Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst

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INTRODUCTION

The mammalian ovum is surrounded by a secondary membrane, the zona pellucida, which is produced by the follicle cells round the ovarian oocyte. The major constituents of this membrane are neutral or weakly acidic mucoproteins, and it is sensitive to dissolution by a variety of proteases, depending on species (Gwatkin, 1964), especially the bacterial protease 'Pronase' (Mintz, 1962). In the rabbit and rat the zona is impermeable to compounds with a molecular weight above 1200 (Austin & Lovelock, 1958). Since in all mammals, an intact zona surrounds the cleavage stages and early blastocyst, this membrane must be removed, or penetrated by the trophoblast cells before implantation can take place. This could result from the interaction of chemical or mechanical factors produced either by the conceptus or by the uterus, or from a combination of some or all of these.

Where marked expansion of the blastocyst occurs before implantation, as in the rabbit, the zona becomes extremely attenuated and almost invisible in the living conceptus. In the mouse, rat, guinea-pig and hamster, however, the diameter of the zona at the time of implantation is not greatly larger than that of the tubal ova, and the membrane is only slightly thinned, therefore facilitating examination and photography. In the case of the mouse, especially, the development of in vitro culture methods for ova and blastocysts has made direct observations of the method of escape from the zona possible. These studies indicate that in this species at least uterine factors play a minimal role.

Whitten (1957) observed lobes extruded from the trophoblast and penetrating the zona of mouse blastocysts in vitro and this observation was later confirmed and extended by Mintz (1962), who showed that these lobes were produced even after enzymic dissolution of the zona, and that the mouse blastocyst could escape from the zona in vitro. Similar escape of the mouse blastocyst from its zona in other media has been reported (Brinster, 1963; Cole & Paul, 1965). The

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present series of observations resulted from the finding that Waymouth's medium MB752/1 (Waymouth, 1959), supplemented with serum, supported the development of mouse ova through blastocyst formation, escape from the zona, and subsequent differentiation of the trophoblast cells (Cole & Paul, 1965).

**MATERIALS AND METHODS**

Blastocysts were isolated from superovulated random bred albino mice (Porton strain) and cultured as previously described (Cole & Paul, 1965). Previous cinemicrographic studies on mouse ova (Kuhl & Friedrich-Freksa, 1936; Borghese & Cassini, 1963) were carried out with the ova cultured in closed chambers in conditions which we found did not allow escape from the zona to occur. In order to overcome the disadvantages of these chambers a pattern shown in Text-fig. 1 was adopted. These chambers were constructed from parts of 'Falcon' polystyrene tissue culture dishes bonded with Araldite or polystyrene cement, and were used only once. After construction the assembly was washed with non-toxic detergent (Decon 75) and sterilized with alcohol or by ultraviolet irradiation. The gas phase in the chamber was maintained at 5 % CO₂ in air by continuous slow flushing with this mixture through the ports shown, or where this was not convenient, by use of Pardee's diethanolamine manometric buffer (Umbreit, Burris & Stauffer, 1957) in the outer reservoir.

The records were made on Gevapan 26R film with a Bolex H16 camera controlled by an Emdeco time lapse unit. A Baker Interference microscope enclosed in a 37 °C warm-box was used, set also for phase contrast or normal bright field illumination depending on the culture. A green filter was used to protect the cultures, which were only illuminated during the photographic exposures.
RESULTS

The blastocysts used for this series of observations were isolated from the uteri by flushing 96–98 h after the estimated time of ovulation, following the injection of H.C.G. Mouse blastocysts examined immediately after flushing from the uterus in satisfactory conditions, show a variety of forms (Plate 1). Some appear fully expanded with the trophoblast flattened and closely adherent to the zona, while others show the cell mass tightly contracted, with little or no blastocoelic space visible, and intermediates between these extremes are also found. The fully contracted blastocysts can easily be mistaken for late morulae or degenerating forms on superficial examination. This range of appearances parallels that undergone by individual blastocysts during the cycles of contraction and re-expansion described below, and indicates that similar cycles occur in utero. In inadequate media, or as a result of damage or physiological stress, e.g. osmotic or high temperature shock, all blastocysts become contracted within the zona and are unable to recover, indicating that the uptake of water is an active process.

Previous workers have demonstrated the existence of a rhythmic series of contractions and expansions of blastocysts of the rabbit (Lewis & Gregory, 1929) and of the mouse (Kuhl & Friedrich-Freska, 1936; Borghese & Cassini, 1963), but have been unable to relate this to any subsequent behaviour of the blastocyst. However, our observations suggest that these cycles are an essential and normal part of the mechanism by which the mouse blastocyst escapes from the zona in utero.

Because of the existence of these cycles, evaluations of blastocyst development based on size after flushing are of limited value, especially as in many investigations media not containing protein, or even saline solutions, which have a rapidly deleterious effect, have been used.

The penetration of the zona

The relationship between the volume changes of the blastocyst and zona and the time of initial penetration of the zona in individual blastocysts are shown in Text-figs. 2 and 3. The calculations on which these figures are based assume that the blastocysts are spherical throughout the period of the observation. Contractions of the blastocysts are relatively rapid, each occupying only 4–5 min, i.e. 2–3 % of an individual cycle, while the re-expansion takes place approximately linearly, but much more slowly, occupying 2–3 h. The zona pellucida also shows a slight elasticity, contracting rather slowly over approximately 2 h following the sudden contraction of the blastocyst. In the initial cycles of contraction and re-expansion the changes in the blastocyst occur to the extent of approximately 45 % of its maximum total volume, and in the zona to approximately 25 %. The final expansion observed in this sequence is greater than those observed earlier and, although the blastocyst is able to contract as tightly as
before, the zona appears to be unable to recover its original dimensions. It is not yet known whether an irreversible change in the zona precedes and allows this large expansion or whether it is caused directly by the mechanical effects of stretching by the blastocyst. For a period of 30 min beginning 8 h 12 min (indicated by (a)) after the first observation one blastocyst underwent a sequence of partial gyrations, apparently while it was expanding within the zona pellucida. Similar spontaneous movements have been seen in blastocysts from which the zona has been artificially removed. The cellular projections first penetrated the zona 8 h 42 min (indicated by (b)) after the beginning of filming.

A similar sequence of contractions and re-expansion occurring in a second blastocyst is shown in Text-fig. 3. The temporal sequence is similar and the large late expansion also occurred, the blastocyst increasing its volume by 75 % over the previous maximum, but no gyration or penetration of the zona was observed in this case. Cycles of volume change may also occur in blastocysts artificially freed of the zona, and in early blastocysts which have not fully expanded within the zona pellucida. The time sequence of the behaviour of such a blastocyst is shown in Table 1.

A more detailed analysis of the penetration of the zona pellucida is illustrated
Fig. A. Mouse blastocysts flushed from the uterus 98 h after the estimated time of ovulation photographed after 30 min in culture. The blastocysts recorded are indicated: (a) Table 1, (b) Text-fig. 2, (c) Text-fig. 3, (d) Plate 2. Bright field illumination, copied from a 16 mm ciné frame.

Fig. B. The initial penetration of the zona pellucida; (a) 97 h 45 min after the estimated time of ovulation (1.5 h in culture), (b) 99 h 45 min (c) 101 h 40 min, (d) 101 h 45 min, (e) 102 h 37 min, (f) a newly released blastocyst from the same culture. Phase contrast, copied from 16 mm ciné frames.
Successive stages in the escape of a mouse blastocyst from the zona pellucida beginning 98 h. after the estimated time of ovulation and ending 17\(\frac{1}{2}\) h later. Full explanation in text. Bright field illumination.

R. J. Cole
Mouse trophoblast and zona

in Plate 1, fig. B. The zona was first penetrated 97\frac{3}{8} h after the estimated time of ovulation (1\frac{3}{8} h filming) when a small mobile process was extruded (a). This was withdrawn (b) after 2 h but left no obvious gap in the zona. After a further 55 min the mobile processes reappeared, and over the next hour grew into a large fluid filled ‘bleb’ (c). When this was suddenly withdrawn (d), 4 h after the first penetration, an obvious break in the zona was left open. These blebs rapidly reappeared (e), 4 h 42 min after the initial penetration, and the blastocyst contracted sharply 3 h later. This sequence of film suggests that the fluid within these processes is not continuous with the blastocoelic fluid but is contained

within individual cells. If this is the case then the initial breakage in the zona is unlikely to be a simple mechanical result of increasing blastocoelic pressure, but rather due to the function of specific cells. A newly escaped blastocyst from the same culture is shown in (f). The appearance of this zona left behind in vitro, with the characteristic tear through which the blastocyst has passed, is very similar to that of empty zona which can be flushed from mouse uteri at the time of implantation.

Escape from the zona pellucida

The escape of a blastocyst from the zona following the initial penetration is shown in Plate 2. At the beginning of the record, 98 h after the estimated time of
ovulation (1A) the blastocyst had penetrated the zona, adjacent to the margin of the embryonic cell mass, which is clearly shown. Expansion of the extruded part of the blastocyst continued (1B, 45 min; 1C, 1 hr 36 min) for 2 h 45 min (1D), when a slight contraction occurred (2A, 2 h 47 min). The blastocyst expanded again (2B, 4 h 14 min) and then underwent a relatively slow contraction (2C, 4 h 26 min) when the blastocyst had fully re-expanded (2D, 6 h 14 min) the embryonic cell mass was clearly outside the zona. These cycles of contraction and expansion continued (3A, 6 h 17 min; 3B, 8 h 36 min; 3C, 8 h 38 min; 3D, 9 h 53 min). A slight contraction occurred at 9 h 56 min but is not illustrated.

After 14 h 4 min (4 A) the blastocyst was again fully expanded and the break in the zona considerably enlarged. A further contraction (4B, 14 h 8 min) was followed by a continuous expansion (4C, 15 h 5 min; 4D, 15 h 59 min). After 16 h 53 min (5 A) the final phase of the expulsion of the blastocyst began, apparently as a result of the elasticity of the zona. The volume of the extruded portion of the blastocyst is then larger than that still retained within the zona (5B, 17 h 18 min). The blastocyst was considerably elongated at the time of release (5C, 17 h 24 min), but re-assumed a more spherical form (5D, 17 h 45 min).

Table 1. *Time course of behaviour of early blastocyst*

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Time Interval</th>
<th>Interval between complete contractions</th>
</tr>
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<tbody>
<tr>
<td>Initial observation (see Pl. 1)</td>
<td>0 0</td>
<td>—</td>
</tr>
<tr>
<td>Slight contraction followed by re-expansion</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Slow partial contraction</td>
<td>1 52 09 20</td>
<td>7h. 30 min</td>
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<tr>
<td>Slight contraction followed by re-expansion</td>
<td></td>
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</tr>
<tr>
<td>Complete contraction followed by re-expansion</td>
<td>1 13 56 54 54</td>
<td>—</td>
</tr>
<tr>
<td>Complete contraction followed by re-expansion</td>
<td></td>
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<tr>
<td>Slight contraction</td>
<td>2 17 52</td>
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DISCUSSION

It is now well established that in the mouse and rat uterine factors play a minimal role in the release of the blastocyst from the zona pellucida, although in the hamster this process appears to be progesterone dependent (Orsini, 1963). In addition to the in vitro conditions described, mouse blastocysts can free themselves from the zona when implanted into the anterior chamber of the eye (Runner, 1947) and when contained in diffusion chambers implanted into the peritoneal cavity of male mice (Bryson, 1964). Severe interference with progestational balance and the oestrogen surge in rats by hypophysectomy, ovariectomy and adrenalectomy does not prevent shedding of the zona, though this may be slightly delayed (Mayer, 1966). It has been suggested that herniation of the mouse zona observed in vitro is an artifact due to abnormal water uptake and turgor of the blastocyst. This investigation suggests that the initial penetration of the zona, while depending on the turgor maintained by the blastocoelic fluid, is a specific process. This conclusion is supported by Blandau's (1961) observations on the loss of the zona by the guinea-pig blastocyst in vitro. In this species, the abembryonal pole cells proliferate in the 4–5 h preceding implantation to form the implantation cone. Many cytoplasmic processes are then extended from this region through the zona, which becomes locally thinned and open, leaving the rest of the membrane to be sloughed off intact. Similar attachment cones occur in ground squirrels and chipmunks (Mossman, 1937) but not in the mouse, although thickening of the abembryonal trophoblast occurs after escape from the zona. Wilson (1963) has described specialized ‘primary invasive cells’ in mouse blastocysts originating in the embryonic mass but it is not known if these are associated with penetration of the zona.

The mechanisms underlying the uptake of water which leads to the cyclical re-expansion of the mouse blastocyst are probably similar to those responsible for blastocyst expansion in other mammals. Studies of blastocyst expansion in the rabbit (Lutwak-Mann, Bournsell & Bennett, 1960) have suggested that the water uptake associated with this change is the result of active transport, and in most vertebrate systems active water movement appears to be secondary to solute movement (Diamond, 1965).

In attempting an analysis of the physical factors underlying water uptake by the mammalian blastocyst the following parameters must be considered: the nature of the outer and inner surfaces of the zona, the outer and inner surfaces of the trophoblast cells and any intercellular material between these cells; the ionic and osmotically active components of the fluid compartments including the uterine fluid (or culture medium), fluid incorporated into the zona, fluid between the zona and the outer surface of the blastocyst, fluid within the cells of the blastocyst, and the blastocoelic fluid. Since rhythmic contraction and re-expansion can occur in mouse blastocysts from which the zonae have been artificially removed, and also in partly freed blastocysts with a large part of their tropho-
blastic surface in direct contact with the medium, the zona, and the fluid between it and the trophoblast, are probably neutral. Filtration effects can be excluded since the measurements presented here show that water can be accumulated within the mouse blastocyst against the elastic resistance of the zona. In the rabbit blastocyst in utero expansion appears to take place against the resistance of the uterine musculature.

Mechanisms which may be involved include passive water drag and electro-osmosis (Kedem, 1965), double membrane effects (Durbin, 1960) and pinocytosis (Chapman-Andresen, 1962).

Mouse trophoblast cells in culture, when attached and spread, show vigorous pinocytosis (Cole & Paul, 1965). However, pinocytosis is not obvious in unattached blastocysts, either within or free from the zona. No decision as to the most likely of these mechanisms for water uptake in the mouse blastocyst can yet be made but advances in handling and culture techniques now make experimental analysis possible.

The loss of water, resulting in contraction of the blastocyst, is at least 50 times more rapid than uptake. It has been suggested that this sudden water loss is due to a tear in the blastocyst but such a break has yet to be described. If ionic pumping systems are responsible, directly or indirectly, for water uptake this rapid loss could be due to a sudden depolarization of the cell membrane. Our analysis suggest that contraction of the blastocyst occurs as soon as a critical volume is reached, without any plateau, so that this behaviour could be initiated as a result of the stretched trophoblast cells reaching a critical surface/volume ratio and could perhaps be reinforced by the induction of streaming potentials (Diamond, 1965). It is not known, however, whether the blastocoelic fluid escapes through or between the cells.

It has been considered that blastocyst expansion provides a larger site over which the implanting conceptus can come into contact with the maternal tissue and that whatever the mechanisms of water uptake, they have evolved to facilitate this process. However, it is also possible that water uptake is a secondary but necessary consequence of the need to incorporate certain solutes from the uterine fluid. The maximum size attainable by a functionable blastocyst would be limited by the number of cells comprising the conceptus at the time of implantation and by the relationship between the number of eggs shed and the size of the available implantation sites. One function of the relatively tough zona pellucida in the mouse and rat may therefore be to limit excessive blastocyst expansion resulting from water uptake during a critical stage of development. Direct analysis of the fluid contained within the mouse blastocyst is obviously limited by the small amounts of material available. In the rabbit, however, studies of this type have been carried out. In general, it appears that amino acids (Lutwak-Mann & Hay, 1965), glucose, lactate and bicarbonate, and sodium, potassium and chloride ions (Lutwak-Mann et al., 1960) are not present in amounts widely different from those in the uterine fluid.
SUMMARY

1. The behaviour of mouse blastocysts during penetration and shedding of the zona pellucida was studied \textit{in vitro} by time lapse cinemicrography.

2. Blastocysts obtained by super-ovulation were filmed in Waymouth’s medium MB 725/1 in a specially designed disposable plastic chamber with a gas space buffered to maintain 5\% CO$_2$ in air.

3. Mouse blastocysts undergo cycles of rapid contraction (4–5 min) followed by slower re-expansion (2–3 h), both before and after penetration of the zona. The zona also exhibits slight elasticity unless excessively stretched.

4. The initial penetration of the zona was followed in detail and appears to be the result of specific cellular activity, not non-specific herniation due to increasing blastocoelic pressure. Rotation of the blastocyst was also observed.

RÉSUMÉ

Observation cinémicrographique du trophoblaste et de la zone pellucide de blastocystes de souris

1. Le comportement de blastocystes de souris a été étudié par des observations cinémicrographiques successives pendant leur pénétration et au moment de la perte de la zone pellucide.

2. Les blastocystes, obtenus par super-ovulation, ont été filmé dans une chambre en plastique spécialement conçue et contenant du milieu de Waymouth (MB 725/1) ayant une concentration de 5\% en CO$_2$.

3. Avant et après la pénétration de la zone pellucide, les blastocystes de souris se contractent (4–5 min) à intervalles réguliers, entre ces contractions on observe des périodes de ‘réexpansion’ de 2 à 3 h. La zone possède également un certain degré d’élasticité, sauf lorsqu’il est excessivement tendu.

4. L’implantation initiale de la zone pellucide a été suivie en détail; elle semble résulter d’une activité cellulaire spécifique plutôt que d’une augmentation de la pression blastocoelique. La rotation du blastocyste a également été observée.

I wish to thank Professor John Paul for providing facilities and for his advice. This work was supported by grants to Professor Paul from the Rockefeller Foundation for equipment, from grant CA 05855, National Cancer Institute, U.S. Public Health Service, and from the Medical Research Council.

I am grateful for the excellent technical assistance of Mrs Pauline Walker and George Lanyon.
REFERENCES


(Manuscript submitted 10 August 1966, revised 16 January 1967)