature, salinity and egg quality (Lu, 1986; Downing and Allen, 1987), ploidies could vary greatly among species or in the same species with different experimental conditions. Stephens (1989) obtained a high percentage of tetraploids when she blocked PB1 at 18°C. Possibly at low temperature (18°C), the two groups of dyads from meiosis I moved too slowly to unite, primarily forming unmixed tripolar segregation and producing tetraploids.

In summary, CB-induced inhibition of PB1 in fertilized eggs of the Pacific oyster produced complicated patterns of chromosome segregation, yielding triploids, tetraploids and aneuploids in specific distributions. This study provided cytological evidence that blocking PB1 could produce meiosis I triploids that are genetically different from meiosis II triploids. Because meiosis I triploids exhibited faster growth rates than normal diploids and meiosis II triploids (Stanley *et al.*, 1984; Yamamoto *et al.*, 1988; Beaumont and Kelly, 1989; Jiang *et al.*, 1991), it may be necessary to re-evaluate blocking PB1 as an alternative method for triploid production. Although blocking PB1 usually produces lower survival and lower levels of triploids than blocking PB2, faster growth rate may compensate for the loss of eggs that are abundant in marine bivalves. It would be beneficial to test whether meiosis I triploids are indeed more heterozygous than meiosis II triploids (Stanley *et al.*, 1984), and whether treatments for blocking PB1 can be improved to produce more meiosis I triploids and fewer of other ploidies.

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