The reaction of the mouse blastocyst and its zona pellucida to enzymes in vitro

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SUMMARY

Mouse blastocysts were cultured for 24 h in the presence of various substances at concentrations as high as possible, consistent with blastocyst survival. Lysis of the zona pellucida was produced by trypsin and pronase, but not by hyaluronidase, collagenase, β-glucuronidase, ribonuclease, neuraminidase or oestrogen. This suggests that the zona lysin secreted by the mouse uterus at the time of implantation consists of a proteolytic enzyme.

INTRODUCTION

Lysis of the zona pellucida, the mucoprotein coat surrounding the pre-implantation embryo, occurs in the mouse at about the time that implantation begins, probably through the action of some factor emanating from the oestrogen-sensitized uterus (McLaren, 1970). Decreased pH is unlikely to be responsible, since it has been shown in vitro that a pH low enough to lyse the zona is incompatible with the survival of the blastocyst (Bowman & McLaren, 1969). We must therefore assume that the lysis is induced by an enzyme.

Some studies have already been made on the effect of enzymes on the zona of the mouse (Smithberg, 1953; Mintz, 1962; Gwatkin, 1964) and other rodents (Chang & Hunt, 1956; Stambaugh & Buckley, 1968). With mouse eggs, pronase and ficin have been consistently found to produce lysis of the zona; trypsin, chymotrypsin and papain have been reported as effective by some authors, ineffective by others; while enzymes other than proteases have been consistently ineffective.

All the earlier work, however, has been concerned with one-cell eggs, stimulated by interest in the mechanism of sperm penetration of the zona, or has involved relatively short exposure of the egg to enzymes, with a view to developing a convenient technique for zona removal. These conditions throw little light on zona loss in the uterus. In vivo, the egg is present in the uterus for about 24 h before zona lysis takes place, and is at the blastocyst stage of development. We have therefore re-examined the effect of certain enzymes on the zona of

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blastocysts maintained in culture for 24 h, over the period when the zona would normally be lost. Wherever possible, we have used the enzymes at a range of concentrations, including the highest compatible with blastocyst survival.

**MATERIALS AND METHODS**

Embryos were recovered by flushing the uteri of females of the randomly bred Q strain of mice at about mid-day, 3½ days post coitum, i.e. half a day before implantation would normally begin. They were cultured in modified Brinster's medium, as described by Bowman & McLaren (1970), at a temperature of 36 °C and a pH of 7·3, in an atmosphere of 10 % CO₂ in air. For each enzyme a concentrated stock solution was made up in distilled water, and diluted with culture medium to give the required final concentration. The following enzymes were tested: hyaluronidase (from bovine testes, Sigma), collagenase (from *Clostridium histolyticum*, Koch-Light), β-glucuronidase (from liver, Fluka), ribonuclease (from bovine pancreas, Calbiochem), neuraminidase (from *Chole rae*, British Drug Houses), trypsin (British Drug Houses) and pronase (from *Streptomyces griseus*, Calbiochem). No attempt was made to adjust the pH to that optimal for enzyme action, but in each case the enzyme was known to be effective at the pH used. Oestrogen (1,3,5 (10)—Estratrien-3, 17β-diol, Koch-Light) was also tested, using a 10⁻² M solution of oestradiol-17β in absolute ethanol diluted in culture medium to the required final concentration.

**Table 1. The effect of enzymes on the survival of mouse embryos and on lysis of the zona pellucida**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Range of concentrations tested</th>
<th>No. of concentrations tested</th>
<th>No. of embryos Total</th>
<th>Survived</th>
<th>No. of zonas lysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase</td>
<td>0·0125–0·05 %</td>
<td>3</td>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0·1–0·2 %</td>
<td>2</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Collagenase</td>
<td>0·05–0·25 %</td>
<td>3</td>
<td>34</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0·5 %</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>0·125–2·0 %</td>
<td>5</td>
<td>49</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>0·0005–0·05 %</td>
<td>3</td>
<td>46</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>0·01–50 units/ml</td>
<td>10</td>
<td>64</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Oestrogen</td>
<td>10⁻⁸ M</td>
<td>1</td>
<td>18</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁻⁹ M</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10⁻¹⁰ M</td>
<td>1</td>
<td>13</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5 × 10⁻³ M–10⁻³ M</td>
<td>2</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0·03–0·25 %</td>
<td>4</td>
<td>34</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>0·5 %</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Pronase</td>
<td>0·0005–0·001 %</td>
<td>2</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0·002 %</td>
<td>1</td>
<td>8</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>
RESULTS

The results are shown in Table 1. Both pronase and trypsin were effective in lysing the zona at all concentrations tested. At the highest concentration of pronase (0.002%), zonae were still present after 2 h of culture, but had gone by 9 h. For comparison, at the much higher pronase concentration normally used for zona removal (0.5%), lysis takes about 10 min at room temperature. The surviving pronase-treated blastocysts looked normal, in contrast to the surviving trypsin-treated blastocysts, which were beginning to degenerate. None of the other substances tested proved capable of producing zona lysis, even at grossly non-physiological concentrations.

DISCUSSION

Only the two proteolytic enzymes, pronase and trypsin, induced lysis of the zona. This is in general agreement with previous experience, though Gwatkin (1964), using a similar range of concentrations to that which proved effective in the present work, observed no zona lysis with trypsin. On the other hand, Smithberg (1953) and Mintz (1962), who both found trypsin effective, do not mention the concentrations used.

Oestrogen was tested because its presence appears to be a necessary condition for loss of the zona, and there is still doubt as to whether it affects the blastocyst directly (see McLaren, 1969). The present results suggest that, in so far as the zona is concerned, oestrogen acts by way of the uterus, rather than directly on the blastocyst.

Although the decidual reaction in the rat is associated with the breakdown of collagen in the uterine stroma (Fainstat, 1963), the bacterial collagenase tested produced no zona lysis, even at a concentration which killed the blastocysts. Hyaluronidase has been discussed in connexion with dissolution of the zona because of its high concentration in spermatozoa, but the findings of Stambaugh & Buckley (1968) and Chang & Hunt (1956), as well as the present study, suggest that it is the trypsin rather than the hyaluronidase content of sperm which is responsible for their transit through the rodent zona. Neuraminidase was tested on account of the high sialic acid content of the mouse zona, but produced no lysis. Ribonuclease and β-glucuronidase, known to be present in rat and human endometrium, also proved ineffective. This is perhaps not surprising, since neither shows an increase in activity at the time of implantation (Wood & Psychoyos, 1967; Wood, Williams, Barley & Cowdell, 1969).

Our results suggest that lysis of the mouse zona is brought about by a proteolytic enzyme secreted by the endometrium under the influence of oestrogen. Although it was not possible to obtain a sample to test, acid cathepsin D appears to be a likely candidate. It is involved in the breakdown of extracellular collagen in the connective tissue matrix of the post-partum involuting rat and human uterus (Woessner & Brewer, 1963; Woessner, 1965), and has a higher
activity in human endometrium during the secretory than during the proliferative phase of the menstrual cycle (Wood et al. 1969). Of greater relevance to the rodent zona is the fact that acid cathepsin D shows a peak of activity in the rat endometrium in early pseudopregnancy (Wood & Psychoyos, 1967; Wood, 1969), at about the stage that zona lysis would be expected in the pregnant animal.

**Résumé**

La réaction à des enzymes in vitro du blastocyste de souris et de sa zone pellucide

Des blastocystes de Souris ont été cultivés pendant 24 h en présence de substances variées dont les concentrations étaient aussi fortes que possible tout en étant compatibles avec la survie du blastocyste. La lyse de la zone pellucide a été produite par de la trypsine et de la pronase, mais non par de l'hyaluronidase ni par de la collagénase, β-glucuronidase, ribonucléase, neuraminidase, ni de l'oestrogène. Ceci suggère que la lysine de la zone secrétée par l'utérus de la souris au moment de l'implantation est une enzyme protéolytique.

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**REFERENCES**


(Manuscript received 29 January 1970)