TIMING OF THE FIRST CLEAVAGE DIVISION
OF HAPLOID MOUSE EGGS, AND THE
DURATION OF ITS COMPONENT STAGES

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SUMMARY

Mouse eggs were activated by treatment with hyaluronidase which removed the follicle cells, followed by culture in vitro, and examined at the first cleavage mitosis. Second polar body extrusion usually occurred and haploid parthenogenesis was initiated. Air-dried chromosome preparations were made between 11 and 15.5 h after activation. Out of the 308 eggs examined 74 had already progressed to the 2-cell stage; the remaining 234 at the 1-cell stage were examined in detail. All chromosome preparations of the first cleavage mitosis were classified into groups corresponding with the stages of prometaphase, metaphase (early or 'pre-chromatid', 'chromatid' and 'late chromatid') and anaphase.

An indirect estimate was made of the duration of the first cleavage mitosis and of its component stages from the incidence of stages observed at different time intervals after activation. Similar eggs were also observed at 37 °C by time-lapse cine-photography and the interval between the disappearance of the pronucleus to the beginning of telophase of the first cleavage division was determined. The results of timing studies on the haploid eggs were compared with results obtained from similar observations on the first cleavage division of fertilized eggs which would of course normally be diploid.

Artificially activated eggs with 2 pronuclei, resulting from second polar body suppression, were also examined, and serial chromosome preparations during mitosis showed that the 2 pronuclear chromosome groups unite on the first cleavage spindle and divide to give a heterozygous diploid 2-cell embryo.

INTRODUCTION

It is possible to activate mouse eggs by various experimental means, for example, by the electrical stimulation of the ampullar region of the oviduct containing ova (Tarkowski, Witkowska & Nowicka, 1970), and by the removal of the cumulus cells with hyaluronidase, followed by culture in vitro (Graham, 1970, 1971, 1972; Kaufman, 1973b). The advantages and disadvantages of these methods have recently been reviewed by Tarkowski (1971); previous work on vertebrates has been critically reviewed by Beatty (1957, 1967) and mammalian work by Austin (1961) and Fechheimer (1968) and some of the mechanisms leading to parthenogenetic and heteroploid development have also recently been discussed (Beatty, 1972).

The development and cell numbers of diploid and polyploid embryos at day 3.5 were investigated by Beatty & Fischberg (1951), and their general conclusion was that the mean number of cells in polyploid embryos relative to diploids was approximately inversely proportional to the ploidy of the embryo. Edwards (1958) repeated this work but considered the cleavage number to be a more useful guide for comparing develop-
mental stages. However, in Edwards's analysis haploids as well as diploids were examined. Gynogenetic haploids recovered after irradiation or nitrogen mustard treatment of the sperm were all very retarded when compared to diploids, while very few haploids recovered after colchicine treatment of the eggs prior to insemination were observed to have cleaved at all. All of the gynogenetic haploids showed signs of abnormal cleavage, so that the relationship between ploidy and cleavage rate did not appear to relate to haploids. Edwards suggested that these differences might be due to a delay in the onset of the first cleavage division rather than different rates of cleavage between haploids (and triploids) when their development at day 3.5 was compared to diploids.

Tarkowski et al. (1970) found that the development of parthenogenetic embryos was retarded in the preimplantation period, and that the normal appearance of the embryos did not seem to be related to their degree of ploidy. The causes of the apparent failure of development of parthenogenetic embryos would seem to be multifactorial (Tarkowski, 1971; Graham, 1971) but it is possible that asynchrony with the uterus at the time of, or just after, implantation may be a very important component. It was with this in mind that the present investigation was initiated, to compare the time course of events at the first cleavage division of the haploid parthenogenone to confirm or deny that this division is delayed compared to similar events in fertilized eggs.

During the course of this and previous investigations on the activation of mouse eggs (Kaufman, 1973b) large numbers of haploid chromosome preparations were examined by the air-drying technique at all stages of the first cleavage mitosis. As a result of this it was possible to make observations on the normal chromosomal morphology in well spread preparations. The main stages of this mitosis are described and illustrated as an aid to those who are unfamiliar with the morphology of this atypical mitotic division.

In addition to the haploid parthenogenones, which constituted about 98% of the activated eggs (of those activated between 18 and 21 h after the HCG injection of superovulation), other types of parthenogenones were also obtained. About 1% of the activated eggs had 2 pronuclei resulting from second polar body suppression or failure of its extrusion, and these eggs were examined in detail prior to their first cleavage division. Because of the controversial nature of their fate, this aspect is discussed in some detail, and evidence presented to suggest that they develop into heterozygous diploid parthenogenones instead of undergoing a form of 'delayed immediate cleavage' to give haploid 2-cell embryos as suggested by Graham (1971, 1972).

MATERIALS AND METHODS

Activation of eggs

Female (C57Bl x A/J)F1 mice 8–10 weeks old were superovulated with 10 I.U. pregnant mares serum gonadotrophin (PMSG) followed at a 48-h interval by 10 I.U. of human chorionic gonadotrophin (HCG), and killed between 19 and 21 h after the HCG injection (ovulation occurs approximately 12 h after the HCG). Oocytes were liberated from the ampullae into a modified Krebs–Ringer bicarbonate culture medium containing 4 mg/ml bovine serum albumin (Whittingham, 1971) and 100 I.U./ml hyaluronidase (Koch–Light, ovine testes), and incubated at 37 °C in 5% CO₂ in air. After 10 min the eggs were isolated from this medium and
transferred to hyaluronidase-free medium and culture continued for a further 6 h. Eggs were examined under the ×50 magnification of a Wild dissecting microscope to determine the overall frequency and types of parthenogenones induced. Approximately 98% of the activated eggs in this time group had a single pronucleus and second polar body (Route 1a, haploid parthenogenesis, Beatty, 1957) and the overall activation frequency was in the region of 75–80% (Kaufman, 1973b).

All eggs with a single pronucleus and second polar body were washed in fresh medium and separated into 8 approximately equal batches. Each batch of eggs was transferred to a 30–50 µl droplet of medium under light liquid paraffin in separate plastic Petri dishes to facilitate further observation. The Petri dishes were returned to the incubator and culture continued. Single Petri dishes were then removed from the incubator at regular intervals from 11 to 15·5 h after the time of addition of eggs to the hyaluronidase medium (taken as time zero) and the eggs examined under the ×50 magnification of the dissecting microscope to determine the number of 1-cell and 2-cell eggs present. On average 8–10 eggs were present in each droplet for each experiment, the whole experiment was repeated on 4 occasions, and the results from each time interval were pooled.

**Fixed preparations.** All 1-cell eggs were isolated from the droplets of medium, washed in fresh medium and air-dried chromosome preparations made by the method described by Tarkowski (1966) and stained with 2% Giemsa (Giemsa stain R66, G. T. Gurr) at pH 7·0 for 1 h. Each chromosome preparation was classified according to its mitotic stage. A brief description of the classification used follows this section, and typical chromosome preparations appropriate to these stages are illustrated (Figs. 2–6).

**In vitro fertilisation and analysis by time-lapse cine-photomicrography of living eggs.** Detailed descriptions of the methods involved in these 2 operations have been published elsewhere (Kaufman, 1973a). However, only one aspect of the in vitro fertilization results has been reported previously, namely the 50% point for their entry into mitosis, which occurred at 17·48 h after the addition of eggs to the sperm suspension. The regression line based on the data for rate of entry of these eggs into mitosis is given here for the first time, and the comparable line for the haploid is presented in Fig. 1 (p. 557), and allows a comparison to be made between the timing of the 2 in vitro systems.

**Classification of the stages in the first cleavage mitosis**

This classification is based on that of McGaughey & Chang (1971), and has been reported in Kaufman (1973a). Because each stage is fairly precisely delineated, it allowed detailed analysis of observations derived from air-dried preparations. Figs. 2–8 illustrate some of the stages.

**RESULTS**

**Duration of the haploid first cleavage mitosis from observations on fixed preparations**

An analysis of the stages of mitosis in 308 eggs progressing through the first cleavage mitosis to the 2-cell stage observed at various times between 11 and 15·5 h after their addition to the hyaluronidase medium is presented in Table 1. When these data are re-expressed to show the proportion of eggs at the various stages in each time interval, they provide the information given in Table 2.

The total duration of the first cleavage mitosis was calculated to be 155·7 min by the method described previously (Kaufman, 1973a). From the overall duration the proportionate lengths of each stage can be derived, so that the duration of prometaphase was calculated to be 10·3 min, the early stage of metaphase (stage III), 77·9, the 'chromatid' or intermediate stage of metaphase (IV), 36·5, the 'late chromatid' stage (V), 10·3, and anaphase, 20·7 min.
Table 1. Numbers of eggs at different stages of mitosis when observed at various times between 11 and 15.5 h after activation

<table>
<thead>
<tr>
<th>Hours after induction</th>
<th>Stages of the first cleavage mitosis</th>
<th>Total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>11</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>11.75</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td>12.25</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>14.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2. Data from Table 1 rewritten to show the proportionate incidence of the various stages in each time group

<table>
<thead>
<tr>
<th>Hours after induction</th>
<th>Stages of the first cleavage mitosis</th>
<th>Total eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>11</td>
<td>1.000</td>
<td>0</td>
</tr>
<tr>
<td>11.75</td>
<td>0.861</td>
<td>0.111</td>
</tr>
<tr>
<td>12.25</td>
<td>0.590</td>
<td>0.077</td>
</tr>
<tr>
<td>13</td>
<td>0.250</td>
<td>0.021</td>
</tr>
<tr>
<td>14</td>
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<td>0.023</td>
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<tr>
<td>14.5</td>
<td>0</td>
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</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0.232</td>
</tr>
</tbody>
</table>

Proportionate length of each stage, and its duration, min

<table>
<thead>
<tr>
<th>Proportionate length of each stage, and its duration, min</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Duration of comparable stages in fertilized eggs</td>
<td></td>
</tr>
<tr>
<td>Duration of the haploid first cleavage mitosis from analyses of time-lapse cine-photomicrographic records</td>
<td></td>
</tr>
</tbody>
</table>

Activated haploid eggs were observed over a period of 8–10 h at 37 ± 0.25 °C from about 10 h after the inducing stimulus. As the interval between frames was 30 s the starting point of mitosis (when the pronuclear outline disappeared from view), and the end point (the beginning of telophase) could be assessed with considerable accuracy,
namely to within 1-2 frames in nearly all cases. In a few eggs it was technically impossible to determine either the beginning or end point (because the pronucleus was out of the plane of focus or because the cleaving egg was viewed end-on and the cleavage furrow could not be observed). These were excluded from the series.

Twenty-four eggs progressed to the 2-cell stage in a mean time of 145.7 ± 1.8 min.

**Timing studies on in vitro fertilization**

Previously published data (Kaufman, 1973a) from observations on (C₅₇Bl x A₁G)F₁ eggs fertilized in vitro by (C₅₇Bl x A₁G)F₁ sperm enabled the 50% point for eggs entering the first cleavage mitosis to be calculated. This point was achieved 17.48 h after the eggs were added to the sperm suspension. The comparable data for the activated group (see the first section, above) was 12.5 h after activation. The regression lines for the rate of entry into mitosis in the 2 groups are shown in Fig. 1.

**The fate of parthenogenetic eggs with 2 pronuclei**

In addition to the haploid parthenogenones already described, eggs with 2 pronuclei were also encountered (these resulted from second polar body suppression after anaphase) and about 20 examined by the air-drying technique at the first cleavage mitosis.

To avoid confusion it is necessary to subdivide Beatty’s (1957) route 1 c, leading to diploid parthenogenesis, into 2 groups. In group 1 c i, a single diploid female pronucleus is formed as a result of suppression of the 2nd polar body, and this was the pathway taken by rabbit eggs subjected to a temperature of 10 °C for 24 h (Chang, 1954). In
the second group, I c ii, suppression of the second polar body results in the formation of 2 haploid pronuclei, and the fate of these pronuclei is now discussed. Observation showed that these haploid groups act in the same way as the male and female pronuclear groups at fertilization by uniting on the first cleavage spindle, subsequently dividing at about the same time as in haploid parthenogenones to give diploid heterozygous embryos.

Graham (1971) isolated 18 of type I c ii parthenogenones 8 h after in vitro activation and made 2-hourly observations on them. He noted that the 2 pronuclei came together but did not form a common metaphase plate and that the 2 pronuclei eventually moved apart before cleavage occurred. Chromosome analysis on similar eggs (Graham, 1972) after their overnight culture in colcemid further suggested that haploid embryos resulted from this abnormal division as all 6 eggs examined had 2 groups of 20 chromosomes each (in 2 eggs one group contained 20 chromosomes while the other had 19 chromosomes). Such a result would, however, be expected even if fertilized eggs were subjected to colcemid treatment before the first cleavage spindle was allowed to form, as was the case with these eggs, and a colcemid environment maintained prior to chromosome analysis.

A few eggs of this type were obtained in addition to the haploid parthenogenones which were the main products of activation in the present series (Kaufman, 1973 a) and half-hourly observations made on them. Air-dried preparations were made on these eggs at varying time intervals after the 2 pronuclear outlines were observed to disappear. In this way serial chromosome preparations confirmed that the 2 pronuclear chromosome groups unite on the first cleavage spindle, and divide to give a diploid 2-cell embryo. Chromosome preparations of the critical stages of prometaphase and the 'chromatid' stage of metaphase in these embryos are illustrated in Figs. 7 and 8, respectively.

DISCUSSION

The timing of the stages of the first cleavage mitosis in fertilized eggs of superovulated and naturally mated mice has recently been reported (Kaufman, 1973 a). A comparison was made between the results obtained from direct analysis (by time-lapse cine-photomicrography) and indirect analyses of the mitotic events occurring after the breakdown of the male and female pronuclear membranes to the onset of cytokinesis. Mouse eggs which have been activated and induced to develop parthenogenetically have also been analysed by the use of similar direct and indirect approaches. With the data from this investigation it is now possible to compare the duration of the individual stages of the first cleavage mitosis and determine why the duration of mitosis in the haploid takes approximately 30 min longer than in the fertilized egg. Before this comparison can be made, however, one important morphological characteristic of the haploid mitosis must be considered. At fertilization chromosomal condensation takes place in the 2 pronuclei prior to syngamy, and 2 stages of prometaphase can be distinguished. After the brief initial stage (IIa) when filamentous chromatin fibres become apparent in both haploid and diploid systems, a second and later stage of
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prometaphase (IIb) can be distinguished only in eggs with 2 or more pronuclei. At this stage considerable condensation of the chromosomes of the 2 groups has occurred, though the 2 pronuclear groups are normally slightly asynchronous prior to the synzygy which is observed at syngamy or early metaphase (stage III) (McGaughey & Chang, 1971; Donahue, 1972), except in activated eggs with 2 pronuclei where the 2 groups seemed always to be synchronous (Fig. 7). In the haploid it is impossible to distinguish between the late pronuclear or condensed phase of prometaphase (IIb) and early metaphase (III), and for convenience in this context all haploids at these morphological stages have been classified as stage III. The direct analyses served to confirm the indirect data on the total duration of mitosis in these eggs. In haploid and diploid (fertilized) eggs early prometaphase (stage IIa) was of similar duration being 10.3 min in the haploid and 10.4 min in fertilized eggs. Similarly stage III in the haploid (77.9 min) was of approximately equal duration in the diploid (79.9 min) when for the reasons explained earlier, the duration of stages IIb and III are combined. For as yet unexplained reasons the duration of the intermediate (IV) and late (Va) stages of metaphase and the duration of anaphase (Vb) are quite different in the haploid compared to the diploid; the overall duration in the haploid was 67.5 min compared to 26.5 min in the diploid. The late stage of metaphase where chromatid separation probably takes place on the equator of the spindle (Va) prior to anaphase movement is slightly longer in the diploid (13.3 min compared to 10.3 min in the haploid) whereas the chromatid stage of metaphase (IV) and the stage of anaphase (Vb) take between 3 and 5 times as long in the haploid compared to the diploid. Stage IV in the haploid took 36.5 min compared to 7.3 min in the diploid, and stage Vb took 20.7 min in the haploid compared to 5.9 min in the diploid.

It remains to be seen whether diploid parthenogenones follow the timing schedule of fertilized eggs or parthenogenetic haploid eggs despite their difference in ploidy (manuscript in preparation).

The onset of mitosis in the haploid was compared to that of the diploid, using in vitro fertilization as the nearest comparable model. In both cases the starting point for timing studies was the time of addition of eggs to the stimulating medium — hyaluronidase medium for the induction of haploids, and a fresh epididymal sperm suspension for the diploids. The major difference between the 2 groups of eggs was in the time after superovulation that the females were killed. Recently ovulated eggs (HCG plus 14 h) were exclusively used for in vitro fertilization studies and relatively aged eggs (HCG plus 18–21 h) for the haploid timing studies. Freshly ovulated eggs could not be activated by simply removing the cumulus cells with hyaluronidase and placing them in culture (Kaufman, 1973b), and aged eggs (HCG plus 18–21 h) were often activated rather than fertilized when added to an epididymal sperm suspension. The difference in the time of onset of mitosis in these 2 groups is quite considerable, the 50% point for haploids entering mitosis being approximately 12.5 h after activation, almost exactly 5 h before the diploids in the in vitro fertilization system.

The pronuclei of parthenogenones appeared between 3 and 4 h after activation, while the male and female pronuclei in the fertilized eggs were usually seen between 5.5 and 7.5 h after the addition of eggs to the sperm suspension. The time of penetration
of spermatozoa from \( F_1 \) mice (from 2 inbred strains) tends to be considerably faster than when spermatozoa are obtained from inbred or crossbred males (Krzanowska, 1966), and for all the present studies both eggs and spermatozoa were obtained from \((\text{C}57\text{Bl} \times \text{A}2\text{G})F_1\) hybrid mice. Apart from the delay for capacitation, and this may be of quite short duration, the timing of fertilization \textit{in vitro} may not be very different from that occurring \textit{in vivo}. In a previous analysis (Kaufman, 1973a) the 50% point for eggs entering mitosis in \((\text{C}57\text{Bl} \times \text{A}2\text{G})F_1\) females mated to \((\text{C}57\text{Bl} \times \text{A}2\text{G})F_1\) males was 28.16 h after the HCG injection of superovulation, and 29.56 h after the HCG injection in CFLP females (Carworth, Europe) mated to CFLP males.

Due to ageing changes in the zona, this becomes permeable to certain stimulating substances including hyaluronidase, and a high proportion of these eggs are activated. This effect could not be reproduced in fresh eggs (Kaufman, 1973b). This contrasts markedly with the fertilization situation where eggs respond to the stimulus of sperm which have penetrated the zona and interacted with the vitelline membrane of the egg. \textit{In vitro} in particular the level of sperm hyaluronidase is likely to be very low as are the levels of other acrosomal enzymes which may be released in the region of the egg. This is due mainly to the small number of sperm reaching the site of fertilization (Zamboni, 1972) but it is impossible to exclude a similar but more specifically local action once the sperm has reached the perivitelline space.

Having observed that the initial cleavage division in haploid eggs is not delayed compared to diploids it is possible that the comparable situation \textit{in vivo} may arise only under conditions of delayed mating when other anomalies such as polyspermy are also more likely to arise (Austin, 1969). The duration of the first cleavage mitosis in the haploid has been shown to be only slightly longer than that of the diploid, so that the pattern of preimplantation development now remains to be investigated to determine whether the apparent retardation of these embryos by the normal time of implantation is a mitotic or intermitotic problem, or due to a more general nuclear and cytoplasmic deficiency state.

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REFERENCES

Haploid first cleavage mitosis in mouse


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Scale for all figures: bar represents 5 μm.

Fig. 2. Haploid mouse egg at the 'pre-chromatid' or early stage of metaphase of the first cleavage mitosis (stage III).

Fig. 3. Haploid mouse egg at the 'chromatid' stage of the first cleavage mitosis (stage IV).

Fig. 4. Haploid mouse egg at late metaphase (an early example of stage Vα) where the 2 chromatids are seen to be separating.
Haploid first cleavage mitosis in mouse
Fig. 5. Haploid mouse egg at late metaphase (a later example of stage Va than in Fig. 4) where complete chromatid separation has occurred, and a single group of 40 chromatids is present at the equator of the first cleavage spindle.

Fig. 6. Haploid mouse egg at anaphase of the first cleavage mitosis (stage Vb).
Haploid first cleavage mitosis in mouse
Fig. 7. Parthenogenetic mouse egg with 2 pronuclei (due to suppression of the second polar body) at the late prometaphase stage (an early example of stage IIb) where the 2 groups of chromosomes appear to be synchronous.

Fig. 8. Parthenogenetic mouse egg with two pronuclei (due to suppression of the second polar body) at the 'chromatid' stage of metaphase (stage IV) after the two haploid chromosome groups have united to give a diploid group of 40 chromosomes.