The effect of progesterone and oestradiol on blastocysts cultured within the lumina of immature mouse uteri

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SUMMARY

1. Ovariectomy-delayed blastocysts flushed from mice treated with medroxyprogesterone acetate were injected into the lumina of immature mouse uterine horns. The horns were then maintained as organ cultures for 1–3 days in media that contained either (a) ethanol or ethanol plus (b) progesterone, (c) oestradiol or (d) progesterone and oestradiol.

2. Trophoblast invasion of the uterine stroma occurred for 13, 10 and 6% of the eggs injected, in media that contained progesterone, progesterone plus oestradiol, and oestradiol, respectively. The uterine stromal tissue did not deciduate.

3. Embryonic development in invading eggs usually became abnormal during the migration of the distal endoderm. Further development was impeded by the progressive collapse of the yolk cavity which allowed trophoblastic giant cells to intermingle with proximal endodermal cells.

4. The optimal oxygen concentration for trophoblastic invasion and embryonic development in the presence of progesterone and oestradiol was between 26 and 40%.

INTRODUCTION

Egg implantation is a complex interactive process during which, in the mouse, the egg invades through the uterine epithelium and embeds itself within the stromal tissue. The free egg and the developing embryo are subordinate to maternal control in as much as a normal blastocyst can not implant or continue development after implantation unless certain precise maternal conditions prevail. The study of egg–uterine interactions in vitro would allow the reduction and control of some of the maternal variables involved. This approach provides an alternative basis for analysing normal implantation and has been used by Glenister (1961a,b) to study implantation in the rabbit. In this method blastocysts invaded strips of endometrium and commenced embryonic development in an organ culture system.

In the present study the mouse has been used as the experimental animal. Entire uterine horns can be maintained in organ culture (Trowell, 1959), with the attendant advantage that normal tissue relationships are maintained.

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As ovarian hormones are required for implantation in the mouse an *in vitro* implantation system is then also a test system for hormonal action *in vitro*.

The morphology of implantation *in vivo* has been studied both with the light (Boyd & Hamilton, 1952; Snell & Stevens, 1966; Finn & McLaren, 1967) and the electron microscope (see Potts, 1968; Nilsson, 1970) and this provides adequate basis for comparison. The slower rate of development *in vitro* may be useful for the study of various aspects of trophoblast invasion in greater detail.

The study of early embryogenesis *in vitro* would provide further opportunities to analyse the normal control mechanisms. Until recently it was difficult to obtain post-blastocyst development of mouse eggs *in vitro*. Cole & Paul (1965) and Gwatkin (1966) found that a very small minority of blastocysts cultured in a basic salt solution supplemented with amino acids and a source of macromolecular protein would develop to an early egg cylinder stage. The majority of eggs developed in two dimensions only, forming flat outgrowths. Gastrulation was occasionally observed after blastocysts were placed in diffusion chambers that were situated in the peritoneal cavity (Bryson, 1964). However, normal tissue relationships were not maintained and development was again in two, rather than three, dimensions. More recently Jenkinson & Wilson (1970) have more regularly obtained early egg cylinder development after the culture of blastocysts injected into bovine lens tissue, and Hsu (1971) has reported development of mesenchymal tissues after the culture of blastocysts on a collagen base.

The present study provides another method for obtaining early egg-cylinder development *in vitro* in a situation where some uterine-embryonic as well as uterine-trophoblast interactions can be studied.

**MATERIALS AND METHODS**

Uterine horns from immature Q strain mice (weighing 7–10 g) were maintained as organ cultures. Details of the method are soon to be published. Before culture 4–20 (usually 6–10) blastocysts were injected into each horn. Prior to injection, a cut was made into the lumen of each uterine horn just below the utero-tubal junction, to allow the through passage of injected media. This prevented injected eggs being forced back out of the cervical end. The blastocysts were flushed (usually on the 5th or 6th day of pregnancy, but also up to 12th day) from Q strain mice that had been ovariectomized and given 2·5 mg of 6α-methyl-17α-acetoxyprogesterone (medroxyprogesterone acetate as Depo Provera; Upjohn) subcutaneously on the afternoon of the 3rd day of pregnancy.

Trowell's T8 (Trowell, 1959) medium (with 0–20% foetal serum) was used both to flush donor uteri and to inject blastocysts into the horns. Blastocysts were injected from a fine glass pipette controlled by a micromanipulator.
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(Singer) and activated by a micrometer syringe (‘Agla’: Burroughs Wellcome). An air bubble drawn into the pipette before the blastocysts was seen (with a dissecting microscope) to pass into the uterine horns. After injection both ends of the horns were anchored on to the supporting grid by pushing stainless-steel needles through the horn and into the mesh of the grid. The needles were used to stretch the horns taut and to prevent expulsion of the blastocysts.

After culture the uterine horns were fixed, usually in Bouin’s fluid (some were fixed in formol-saline), embedded in wax and sectioned at approx. 6 µm. Sections were stained with haematoxylin (Harris’s) and eosin (HE) and selected treatments were restained with Mallory’s trichrome (Hughesdon’s modification, see Carleton & Drury, 1957) or with methylgreen and toluidine blue stains. Mallory’s trichrome stain was used after fixation with formol-saline to identify pieces of remaining zona pellucida (McLaren, 1970) and after either fixative to show the distribution of uterine connective tissue. The methylgreen and toluidine blue stain was used to demonstrate W-bodies (Finn & McLaren, 1967). Egg and uterine survival were subjectively estimated as the percentage of cells alive after culture. Uterine survival is given as a percentage and eggs were designated as ‘alive’ (greater than 50% of their cells alive) and ‘dead’ (less than 50% of their cells alive), according to the same criteria trophoblast and embryonic tissue was designated as ‘alive’ or ‘dead’.

To test the effect of hormones on eggs cultured within the lumina of uteri

Progesterone and oestradiol were added to Trowell’s T8 culture medium (Flow Laboratories) that contained 20% foetal calf serum, 100 i.u. of penicillin and 2 µg of ‘Fungizone’ (Amphotericin B, Squibb) per ml. There were four treatment groups, namely progesterone (5 µg/ml of medium), oestradiol (5 µg/ml), progesterone plus oestradiol (5 µg + 5 µg/ml) and a control medium containing 0.125% of ethanol. Ethanol made up 0.063% of the media containing a single hormone and 0.125% of the medium containing both hormones.

There were 15 independent replicated experiments. Culture times were 1-3 days (1 experiment), 2 days (11 experiments) and 3 days (3 experiments). The incubator temperature was 35 °C. All treatments within an experiment were gassed at the same pressure (1.0-2.5 lb/sq.in., i.e. 6895-17237 N m⁻²) and oxygen concentration (22-30%) with about 5% carbon dioxide and the remainder nitrogen.

To test the effect of the oxygen concentration on eggs cultured within the lumina of uteri

The culture medium contained 5 µg/ml of both progesterone and oestradiol and was the same as that used in the equivalent hormonal treatment group. Fifteen of the 56 experiments were the same as used to test the effect of progesterone plus oestradiol on eggs cultured in the lumina of uteri.

The culture times were 1-3 days (one experiment), 2 days (49 experiments) and
3 days (6 experiments). The oxygen concentration varied from 20–95 % and the carbon dioxide concentration varied from around 3 to 5 %, with the remainder nitrogen. The gas pressure after culture varied from 0·0 to 5·0 lb/sq.in. (usually 0·5–2·0) and the oxygen concentration was measured after pyrogallol absorption with the method of Fainstat (1968).

Statistical methods

Discrete data were analysed after their arrangement into contingency tables. When observed values in $2 \times 2$ tables were 5 or less, Yates's correction (Snedecor, 1956) or Fisher's exact method (Fisher, 1958) was used. The results of 'The effect of hormones on eggs cultured within the lumina of uteri' section were analysed by $2 \times 2$ tables using three comparisons. These were (1) progesterone-treated groups (i.e. progesterone and progesterone plus oestradiol groups) versus non-progesterone-treated groups (i.e. ethanol and oestradiol groups); (2) within progesterone-treated groups comparison (i.e. progesterone group versus progesterone plus oestradiol group) and (3) the oestradiol versus the ethanol-treated group. For analysis of the results from 'The effect of the oxygen concentration on eggs cultured within the lumina of uteri' section, $2 \times n$ contingency tables were tested for heterogeneity. A significant chi-square value then indicated real differences over the oxygen range tested. To establish if trophoblast activity was correlated with embryonic development, eggs were grouped into $2 \times 2$ tables and the significance of the association between successive stages of activity and development tested for in the normal way. Associations that had significant chi-square values then had measures of intensity calculated with the 'Log Odds' method (McLaren, 1952) and comparisons were made between measures of intensity.

Data that showed continuous variation were analysed by analyses of variance, correlation and regression techniques. Results as percentages were transformed by the arcsin $\sqrt{\text{percentage}}$ method (Snedecor, 1956) before analysis.

RESULTS

The effect of hormones on eggs cultured within the lumina of uteri

1. The number and viability of eggs after culture (Table 1)

Forty-three per cent of all eggs injected at the beginning of culture were found on histological sections. A smaller proportion of the injected eggs were found in the progesterone treated groups than in the nonprogesterone treated groups ($P < 0·001$).

Approximately 9% of all the eggs found were dead. There were higher proportions of dead eggs, and eggs with dead embryonic tissue, from the non-progesterone groups than from the progesterone treated groups ($P < 0·01$ and $< 0·001$, respectively).
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**Table 1. The effect of progesterone and oestradiol on the viability and development of eggs cultured within the lumina of uteri**

<table>
<thead>
<tr>
<th>Treatment ... ...</th>
<th>Ethanol</th>
<th>Progesterone</th>
<th>Oestradiol</th>
<th>Progesterone and Oestradiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of eggs injected</td>
<td>112</td>
<td>113</td>
<td>113</td>
<td>114</td>
</tr>
<tr>
<td>No. of eggs found after culture</td>
<td>57</td>
<td>35</td>
<td>63</td>
<td>40</td>
</tr>
<tr>
<td>% of eggs found that were 'alive'</td>
<td>88</td>
<td>100</td>
<td>84</td>
<td>97</td>
</tr>
<tr>
<td>Trophoblast activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of eggs found with giant cells</td>
<td>51</td>
<td>86</td>
<td>68</td>
<td>90</td>
</tr>
<tr>
<td>% of eggs found that were non-invasive</td>
<td>100</td>
<td>31</td>
<td>83</td>
<td>63</td>
</tr>
<tr>
<td>% of eggs found that were invading the epithelium</td>
<td>0</td>
<td>26</td>
<td>6*</td>
<td>10</td>
</tr>
<tr>
<td>% of eggs found that were invading the stroma</td>
<td>0</td>
<td>43</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>Embryonic development</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of eggs found that were in delay</td>
<td>81</td>
<td>17</td>
<td>63</td>
<td>33</td>
</tr>
<tr>
<td>% of eggs found with proximal endoderm</td>
<td>12</td>
<td>14</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>% of eggs found with distal endoderm</td>
<td>7</td>
<td>17</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>% of eggs found beyond distal endoderm</td>
<td>0</td>
<td>51</td>
<td>13</td>
<td>28</td>
</tr>
</tbody>
</table>

* Two of these eggs were invading the serosal (external) surface of a uterine horn.

### 2. Trophoblast activity

(a) **Giant cells.** Trophoblast giant cell transformation occurred during culture in all treatment groups (Table 1). Transformation occurred first near the abembryonic pole or in the equatorial regions of the eggs. Giant cells were arbitrarily classed as small or large according to the type and size of their nuclei. Small giant cells had nuclei about 1–2 times the size of the nuclei of uterine epithelial cells (Fig. 2) and large giant cells usually had larger nuclei with a different chromatin pattern (Fig. 8). Non-transformed trophoblast cells had dense basophilic nuclei and darkly staining cell walls (Fig. 1). Small giant cells had larger ovoid shaped, palely stained nuclei that contained pinkish nucleoli and there was usually an increased amount of palely stained cytoplasm with faint irregular-shaped cell walls (Fig. 2). In the largest type of giant cell seen the nuclear shape was roundish and the nuclear plasma stained more darkly and usually contained one large nucleolus (Fig. 8).

The proportion of eggs with giant cells was higher for the progesterone- than for the non-progesterone-treated groups ($P < 0.001$). The large giant cells were seen in trophoblast tissue that was invasive and were not present in the ethanol group.

(b) **Non-invasive eggs (Figs. 1 and 2).** Non-invasive eggs appeared on histological sections to be free in the lumina or attached to the luminal epithelium.
The proportion of non-invasive eggs was lower for the progesterone- than for the non-progesterone-treated groups \( (P < 0.001) \) (Table 1) and lower for the progesterone- than for the progesterone- and oestradiol-treated group \( (P < 0.01) \). This proportion was also lower for the oestradiol- than for the ethanol-treated group \( (P < 0.01) \).

(c) Invasion of the uterine epithelium and stroma. Eggs were considered to have passed beyond the attachment phase and to have invaded the epithelium when the latter showed signs of erosion or disruption (Figs. 3–5). Evidence of the rupture of the epithelial basement membrane by the trophoblast was interpreted as invasion of the uterine stroma (e.g. Fig. 7). The trophoblast of eggs that were invading the epithelium was often phagocytosing epithelial and stromal cells and sometimes epithelial cells with an apparently healthy nucleus but no cytoplasm were seen (Fig. 4). Sections were occasionally found on which the trophoblast appeared to have encircled healthy epithelial or stromal cells.

Although some giant cells were seen in the trophoblast tissue overlaying the inner cell mass in a few eggs (Fig. 16), this embryonic pole trophoblast was...
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never seen to be actively invasive. The most advanced embryos had no ecto-
placental cone development.

The proportions of eggs invading the epithelium and the stroma were higher
for the progesterone- than for the non-progesterone-treated groups (P, significant
by inspection and < 0.001, respectively). Eggs in the ethanol-treated group were
non-invasive and treatment with oestradiol only allowed a few eggs to invade
the epithelial and stromal tissues.

3. Embryonic activity

Eggs for which there had been no development of the inner cell mass tissue
beyond that which had occurred at the time of injection were termed 'delay
eggs' (Fig. 1). The single layer of cuboidal or squamous cells that formed the
inner border of the inner cell mass was termed the 'proximal endoderm' (Snell
& Stevens, 1966) (Fig. 5). Eggs with distal endoderm were those in which the
endodermal cells had grown away from an enlarged inner cell mass along the
inside of the trophoblast so that the walls of the yolk cavity were bilaminar
(Snell & Stevens, 1966) (Fig. 14). In the eggs with more advanced embryonic
development it was usually not possible to identify distal endoderm cells. The
yolk cavity had usually collapsed, which allowed trophoblast giant cells to inter-
mingle with cells in the region of the proximal endoderm (Fig. 12). The most
advanced embryonic development was usually disorganized and only in a few

Figures 8–13

Embryos after culture for 2 days in media containing progesterone (Fig. 8), or
progesterone and oestradiol. Figs. 8–10 are from cross-sections of uteri and Figs. 11–
13 from longitudinal sections. All sections were stained with HE. The line on
Fig. 8 represents 25 μm for Figs. 1–20.

Fig. 8. A large trophoblast giant cell with a large nucleolus (arrow) that has invaded
the stromal tissue and is situated directly above a uterine gland (G). 28 % O₂.

Fig. 9. A collapsed blastocyst. The blastocoel cavity (B) is greatly reduced follow-
ing the fusion of the opposite trophoblastic walls. 22 % O₂.

Fig. 10. A contracted blastocyst. The blastocoel cavity is small relative to the size
of the inner cell mass. Note the asymmetrical thickening of the trophoblast wall
(arrow). 20 % O₂. (Initially 31 %.)

Fig. 11. This solid embryo was the healthiest intraluminal embryo in uteri cultured in
41–50 % O₂ group. Embryonic development is probably beyond the proximal endo-
derm stage and the blastocoel cavity has collapsed. The trophoblast tissue present
(T) is largely unhealthy. 42 % O₂.

Fig. 12. Two solid embryos with development beyond that of the distal endoderm
that have fused together at their abembryonic poles. The common trophoblast has
invaded the stroma. N = nuclei of large giant cells. 31 % O₂.

Fig. 13. An embryo invading the epithelium that appears to have two inner cell
masses. The 'mass' on the left was probably formed by proliferation of the
abembryonic trophoblast and/or incorporation of tissue after fusion with the
unhealthy egg shown on the extreme left of the picture. Note the damaged epithe-
lium adjacent to the abembryonic hemisphere. 56 % O₂.
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Embryos was it possible to distinguish extra- from embryonic ectoderm and to identify endoderm cells.

Stages of embryonic development after culture. The proportion of eggs that were in delay was lower (Table 1) in the progesterone- than in the non-progesterone-treated groups \((P < 0.001)\), and lower in the oestradiol- than in the ethanol-treated group \((P < 0.05)\).

The proportion of eggs whose embryonic development had progressed beyond the distal endoderm stage was higher in the progesterone- than in non-progesterone-treated groups \((P < 0.001)\) and higher in the progesterone than the progesterone- and oestradiol-treated group \((P < 0.05)\). This proportion was higher in the oestradiol than in the ethanol-treated group, where there were no eggs beyond the distal endoderm stage \((P < 0.02)\). With the exception of a few 'dead' eggs in the oestradiol-treated group, eggs 'died' before they became invasive and while they were at the delay stage.

4. The correlation between trophoblast activity and embryonic development

The null hypothesis held that any stage of trophoblast activity would be equally likely to occur with any one stage of embryonic development. Non-invasive eggs were divided into those that were probably free (in the lumina of uteri) and those that were probably attached to the epithelium.

Eggs were both free and in a state of delay when injected at the beginning of culture. This particular association was significant after culture for the three treatment groups given hormones \((P < 0.001\) in each case) and non-significant

**Figures 14–20**

Embryos in uteri and uterine tissue after culture for 2 days in media containing oestradiol (Figs. 14, 15) or progesterone and oestradiol. (Figs. 14, 15 and 20 were from cross-sections of uteri and Figs. 16–19 from longitudinal sections.) All sections were stained with HE. The line on Fig. 14 represents 25 \(\mu\)m for Figs. 1–20.

Fig. 14. An embryo with development beyond that of the distal endoderm (DE). The invasion of the epithelium is proceeding slowly relative to the state of the embryonic development. 25 % \(O_2\).

Fig. 15. Another embryo in the uterus shown in Fig. 14. The trophoblast cells have damaged but not breached the epithelium.

Fig. 16. Another embryo invading the epithelium in the uterus shown in Fig. 13. There are giant cells (GC) situated immediately adjacent to the inner cell mass (ICM). Note the thickening and folding of the abembryonic trophoblast, and the disorganization of the adjacent epithelium.

Fig. 17. The healthiest embryo in the 41–50 % \(O_2\) group. This embryo was attached to the longitudinal muscle of the uterus shown in Fig. 11. \(DE = \) distal endoderm cells.

Fig. 18. The epithelial nuclei are rod-shaped and positioned apically. 95 % \(O_2\).

Fig. 19. Epithelial nuclei positioned centrally in the cells. 33 % \(O_2\).

Fig. 20. Epithelial nuclei are ovoid in shape and positioned near the base of the cells. 26 % \(O_2\).
for the ethanol-treated group. The intensity of this association was stronger for the progesterone-treated groups than for the non-progesterone-treated groups ($P < 0.02$).

Again the association between attachment to the epithelium and the possession of proximal endoderm was significant for three treatment groups given hormones ($P < 0.05–0.001$) and non-significant for the ethanol-treated group. Epithelial invasion was protracted in the oestradiol-treated group (Figs. 14, 15) and only in this group was the association between the invasion of the epithelium and the possession of distal endoderm significant ($P < 0.02$).

The association between invasion of the stroma and the possession of embryonic tissue beyond the distal endoderm stage was significant for all groups treated with hormones ($P < 0.01–0.001$). The association was complete for the group given progesterone and oestradiol.

5. **The form of eggs according to their trophoblast activity after culture**

Expanded eggs are those with an ovoid shape and a relatively large blastocoeo or yolk cavity (e.g. Fig. 2). Most non-invasive eggs were of this form. Eggs in which the size of the blastocoeo or yolk cavity was reduced relative to the size of the embryonic tissue are either collapsed (i.e. the trophoblast walls have folded in and occasionally fused (Fig. 9)) or contracted (i.e. although the eggs were ovoid in shape the circumferential distance was shortened (Fig. 10)). The trophoblast of contracted eggs sometimes showed degenerative changes where the nuclear membranes were crinkled, the internuclear distance was shortened and in the cytoplasm vacuoles were apparent. Often local proliferation of the abembryonic trophoblast filled in part of the blastocoeo (or yolk) cavity (Figs. 13, 16). Collapsed eggs were more common among early stages and contracted eggs, more so during later stages of trophoblast activity. When the yolk cavity was either very small or absent, the eggs were considered to be solid in form. Most eggs invading the stroma and those that were ‘dead’ were of this type.

6. **Loss of zonae pellucidae in culture from 5th day eggs**

Approximately half of the eggs flushed on the 5th day of pregnancy from ovariectomized-progestin treated mice were in their zonae. Eggs flushed at later stages of pregnancy had usually lost their zonae.

Pieces of zona were found near 4 (of 43), 4 (10), 3 (15) and 8 (24) eggs that were examined after culture in media that contained ethanol, progesterone, oestradiol and progesterone and oestradiol, respectively. Zona-loss appeared to have resulted from its dissolution in situ without prior hatching.
### Table 2. The effect of the oxygen concentration on the viability and development of eggs cultured within the lumina of uteri

<table>
<thead>
<tr>
<th>Oxygen concentration (%)</th>
<th>22–25</th>
<th>26–30</th>
<th>31–40</th>
<th>41–50</th>
<th>51–70</th>
<th>71–95</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of eggs injected</td>
<td>123</td>
<td>139</td>
<td>46</td>
<td>58</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>No. of eggs found after culture</td>
<td>41</td>
<td>89</td>
<td>31</td>
<td>9</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>% of eggs found that were ‘alive’</td>
<td>73</td>
<td>83</td>
<td>55</td>
<td>56</td>
<td>48</td>
<td>33</td>
</tr>
<tr>
<td>Trophoblast activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of eggs found with giant cells</td>
<td>100</td>
<td>79</td>
<td>84</td>
<td>44</td>
<td>65</td>
<td>63</td>
</tr>
<tr>
<td>% of eggs found that were non-invasive</td>
<td>71</td>
<td>31</td>
<td>45</td>
<td>100*</td>
<td>65</td>
<td>87</td>
</tr>
<tr>
<td>% of eggs found that were invading the epithelium</td>
<td>20</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>% of eggs found that were invading the stroma</td>
<td>10</td>
<td>61</td>
<td>48</td>
<td>0</td>
<td>17</td>
<td>3</td>
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<td>Embryonic activity</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% of eggs found that were in delay</td>
<td>39</td>
<td>21</td>
<td>16</td>
<td>33</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>% of eggs found with proximal endoderm</td>
<td>51</td>
<td>20</td>
<td>29</td>
<td>33</td>
<td>26</td>
<td>70†</td>
</tr>
<tr>
<td>% of eggs found with distal endoderm</td>
<td>7</td>
<td>16</td>
<td>6</td>
<td>33</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>% of eggs found beyond distal endoderm</td>
<td>2</td>
<td>43</td>
<td>48</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

* Two of these eggs were attached to the serosal (external) surface of a uterine horn.
† Assessment of embryonic development was approximate in this group and in others where a high proportion of the eggs were dead.

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1. **The number of eggs after culture** (Fig. 21)

   Forty-eight per cent of all eggs injected prior to culture were found on histological sections. No consistent effect of oxygen concentration was observed.

2. **Egg viability after culture**

   The proportions of the eggs that were ‘alive’ varied significantly over the 22–95 % O₂ concentration range \((P < 0.001)\). This was mainly due to disproportionately large numbers of ‘dead’ eggs in the 22–25 % and 71–95 % O₂ groups (Fig. 21). The proportion of eggs with ‘dead’ trophoblast was higher than the proportion that were dead \((P < 0.05)\) over the 22–95 % O₂ range, while in the 71–95 % O₂ group some eggs had their embryonic tissue selectively killed. The majority of ‘dead’ eggs died before their trophoblast became invasive.

3. **Trophoblast activity** (Fig. 22)

   The trophoblast of some eggs that were invading the epithelium made contact with condensed ‘cells’ that resembled the ‘primary invasive cells’ of Wilson (1963) (later termed W-bodies by Finn & McLaren, 1967) (Figs. 3–5). The area
of stromal invasion in the presence of high oxygen concentrations was often small and occasionally the trophoblast died after invasion.

The proportions of eggs that were invading the stroma varied significantly \((P < 0.001)\). Sixty per cent of the eggs in the 26–30\% \(O_2\) group invaded the stroma while none did in the 41–50\% \(O_2\) group.

4. Embryonic development (Fig. 23)

Because of the doubtful status of some eggs, they were divided into early (those in delay plus those with proximal endoderm) and late (those with distal endoderm plus those beyond the distal endoderm stage) developmental stages.

Heterogeneity tests for the proportions of eggs that were in the early and in the late stages of development were significant \((P < 0.001)\). Fifty-eight per cent of eggs in the 26–30\% \(O_2\) group were at a late stage of development and probably all the eggs in the 71–95\% \(O_2\) group were at an early stage.

*The survival of uteri, their reaction to invading trophoblast and the orientation of eggs within their lumina*

The mean percentage of uterine cells viable after culture was higher for the hormone-treated groups (namely 68–70) than in the ethanol-treated group (namely 62). The mean for this estimate over the 22–95\% \(O_2\) range was 76\%.
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Fig. 22. The effect of the oxygen concentration on trophoblast activity. □, Percentage of injected eggs that were alive and probably free in the lumen. □, Percentage of injected eggs that were alive and probably attached to the epithelium. □, Percentage of injected eggs that were alive and invading the epithelium. ■, Percentage of injected eggs that were alive and invading the stroma.

The regression of uterine survival on the O₂ concentration was significant \( r = +0.29, 51 \text{ d.f.}, P < 0.05 \).

When stained with Mallory's trichrome an increased concentration of uterine stromal connective tissue could be seen in front of the invading trophoblast tissue. Uterine stromal decidual cells were not seen in any experiment.

The nuclei of epithelial cells from uteri cultured at O₂ concentrations of around 56% and higher were often positioned apically and there was a prominent subnuclear epithelial band (Fig. 18), whereas for lower O₂ levels nuclei were usually positioned centrally (Fig. 19) or in the basal region of the epithelial cells (Fig. 20).

During the experiment to determine the optimal oxygen concentration the positioning of 64 eggs, relative to the position of the mesometrium, in 17 cultured uteri was examined. There was no significant tendency \( P < 0.2 \) for eggs in the same uteri to have their embryonic poles orientated the same way. The embryonic tissue was, however, positioned at the opposite pole to that at which invasion occurred.
Fig. 23. The effect of the oxygen concentration on embryonic activity. □, Percentage of injected eggs that were alive and probably in delay. ■, Percentage of injected eggs that were alive and probably with proximal endoderm. □, Percentage of injected eggs that were alive and with distal endoderm. ■, Percentage of injected eggs that were alive and beyond distal endoderm.

DISCUSSION

In the presence of hormones some blastocysts cultured for 2–3 days within the lumina of immature uteri invaded the uterine stroma. Trophoblastic activity was accompanied by embryonic development that reached the 'egg cylinder' stage.

The proportion of injected eggs that were found after culture depended both on the hormonal treatment and on the oxygen concentration. It seems paradoxical that a smaller proportion of eggs were found (and yet a higher proportion of these were alive) in the two progesterone-, than in the ethanol- and oestradiol-treated groups as progesterone treatment provided the strongest stimulus (or created the most suitable conditions) for egg development. One explanation is that uteri were metabolically most active under the influence of progesterone and that any dead or partially dead eggs they contained would have undergone cytolosis more rapidly than in non-progesterone-treated uteri.

Variations in the oxygen concentration had a pronounced effect on the proportions of injected eggs found after culture and their viability. The highest
levels of oxygen killed most eggs and sometimes selectively killed the inner cell mass tissue, and embryonic development probably did not progress beyond the proximal endoderm stage for the 71–95% oxygen range. Glenister (1970) found that different tissues had their own optimal oxygen concentrations for development in rabbit embryos that were invading endometrial tissue in culture. High oxygen levels were selectively lethal to embryonic tissues.

Giant-cell transformation of the trophoblast occurred more frequently in the progesterone than in the non-progesterone-treated groups. Transformation to small giant cells was observed in about one half of the eggs in the ethanol control group. Large giant cells with dark-staining nucleoplasm and a distinctive large single nucleolus did not develop in this group. Although giant-cell transformation occurs without the addition of hormones in vitro (Gwatkin, 1966) and in extra-uterine sites in vivo (Fawcett, Wislocki & Waldo, 1947; Bryson, 1964; Kirby & Cowell, 1968) transformation of eggs within uteri may require hormones. Blastocysts in mice where implantation has been delayed by ovariectomy or by lactation do not transform (Dickson, 1963, 1966a; McLaren, 1968). Implantation and therefore transformation is induced experimentally by oestrogen (Whitten, 1955; Yoshinaga & Adams, 1966) and it is associated with proliferation of the abembryonic pole trophoblast (Dickson, 1966b) and early invasion (Snell & Stevens, 1966).

The present findings may then differ from the in vivo situation in several respects. Transformation in vivo occurs in the presence of both progesterone and oestrogen, whereas in vitro it began without hormones, and large giant cells were formed in the presence of either progesterone or oestradiol alone. Kirby (1971) however, illustrated ‘enlargement’ of lateral and abembryonic trophoblast cells in blastocysts from non-treated ovariectomized mice. The requirements for transformation into large giant cells in vitro were found by Gwatkin (1966; 1969) to include specific amino acids and protein macromolecules in addition to an energy source and a basic salt solution. The uterine epithelium seems capable of selective resorption and absorption that may be hormone controlled (Nilsson, 1970) and perhaps the direct supply of media within the lumina along with the eggs, as in the present experiments, started trophoblast transformation.

Control of ‘implantation’

The trophoblast was only invasive in the presence of hormones and more so with progesterone than with oestradiol. Thus implantation in vitro was dependent on hormones and in this respect resembles the in vivo situation. However, the qualitative and quantitative hormonal requirements and their sequence of action for normal implantation are more precise than these in vitro findings would indicate. Pro-oestrus oestrogen ‘primes’ the uterus (Finn, 1966; Martin & Finn, 1971) for the subsequent action of progesterone and further oestrogenic activity intervenes on or before the 4th day of pregnancy, and this is followed
by a period of uterine receptivity during which eggs can implant (see Smith, 1966; Humphrey, 1967; Bloch, 1968; Grant, 1968; Bindon, 1969; Finn & Martin, 1969). Implantation with either progesterone or oestradiol alone and implantation with both progesterone and oestradiol in uteri which had not received correct hormonal sequential treatment would be unexpected in vivo, but occurred in vitro. This relative lack of specificity may have resulted from differences due to the experimental situation, hormonal contamination of the foetal calf serum used in the media or from a genuine difference in the hormonal requirements due to the immaturity of the uteri used.

The culture method placed limitations on the range of response available to the uterus, and this may have affected the hormonal actions controlling implantation. In vivo, hormones act to ensure that the uterus is very selective in allowing eggs to implant and continue development. There are mechanisms for the elimination and destruction of embryos that may not operate in vitro. For example, high doses of oestradiol (as used in the present work) administered to pregnant mice can prevent implantation (Martin, 1963). Oestrogens activate the myometrium (Reynolds, 1949), which may in vivo lead to the expulsion of eggs (Adams, 1970). Cultured uteri were stretched taut and pinned down, a procedure which would prevent the occurrence of expulsive contractions. Under these conditions oestradiol in the absence of progesterone allowed a small number of eggs to implant.

There are indications, however, that the epithelium was more resistant to invasion in the oestradiol treated group than in the progesterone-treated groups (Figs. 14, 15). The stage of embryonic development reached before the epithelium was breached was more advanced in the oestradiol group than in the two progesterone-treated groups, and only in the presence of oestradiol alone was the association between possession of distal endoderm and invasion into the epithelium significant. Eggs with distal endoderm in the progesterone-treated groups had usually invaded through the epithelium and into the stroma. Oestrogen stimulates epithelial mitoses in vivo (see Martin & Finn, 1971) and the epithelium in the oestradiol treated uteri may have been in a growth phase.

In vivo, androgenic impurities (Grant, 1972) as well as very small doses of oestradiol (Humphrey, 1967) allow progesterone to support implantation in mice ovariectomized before the fourth day of pregnancy. It seems possible that in vitro as in vivo that small amounts of androgenic or oestrogenic impurities could have combined with progesterone to allow implantation.

The levels of oestradiol and progesterone used in these experiments were high (5 μg/ml of either or both hormones). The rate and size of trophoblast outgrowth from blastocysts placed in plastic Petri dishes was, however, not influenced by the addition of both progesterone and oestradiol, each at a concentration of 5 μg per ml (Grant, 1972). The addition of proteins to the media, in the form of foetal calf serum, may bind some of the added steroids (Heap, 1969).
Morphology of implantation

In vivo, eggs implant in an antimesometrial crypt with the embryonic tissue directed mesometrially (see Snell & Stevens, 1966). Eggs in the present work positioned themselves randomly with respect to adherent mesometrium left on the uterine horns (which were themselves variably positioned on supporting grids). As in vivo, the embryonic tissue was at the opposite pole to that which first underwent trophoblastic proliferation.

Progesterone treatment of uterine horns cultured in vitro induced luminal closure (Grant, in preparation) and eggs in such horns were often situated in well-formed indentations, while eggs in horns treated with oestradiol or ethanol were often found in the centre of large lumina with little evidence of indent formation.

Fusion between eggs that were in contact in the lumina of uteri was relatively common. Mature blastocysts do not fuse when placed together in vitro (Mulnard, 1971) nor do trophoblast vesicles from which embryonic tissue has been excluded (Gardner, 1971). Kirby (1971), however, observed fusion between two blastocysts in the uterus of an ovariectomized non-treated mouse. Fusion in this study was seen when the blastocysts had developed beyond the delay stage (Figs. 4, 12).

The first evidence of invasion usually occurred near the abembryonic pole and less often in the equatorial regions of the blastocysts. Likewise phagocytosis and W-bodies (Finn & McLaren, 1967) were first noted in these regions. In these respects the in vitro situation parallels that seen in vivo (Snell & Stevens, 1966; Finn & McLaren, 1967). W-bodies (the primary invasive cells of Wilson (1963)) are probably dying cells (Finn & Lawn, 1968; Wilson & Smith, 1970) of maternal origin. Trophoblast processes were observed to encircle apparently viable epithelial cells and to be associated with vacuolated epithelial cells (Fig. 4). Fixation shrinkage sometimes caused encircled cells to be plucked off the epithelial basement membrane (Fig. 6). Finn & Lawn (1968) and Wilson & Smith (1970) have observed tongues of trophoblast tissue penetrating between the lateral borders of epithelial cells, on electron micrographs. The epithelium in vivo is rapidly removed, thus making it difficult to obtain electron micrographs of this stage of trophoblast activity (Potts, 1969). This process was protracted in vitro and may have been further slowed when uteri were treated with oestradiol.

The concentration of connective tissue in the uterine stroma immediately in front of the invading trophoblast may have corresponded to collagen described in this position in vivo (Potts, 1969). In an electron-microscope study Potts noted that the epithelial basement membrane swelled and the associated collagen fibres became more numerous as the trophoblast advanced. The absence of a decidual cell reaction in response to the developing blastocysts may have been due either to the absence of hormonal pretreatment to sensitize the uterus (Finn, 1966; De Feo, 1967) or to the immaturity of the uteri. The
functions of the decidual reaction that accompanies implantation in vivo are still in doubt (see reviews by McLaren, 1965; Finn, 1971). The present study provides evidence that the implantation chamber resulting from decidualization in vivo may protect the developing embryo from myometrial pressure. A number of embryos in vitro were clearly misshapen because of luminal closure.

The solid form of many of the embryos that had developed beyond the distal endoderm stage was associated with the fusion of the abembryonic trophoblast with the advancing embryonic tissues. Compared with normal 6th-day embryos these advanced in vitro embryos have no yolk cavity and proliferated trophoblastic tissue in the region of the abembryonic pole. Although the prominent yolk cavity characteristic of in vivo embryos may sometimes represent a shrinkage artifact (Potts, 1969), normal 5-day post-coitum egg cylinders flushed directly from uteri have a prominent yolk cavity (Grant, 1968). Reichert's membrane was positioned so that there was a definite yolk cavity while the in vitro embryos in the present study had no obvious Reichert's membrane between the trophoblast and the endoderm. Earlier development in vitro foreshadowed the trophoblastic proliferation and the concomitant collapse of the yolk cavity (Figs. 13, 16).

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REFERENCES

**Blastocyst-uterine interactions in vitro**


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