The effect of osmolarity on mouse parthenogenesis

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SUMMARY

Eggs from (C57B1 x AaG)F1 mice were activated by treatment with hyaluronidase, which removed the follicle cells, and cultured in vitro. Observations were made 6–8 h after hyaluronidase treatment to determine the frequency of activation and the types of parthenogenones induced. Cumulus-free eggs resulting from hyaluronidase treatment were incubated for 2 h in culture media of various osmolarities. The frequency of activation was found to be dependent on the postovulatory age of oocytes, while the types of parthenogenones induced were dependent on the osmolarity of the in vitro culture medium and their postovulatory age.

Culture in low osmolar medium suppressed the extrusion of the second polar body (2PB). This decreased the incidence of haploid eggs with a single pronucleus and 2PB and immediately cleaved eggs from 97.5% to 42.3% of the activated population. Where 2PB extrusion had been suppressed, 97.4% of parthenogenones contained two haploid pronuclei. Very few were observed with a single and presumably diploid pronucleus.

Serial observations from 11 to 18 h after hyaluronidase treatment were made on populations of activated eggs as they entered the first cleavage mitosis after 2 h incubation in medium either of normal (0.287 osmol) or low (0.168 osmol) osmolarity. A delay in the time of entry into the first cleavage mitosis similar to the duration of incubation in low osmolar medium was observed.

Further, eggs were incubated in control and low osmolar culture media containing uniformly labelled [U-14C]amino acid mixture to examine the extent of protein synthesis in recently activated eggs subjected to these culture conditions. An hypothesis is presented to explain the effect of incubation in low osmolar culture medium in delaying the first cleavage mitosis.

INTRODUCTION

The recent publications by Tarkowski, Witkowska & Nowicka (1970) and Graham (1970) on experimental parthenogenesis in the mouse have provided a considerable stimulus to workers in the field of mammalian developmental biology. More recently Tarkowski (1971), Graham (1971, 1972), Mintz & Gearhart (1973) and Kaufman (1973c) have proposed various hypotheses in an attempt to explain why mouse parthenogenones fail to develop beyond the early post-implantation period. At the present time it is not clear whether this is due to asynchrony between the parthenogenone and the uterus at the time of implantation (possibly resulting from the slower cleavage rate of these embryos compared to fertilized eggs), failure resulting from the absence of a cytoplasmic component normally provided by the fertilizing spermatozoon, the exposure and subsequent

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expression of recessive lethal genes (see review by Tarkowski, 1971), an incom-
plete zona reaction in these embryos (Mintz & Gearhart, 1973) or possibly a gene
dosage effect resulting from X-chromosome imbalance (Lyon, 1973, personal
communication).

The investigation of any of these hypotheses requires a simple experimental
approach capable of providing a large number of parthenogenones of various
types. Thus a high activation frequency and yield specificity are essential. It was
with these criteria in mind that the present investigation was undertaken.
Kaufman (1973a) showed that the postovulatory age of oocytes was a critical
factor in determining when the activating stimulus was applied. The use of
hyaluronidase medium, being a simplification of Graham's method of activation
(Graham, 1970), consistently provided an activation frequency of between 70
and 80% when eggs at the appropriate stage were stimulated. Approximately
98% of activated eggs were of the haploid type with a single pronucleus and
second polar body when eggs from females killed between 16 and 20 h after HCG
were stimulated, though maximal yields were obtained on stimulating eggs from
females killed between 18 and 20 h after HCG. A similar frequency of activation
was obtained when eggs from females killed approximately 25 h after HCG were
stimulated, though the incidence of the various types of parthenogenones differed
markedly, with a 62% incidence of haploid eggs of the immediate cleavage type.

Graham (1971, 1972) has demonstrated that decreasing the osmolarity of the
culture medium increases the frequency of suppression of second polar body
formation. This experimental approach potentially provides a means of inducing
large numbers of diploid parthenogenones only rarely obtained by the use of
isotonic culture conditions.

The demonstration that activated eggs with a single pronucleus (without a
second polar body) were diploid (Graham, 1972) and the more recent demonstra-
tion that eggs with two pronuclei (without a second polar body) were also
potentially diploid (Kaufman, 1973c) provided a stimulus for the present
investigation into the effect of osmolarity on activated eggs. The present work
complements the study of Brinster (1965) on the effect of osmolarity on the in
vitro development of fertilized mouse embryos, confirming that mouse oocytes
are able to withstand a very wide range in the osmolarity of the environment for
limited periods of time without apparent detrimental effects.

MATERIALS AND METHODS

(a) Activation of eggs. Female (C57Bl × A2G)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 14 and 25% h after the HCG injection (ovulation occurs
approximately 12 h after the HCG injection). Oocytes were liberated from the
ampullae into 0.5 ml of 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) contained in an embryological watchglass, and incubated at 37 °C in 5% CO₂ in air. After 15 min the eggs were isolated from this medium and transferred to another watch-glass containing 0.5 ml of hyaluronidase-free medium under 2 ml of light liquid paraffin. Atretic and fragmented eggs were discarded, presumably ovulated in response to the PMSG injection (Fowler & Edwards, 1957). After approximately 6–8 h the eggs were examined under the 50× magnification of a Wild dissecting microscope to determine the overall activation frequency and types of parthenogenones induced.

(b) Experiments to assess the response of eggs to handling. Female mice were ovulated as described in section (a) and killed at various times after HCG. Oocytes were liberated into culture medium (0.287 osmol) and incubated at 37 °C in 5% CO₂ in air for approximately 6 h. Eggs were then isolated from this medium and transferred to hyaluronidase medium for 5–10 min. This effectively removed the adherent cumulus cells, and allowed the activation frequency and types of parthenogenones induced in response to the stimuli associated with handling to be immediately assessed.

(c) Experiments to determine the effect of osmolarity. In these experiments standard culture medium (see section (a)) was diluted as follows: 1 vol. of water was added to 4 vols. of medium, referred to as 4/5 medium (b), or 2 vols. of water added to 3 vols. of medium, and referred to as 3/5 medium. The measured osmolarities of these media were as follows: undiluted culture medium, 0.287 osmol; 4/5 medium, 0.227 osmol; and 3/5 medium, 0.168 osmol.

Females were killed at various times after HCG and their eggs liberated from the ampullae directly into culture medium containing 100 i.u./ml hyaluronidase as described above. After 10–15 min eggs were isolated and transferred to either (a) undiluted culture medium, (b) 0.227 osmolar or (c) 0.168 osmolar medium. After 2 ½ h eggs from all groups were transferred to undiluted culture medium and examined approximately 6 h later to determine the overall activation frequency and types of parthenogenones induced.

Further groups of eggs from females killed between 20 and 21 h after HCG were incubated in hypertonic culture media for 2 ½ h prior to transfer to undiluted medium. The increased tonicity was achieved by the addition of solid sodium chloride to the medium. The osmolarities of these hypertonic media were 0.336 and 0.402 osmol.

(d) Experiments to assess the time of onset of the first cleavage mitosis. A detailed description of the method employed to assess the time of onset of the first cleavage mitosis in activated eggs with a single pronucleus and second polar body after incubation in undiluted culture medium has been reported elsewhere (Kaufman, 1973 c). A second method was employed with activated eggs which had been incubated in 0.168 osmolar medium.

At approximately 8 h after activation, eggs with a single pronucleus and second polar body and eggs with two pronuclei (without a second polar body) were
isolated from the culture dishes. Eggs of each type were then separated into batches of 10 eggs in 30–50 µl drops of medium under light liquid paraffin in 15 × 60 mm diameter disposable plastic Petri dishes (Falcon) to facilitate further observation (see Kaufman, 1973b). These dishes were returned to the incubator for further culture. At regular intervals from 13 to 18 h after activation the dishes were removed from the incubator and their contents examined under the 50 × magnification of a Wild dissecting microscope. Eggs where the pronuclear outline had disappeared were removed from the culture drops and examined by the air-drying technique (Tarkowski, 1966) to confirm their haploid or diploid status. Disappearance of the pronuclear outline(s) was used as a criterion for entry of the egg into the first cleavage mitosis.

\[ e \] [U-\( ^{14} \)C]amino acid mixture incorporation into proteins. (C57Bl × A2G)F\(_1\) female mice were superovulated and killed 20–21 h after HCG. The general format for this investigation is outlined diagrammatically in Fig. 1. Eggs from three females were treated for 15 min with hyaluronidase medium, the cumulus-free eggs pooled, and assigned to the various treatment groups. Eggs in groups 1–4 were incubated in 0·168 osmolar medium for the first 2\( \frac{1}{2} \) h of culture, then

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### Fig. 1. Format of experiment to assess [U-\( ^{14} \)C]amino acid mixture incorporation into proteins by activated eggs after incubation in low and normal osmolarity culture media.

- **H**: Duration of culture in labelled medium.
- **A**: First period of 1 h 7 min after hyaluronidase treatment.
- **B**: Second period of 1 h 7 min after hyaluronidase treatment.
- **C**: Second period of 2\( \frac{1}{2} \) h incubation.
- **D**: 2 h period of culture prior to selection of activated eggs for analysis.

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<table>
<thead>
<tr>
<th>Groups</th>
<th>0</th>
<th>0:168</th>
<th>0:287</th>
<th>0:287</th>
<th>0:287</th>
<th>1 h 7 min</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
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</tbody>
</table>

- **Time (h)**
transferred to 0.287 osmolar medium, whereas eggs in groups 5–8 were cultured throughout in 0.287 osmolar medium. In groups 1 and 5 eggs were incubated for the first 1 h 7 min in medium containing uniformly labelled [U-14C]amino acid mixture (10 μCi/ml) obtained from the Radiochemical Centre, Amersham (specific activity 57 mCi/m-atom carbon). Eggs in groups 2 and 6 were cultured in labelled medium for the second period of 1 h 7 min of culture. Groups 3 and 7, where eggs were incubated for 2 1/2 h in labelled medium, acted as controls for their respective groups. Eggs in groups 4 and 8 were transferred after 2 1/4 h of culture in unlabelled medium to 0.287 osmolar medium containing the label for 2 1/4 h incubation in this medium. All eggs were washed carefully in unlabelled medium of the appropriate osmolarity after each transfer. After approximately 7 h the contents of all dishes were examined with the 50 × magnification of a dissecting microscope.

All activated eggs were isolated and washed in unlabelled medium at the end of the experiment. Activated eggs were squashed between two discs of glass-fibre paper (Whatman GF/A 2.5 cm diameter). The subsequent procedure for treating these discs was as described by Monesi & Salfi (1967), with minor modifications. Discs were taken through several changes of cold 10 % trichloracetic acid to precipitate the proteins and remove the acid-soluble non-incorporated label. After the final wash in ether, the discs were air-dried and placed in counting vials with 5 ml scintillation fluid (6 g PPO/l of toluene). Samples were counted in a Tracerlab coru/atic 200 liquid scintillation spectrometer. Counts were corrected for background and quenching.

RESULTS

(i) Factors affecting the types of parthenogenones induced

(a) The effect of postovulatory age of oocytes

Groups 1–4 of Table 1 summarize the effect of postovulatory age on the types of parthenogenones induced in eggs isolated from females killed 14, 16–20, 21 and 25–25 1/2 h after HCG, treated with hyaluronidase prior to in vitro culture in standard (0.287 osmolar) medium (Kaufman, 1973a), while groups 5 and 6 demonstrate the effect of handling alone on eggs isolated at 16 and 18–19 1/2 h after HCG.

Groups 1, 5 and 6 (Table 1) represent the ‘background’ level of activation resulting from minimal handling of these eggs. The present data showed that this was only an effective activating stimulus to aged eggs, being totally ineffective with recently ovulated material. The stimuli involved here were probably both mechanical and due to transient fluctuations in temperature during the handling period. It should be emphasized, however, that a pulse of hyaluronidase of 10–15 min seemed to be capable of evoking a maximal response in susceptible groups of eggs in the present series, consistently giving an activation frequency of between 70 and 80 %. In most experiments when activated eggs were allowed
Table 1. The effect of postovulatory age and handling on the activation frequency and types of parthenogenones induced

<table>
<thead>
<tr>
<th>Group</th>
<th>HCG + hours</th>
<th>Activating stimulus</th>
<th>Total no. of eggs</th>
<th>Activated eggs (%)</th>
<th>Overall activation frequency (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 pronucleus + 2PB</td>
<td>Immediate cleavage</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
<td>Handling + hyaluronidase</td>
<td>172</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>16–20</td>
<td>Handling + hyaluronidase</td>
<td>465</td>
<td>257 (98.5)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>Handling + hyaluronidase</td>
<td>600</td>
<td>398 (92.6)</td>
<td>20 (4.7)</td>
</tr>
<tr>
<td>4</td>
<td>25–25 ½</td>
<td>Handling + hyaluronidase</td>
<td>382</td>
<td>96 (33.3)</td>
<td>179 (62.2)</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>Handling</td>
<td>126</td>
<td>33 (100.0)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>18–19 ½</td>
<td>Handling</td>
<td>229</td>
<td>100 (92.6)</td>
<td>2 (1.9)</td>
</tr>
</tbody>
</table>

* Lower value due to low overall activation frequency in '16 h' group (27.0 %, see Kaufman, 1973a).
Table 2. The effect of 2 1/2 hours incubation in culture media of various osmolarities on the types of parthenogenones induced in eggs from females killed 20–21, 15 1/4 and 25 1/2 hours after HCG

All eggs were preincubated in hyaluronidase medium, and observations made 6–8 h later.

<table>
<thead>
<tr>
<th>Group</th>
<th>HCG + hours</th>
<th>Measured osmolarity of medium (osmol)</th>
<th>Total no. of eggs</th>
<th>Activated eggs (%)</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 pronucleus + 2PB</td>
<td>Nil 2PB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Immediate cleavage</td>
<td>1 pronucleus 2 pronuclei</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20–21</td>
<td>0.168</td>
<td>1633</td>
<td>366 (29.8)</td>
<td>154 (12.5)</td>
</tr>
<tr>
<td>2</td>
<td>20–21</td>
<td>0.227</td>
<td>107</td>
<td>68 (86.1)</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>3</td>
<td>20–21</td>
<td>0.287</td>
<td>737</td>
<td>510 (93.6)</td>
<td>21 (3.9)</td>
</tr>
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<td>(control)</td>
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</tr>
<tr>
<td>4</td>
<td>20–21</td>
<td>0.336</td>
<td>375</td>
<td>263 (83.5)</td>
<td>19 (6.0)</td>
</tr>
<tr>
<td>5</td>
<td>20–21</td>
<td>0.402</td>
<td>147</td>
<td>94 (67.9)</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>15:25</td>
<td>0.168</td>
<td>50</td>
<td>0</td>
<td>0</td>
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<tr>
<td>7</td>
<td>15:25</td>
<td>0.227</td>
<td>50</td>
<td>0</td>
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<tr>
<td>8</td>
<td>25:5</td>
<td>0.168</td>
<td>43‡</td>
<td>7 (21.9)</td>
<td>9 (28.1)</td>
</tr>
</tbody>
</table>

* Cleavage rate at 24 h 82.9%.
† Cleavage rate at 24 h 18.8%.
‡ A further 46 eggs fragmented.
to remain in culture for a further 18 h almost 100% cleaved to the 2-cell stage (or to the 4-cell stage in the immediate cleavage class).

The significantly higher incidence of immediate cleavage eggs in the 25–25.5 h group (Table 1, group 4) compared to the incidence in groups 1, 2 and 3 (Table 1) has been discussed elsewhere (Kaufman, 1973a).

(b) The effect of osmolarity

Groups 1–5 of Table 2 demonstrate the effect of incubating cumulus-free eggs from females killed 20–21 h after HCG in media of various osmolarities (range 0.168–0.402 osmol) for 2.5 h directly after hyaluronidase treatment. All eggs were transferred to standard culture medium (0.287 osmol) for a further 4–6 h before observations were made on the activation frequency and types of parthenogenones present. Groups 6 and 7 of Table 2 represent the effect of 2.5 h incubation in 0.168 and 0.227 osmolar media on eggs from females killed 15.5 h after HCG, while group 8 demonstrates the effect of 0.168 osmolar medium on eggs isolated 25.5 h after HCG.

Because of the considerable differences which result when eggs are incubated for 2.5 h in hypo- and hypertonic media, these categories will be considered separately.

(1) Hypotonic media. The closeness of the overall activation frequencies, being 75.2% in the 0.168 osmolar and 73.8% in the 0.227 osmolar groups (Table 2, groups 1 and 2 respectively) and 74.0% in the control series would seem to confirm the hypothesis that hyaluronidase treatment and handling act as activating stimuli, and that the effect of incubation in hypotonic media during the period when eggs are completing their second meiotic division is to modify the pathway of development taken by these oocytes.

Reference to groups 6 and 7 of Table 2 demonstrate that the activation frequency of eggs isolated from females 15.5 h after HCG and added to either 0.168 or 0.227 osmolar medium is very low, suggesting that low osmolarity per se is not an activating stimulus. It is interesting that the two eggs which were activated (see group 6, Table 2) contained a single pronucleus but no second polar body. This class of parthenogenone has been observed by Graham (1971) in his experiments on the effect of osmolarity, though in the present series of experiments this class of parthenogenone was only rarely encountered.

An important feature illustrated in Table 2 is the increase in the incidence of oocytes with two pronuclei from 2.6% in the control series to 56.2% in the 0.168 osmolar group. This increase in response is significant ($P < 0.001$, $\chi^2 = 453.3$). In addition there is an increase in the incidence of activated eggs undergoing immediate cleavage from 3.9% to 12.5%, with a corresponding decrease in the incidence of eggs with a single pronucleus and second polar body from 93.6% to 29.8%.

The response of eggs added to 0.227 osmolar medium was intermediate between that observed in the control group and the 0.168 osmolar group.
A small number of eggs were observed in the 0·168 osmolar group with a single pronucleus (presumed diploid) without a second polar body, and this represented 1·5 % of the total number of activated eggs in this group. No eggs of this type were observed in the control series.

The results of experiments when eggs isolated from females killed 25±1 h after HCG were cultured for 2±1 h in 0·168 osmolar medium are presented in Table 2 (group 8). If only the apparently morphologically normal eggs are considered, the overall activation frequency of 74·4 % is very similar to the 74·0 % of the control series. The incidence of the various types of parthenogenones closely resembles the response to 0·168 osmolar medium observed in the 20–21 h group, with a high incidence of eggs with two pronuclei rather than of the immediate cleavage type as observed in the control series (group 4, Table 1). The incidence of fragmentation in these eggs was very high, being 51·7 % of the total eggs subjected to this treatment. No eggs were observed with a single (presumably diploid) pronucleus resulting from suppression of the second polar body in this time group.

(2) Hyper tonic media. The results from experiments where eggs from females killed 20–21 h after HCG were added to either 0·336 or 0·402 osmolar media are presented in Table 2 (groups 4 and 5 respectively). The eggs cultured in 0·336 osmolar medium appeared morphologically normal at 2±1 and 6 h after hyaluronidase treatment, and 84·0 % were activated. However, eggs which have been cultured in 0·402 osmolar medium all appeared to have an increased perivitelline space after 2±1 h, probably due to a decrease in the vitelline volume. At 6 h an overall activation frequency of 65·3 % was recorded, but the majority of pronuclei were smaller than are usually observed at this time. The majority of activated eggs had a single pronucleus and had extruded the second polar body, which also seemed in most cases to be smaller than usual. In some of the eggs, especially in the non-activated class, the vitelline outline was irregular. Further observations were made on both these groups of eggs at 24 h after activation to determine how many had progressed to the 2-cell stage (or 4-cell stage in the case of the immediate cleavage class). This data is presented in a footnote to Table 2. It will be apparent from this and previous data (see comment at the end of Results section (a)) that of all the media tested (from 0·168 to 0·402 osmol), the only one which definitely impaired development to the first cleavage division was the 0·402 osmolar medium. Significantly fewer eggs cleaved by 24 h after activation when incubated for 2±1 h in 0·402 as compared to 0·336 osmolar culture medium ($P < 0·001$, $\chi^2_{0.00} = 138·69$).

(ii) The effect of low osmolarity on the time of onset of the first cleavage mitosis in activated eggs

Serial observations have been reported elsewhere (Kaufman, 1973c) which enabled the 50 % point for activated eggs with a single pronucleus and second polar body (haploid) entering the first cleavage mitosis to be calculated. This
point was achieved approximately 12.4 h after activation when eggs remained in 0.287 osmolar medium during the total culture period. In this analysis 308 eggs were examined at various times between 11 and 15.5 h after their addition to hyaluronidase medium.

Serial observations from 13 to 18 h after activation were made on parthenogenones with a single pronucleus and second polar body (haploid) and eggs with 2 pronuclei without a second polar body (diploid) when activated eggs from females killed 20–21 h after HCG were incubated for 2.5 h in 0.168 osmolar medium. These results are presented in graphical form in Fig. 2, and all regression lines have been drawn by eye.

The time of entry into mitosis of eggs with a single pronucleus and second polar body represents the combined results from observations made on 128 haploid eggs, while serial observations on 226 eggs with two pronuclei provided the information for the second line.

The difference between the 50 % points for entry into mitosis in these two groups is approximately 20–30 min. Thus haploid and diploid parthenogenones induced under identical conditions enter mitosis at about the same time after activation.

The difference between the 50 % points for entry into mitosis in parthenogenones with a single pronucleus and second polar body between those cultured wholly in 0.287 osmolar medium and those incubated for 2.5 h in 0.168 osmolar
medium is approximately \(2\frac{1}{2}\) h. This difference in time is very close to the period spent in the low osmolar medium by the group of eggs which were delayed in entering mitosis.

(iii) \([U-^{14}C]\)amino acid mixture incorporation into proteins

The combined results from three experiments are presented in Table 3. Groups 5–8 represent controls for groups 1–4, while groups 3 and 7 act as controls for groups 1 and 2 (combined) and 5 and 6 (combined) respectively. The most interesting feature of this table is the reduced level of incorporation observed in groups 1–3 compared to groups 5–7. When these values are examined in more detail, the level of incorporation during the first hour of incubation in low osmolarity medium is just over one half that occurring in group 5. An increased uptake is observed in the second hour of incubation in low osmolarity medium. This is significantly above the level observed in group 1, but still significantly lower than that in the control (group 6). There is probably no significant difference between the levels of incorporation observed in groups 4 and 8.

**DISCUSSION**

The results presented in this paper are a detailed analysis of the effect of osmolarity on mouse eggs obtained at various times after the HCG injection of superovulation. Cumulus-free eggs were incubated in media within the range 0.168–0.402 osmol for a period of \(2\frac{1}{4}\) h directly after activation induced by pre-incubation in hyaluronidase medium. The results presented in Table 1 confirm and extend previous findings that the frequency of activation was dependent on the postovulatory age of oocytes when stimulated (Kaufman, 1973a). When eggs were incubated in media of different osmolarities directly after activation (see Table 2) the proportionate incidence of the various types of parthenogenones induced was considerably altered, though the overall activation frequency was not markedly affected. This either suggests that osmolarity *per se* is not an
activating stimulus, or that maximal rates of activation may already have occurred in response to the hyaluronidase treatment. Experiments with other strains of mice may be required to clarify this issue.

The response which was most marked and is indicated by the greatest difference in the proportionate incidence of the various types of parthenogenones encountered, was observed when eggs were incubated in 0.168 osmolar culture medium directly after activation. As an alternative to diluting all the components of the medium, lowering of the osmolarity may also be achieved by reducing the level of sodium in the medium (while maintaining the Na/K ratio). The effectiveness of this experimental approach remains to be tested.

The earliest time when second polar body extrusion was commonly observed in (C57Bl × A2G)F1 eggs was approximately 2.5 h after activation, so that incubation for this period in media of different osmolarities allows the investigator to test their effect on populations of eggs which would normally be completing meiosis II.

Graham (1971, 1972) has previously reported that decreasing the osmolarity of culture medium increased the frequency of second polar body suppression, and this observation is confirmed by the present findings. The major difference between the results presented in this series and those reported by Graham relate to the widely differing proportions of parthenogenones encountered with a single pronucleus in the absence of a second polar body (presumably diploids). These were only found in very low numbers in the present series, and several hypotheses may be forwarded to explain this variation. Culture conditions and techniques were not strictly comparable to those used by Graham, while there may be considerable strain variation in response to a similar activating stimulus. In the extensive series reported here, 56% of all eggs activated 20–21 h after HCG and incubated in 0.168 osmolar medium developed two pronuclei in the absence of a second polar body (see Table 2, group 1). These embryos are potentially diploid (Kaufman, 1973c).

The largest class of parthenogenones encountered after incubation in low osmolar medium (as described in this paper) will give rise to heterozygous diploid embryos (assuming crossing over has occurred during meiosis). It is likely that further analysis of these diploid embryos may provide more favourable subject material for the analysis of later development than haploid or homozygous diploid embryos.

The observation that parthenogenones were delayed on entering the first cleavage mitosis after incubation in low osmolar medium stimulated the investigation of the underlying mechanism. The results of the labelling experiments presented here demonstrate that the quantitative assessment of protein synthesis is one aspect of the biochemical events which occur during incubation in low osmolarity medium which can be effectively monitored. Incubating activated eggs in low osmolar medium reduced the overall level of uniformly labelled [U-14C]amino acid mixture incorporation into proteins to about half the level
observed in eggs cultured throughout under control conditions. Statistically significantly higher counts were recorded in the second hour of incubation in the labelled low osmolar medium compared to the first, suggesting that a partial accommodation to the low osmolarity medium may take place. Slightly lower counts were recorded in eggs previously cultured for \(2\frac{1}{2}\) h in 0-168 osmolar medium compared to control eggs cultured throughout in 0-287 osmolar medium. The significantly lower rate of protein synthesis in eggs cultured for the first 2\(\frac{1}{2}\) h in low osmolar medium may be due to changes in cell membrane permeability which result in a reduced uptake of amino acid precursors, possibly due to the low sodium concentration in the medium (Schultz & Curran, 1970). Alternatively, incubation in low osmolarity medium may result in a shift in the protein species synthesized. Qualitative analysis of protein from variously treated eggs will be required to distinguish between these two possibilities.

The activation of eggs \textit{in vitro} has the advantage over the \textit{in vivo} approach (Tarkowski \textit{et al.} 1970) in that much larger numbers of eggs can be handled by the investigator at any one time, so that synchronous populations can easily be made available for developmental or timing studies. Various classes of parthenogenones have been transferred to the oviducts of pseudopregnant recipients within 6 to 9 h of \textit{in vitro} activation (Kaufman & Gardner, 1974). A high proportion of the embryos isolated on day 4 appeared to be morphologically normal morulae or blastocysts, or evoked a decidual response when the uteri of recipients were examined on day 6 or 7 of pseudopregnancy. Thus aged eggs isolated 20–21 h after HCG and induced to develop parthenogenetically by \textit{in vitro} activation are fully capable of developing at least to the blastocyst stage on transfer to pseudopregnant recipients. The other great advantage of the \textit{in vitro} approach is its relative ease and reliability, and this may lead in the near future to the establishment of haploid and diploid parthenogenetic cell lines which could be made available for genetic and biochemical research.

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REFERENCES


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