The Action of Various Agents upon the Rabbit Embryo

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WITH THREE PLATES

INTRODUCTION

In the course of an investigation on the uptake of labelled ions by the rabbit embryo and its environment (Lutwak-Mann, Boursnell, & Bennett, 1960), experiments were done on pre-implantation blastocysts obtained from rabbits which had been treated parenterally with certain purine analogues. Histological examination of such blastocysts by the method of Moog & Lutwak-Mann (1958) showed that, as a result of treatment of the pregnant animals, the embryos had incurred severe damage chiefly localized in the embryonic disc. This observation prompted a wider study, reported below, of the action of various agents upon the early rabbit embryo, following their administration to the mother. Our investigation was chiefly concerned with the pre-implantation 6½-day-old blastocyst, but 5- and 7-day embryos were also examined. At the same time we have studied the influence of some of these agents upon ovulation, fertilization, and cleavage, as well as on implantation and later stages of pregnancy. We believe that these findings should be included as they contribute to a fuller understanding of the mode of action of embryotoxic factors.

The viability of cleaving eggs that had been placed for a few hours in the oviducts of rabbits under treatment with purine analogues, was also investigated; these eggs were subsequently transferred to normal permanent recipients, in which their development was studied at later stages of pregnancy (the egg-transfer experiments were carried out by C. E. A.). Some of our findings have already been briefly reported (Hay, Adams, & Lutwak-Mann, 1960).

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EMBRYOTOXIC AGENTS

MATERIALS AND METHODS

Animals

The rabbits were bred and housed singly at the Animal Research Station; only does with an established record of fertility were used. The number of experimental rabbits was 200; 22 untreated animals provided control embryos.

Treatment of rabbits

The substances administered to the experimental animals were (a) hormones (oestradiol benzoate, diethylstilboestrol, dimethylstilboestrol, progesterone, desoxycorticosterone acetate, cortisone acetate); (b) antimitotic agents, comprising 2 colchicine derivatives (Colcemid, N-desacetylmethylcolchicine; Thiolcolciran, N-desacetylthiocolchicine), and several polyfunctional alkylating agents (Thiotepa, triethylene-thiophosphoramid; TEM, triethylenemelamine; Degranol, β-bis-1,6-chlorethylamino-D-mannitol; E 39 soluble, 2: 5-bis-ethylene-imino-3: 6-bis-methoxethoxybenzoquinone; Myleran, 1,4-dimethanesulphonyxbutane); 2 purine and 1 pyrimidine analogues (6-mercaptopurine; 8-azaguainine; 5-bromouracil); (d) vitamin A (special preparation of vitamin A acetate, containing 40,000 i.u. vitamin A per ml.); (e) vitamin antagonists (analogues of vitamin B₁₂, namely B₁₂ methylamide and ethylamide, and B₁₂ anilide; 2 folic acid antagonists, aminopterin and amethopterin); and (f) a group of miscellaneous agents (trypan blue; Neptazane, 2-acetylimino-3-methylthiadiazoline sulphonamide; carbon tetrachloride; carbutamide; cysteamine hydrochloride; X-radiation).

The timing of maternal treatment, and the dosage, represent a crucial but variable, experimental factor, and will be given in detail below. Unless otherwise stated, the agents were given parenterally. Irradiation with X-rays involved total body exposure. In this study the day of mating is referred to as day 0.

Histological technique

Pre-implantation blastocysts fixed in undiluted methanol were prepared as described by Moog & Lutwak-Mann (1958), except that the saline rinse was omitted. While the fixed blastocysts were in methanol, and before they were spread on to the cover-slips, their longest and shortest diameters, at right angles to each other, were measured by means of a square grid inserted into the eyepiece of the microscope. A Leitz screw-micrometer eyepiece was used to measure the embryonic disc of mounted, stained blastocysts; again, the longest and shortest diameters, at right angles to each other, were measured.

Mitotic counts were made in that portion of the abembryonic region of the blastocyst where the trophoblast consisted of only one layer of cells. The resting and dividing cells in 3–4 rectangular areas were counted in each ‘arm’ of the specimen, 450–650 cells usually being counted in each blastocyst. The mitotic index was expressed as the percentage of dividing cells in the total number counted.
Cleaving ova were examined by low-power microscopy; stages of pregnancy other than blastocysts, by direct visual observation.

Chemical methods

The activity of carbonic anhydrase was routinely assayed in the endometria of the treated animals during the progestational phase, by the method of Lutwak-Mann & Adams (1957). Determinations of bicarbonate, glucose, and lactic acid in the blastocyst fluid were made as previously described (Lutwak-Mann, 1954, 1960).

Egg transfer

Details of the procedure are described below (p. 481).

RESULTS

Experiments with untreated rabbits

Earlier observations on the appearance of normal 6-day blastocysts were extended to include embryos of 5 and 7 days so as to provide a wider basis for comparison with blastocysts from treated animals. The embryonic discs of blastocysts aged 5–7 days, were classified into 6 developmental stages (A–F), as set out in Table 1; stage A is the least mature, typical of day 5, and stage F

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description of embryonic disk</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Small disc with rather indefinite outline, composed of 1–2 layers of cells (Plate 1, fig. A)</td>
</tr>
<tr>
<td>B</td>
<td>Round disc with smooth outline and about 3 layers of cells thick (Plate 1, fig. B)</td>
</tr>
<tr>
<td>C</td>
<td>Disc beginning to elongate and grow out in a posterior direction, showing a slight thickening at its anterior edge (Plate 1, fig. C)</td>
</tr>
<tr>
<td>D</td>
<td>Onset of primitive-streak formation with cells beginning to condense in the midline of the zone of outgrowth (Plate 1, fig. D)</td>
</tr>
<tr>
<td>E</td>
<td>Primitive streak present in the posterior part of the disc.</td>
</tr>
<tr>
<td>F</td>
<td>Mesoderm beginning to grow out in a fan-shape at the posterior end of the disc (Plate 1, fig. E)</td>
</tr>
</tbody>
</table>

the most advanced, seen in some late 6-day, and the majority of 7-day, blastocysts (Plate 1, figs. A–E). When individual blastocysts were classified, intermediate stages were also recognized, e.g. in stage B–C the posterior growing edge of the disc could be distinguished, although it had not yet begun to grow out.

The diameters of entire unopened blastocysts, and of the embryonic discs, were measured in 133 embryos aged 5–6½ days and taken from 17 litters; the
mean diameters for each litter are recorded in Table 2. So far as one could tell from direct visual observation, fixation in methanol induced no appreciable change in either shape or size of the blastocysts. On day 5 the embryos were almost spheroidal, the mean diameters of entire blastocysts ranging from $1.3 \times 1.3$ mm. to $2.2 \times 2.1$ mm., and those of the discs from $0.64 \times 0.59$ to $0.90 \times 0.77$ mm. During the 6th day the embryos became ellipsoidal in shape, the mean entire diameters ranging from $2.2 \times 2.2$ mm. to $4.8 \times 4.4$ mm., and the discs from $1.0 \times 0.85$ mm. to $1.35 \times 0.99$ mm. Only two 7-day blastocysts were measured, and their embryonic discs were not substantially larger than those of the bigger 6½-day embryos. Table 2 also shows the percentage number of blastocysts in each stage in 19 normal litters examined on days 5–7; the litters are arranged according to the mean diameters of the entire blastocysts. It can be seen that the first sign of primitive streak formation (stage D) was not reached until the litter had a mean blastocyst diameter of over 4 mm.; this was also true for individual blastocysts. However, blastocysts of over 4 mm. often had discs in stage C.

### Table 2

Size of entire blastocyst and of embryonic disc in relation to developmental stages A–F

<table>
<thead>
<tr>
<th>Age and no. of blastocysts examined in each litter</th>
<th>Mean diameters of entire blastocysts in mm.</th>
<th>Mean diameters of embryonic discs in mm.</th>
<th>Number of blastocysts (%) in stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>$1.3 \times 1.3$</td>
<td>$0.64 \times 0.59$</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>$1.5 \times 1.4$</td>
<td>$0.60 \times 0.52$</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>$1.9 \times 1.8$</td>
<td>$0.78 \times 0.65$</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>$2.2 \times 2.1$</td>
<td>$0.90 \times 0.77$</td>
<td>10</td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>$2.2 \times 2.2$</td>
<td>$1.00 \times 0.85$</td>
<td>...</td>
</tr>
<tr>
<td>9</td>
<td>$2.9 \times 2.8$</td>
<td>$1.21 \times 1.10$</td>
<td>...</td>
</tr>
<tr>
<td>9</td>
<td>$3.2 \times 3.0$</td>
<td>$0.99 \times 0.92$</td>
<td>...</td>
</tr>
<tr>
<td>8</td>
<td>$3.4 \times 3.2$</td>
<td>$1.05 \times 0.96$</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>$3.4 \times 3.2$</td>
<td>$1.06 \times 0.99$</td>
<td>...</td>
</tr>
<tr>
<td>10</td>
<td>$3.6 \times 3.5$</td>
<td>$1.16 \times 1.06$</td>
<td>...</td>
</tr>
<tr>
<td>6</td>
<td>$3.8 \times 3.6$</td>
<td>$1.13 \times 1.03$</td>
<td>...</td>
</tr>
<tr>
<td>7</td>
<td>$3.9 \times 3.5$</td>
<td>$1.12 \times 1.01$</td>
<td>...</td>
</tr>
<tr>
<td>13</td>
<td>$4.4 \times 4.1$</td>
<td>$1.17 \times 0.99$</td>
<td>...</td>
</tr>
<tr>
<td>7</td>
<td>$4.5 \times 4.0$</td>
<td>$1.09 \times 1.05$</td>
<td>...</td>
</tr>
<tr>
<td>10</td>
<td>$4.5 \times 4.2$</td>
<td>$1.28 \times 1.02$</td>
<td>...</td>
</tr>
<tr>
<td>11</td>
<td>$4.8 \times 4.4$</td>
<td>$1.18 \times 1.01$</td>
<td>...</td>
</tr>
<tr>
<td>5</td>
<td>$4.8 \times 4.2$</td>
<td>$1.35 \times 0.99$</td>
<td>...</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>...</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>...</td>
</tr>
</tbody>
</table>

The embryonic discs of only two 7-day blastocysts were measured, one in each litter.

Trophoblastic knobs, described by Schoenfeld (1903), were found in some of the larger 6-day and in 7-day embryos in the double-layered part of the abembryonic hemisphere, close to the edge of the endoderm (Plate 2, fig. F). Often a few
knobs were found together while the rest of the trophoblast was devoid of them. No knobs were seen in blastocysts with a diameter below 4 mm., but there were somewhat larger blastocysts in which no knobs were present. The development of the knobs did not appear to be closely correlated with that of the embryonic disc; they were not associated with discs earlier than stage C, though some embryos in stage F had no knobs.

Nuclear degeneration was commonly observed in the embryonic area, and a moderate amount of it was considered normal (see Glucksmann, 1951). The distribution of the degeneration granules was characteristic for each stage of development, e.g. they were distributed throughout the stage B discs, but were concentrated in their anterior parts in stage C. Globules of material which stained metachromatically with haematoxylin were frequently found associated with the degenerating cells. This material formed small nodules projecting into the blastocyst cavity, stained positively with Alcian blue, and gave the periodic acid Schiff reaction; it seems likely therefore that the material was mucin. These nodules could be seen in unstained methanol-fixed blastocysts, where they appeared black in transmitted light, and frequently also in fresh embryos. The presence of mucin located in the disc area appeared to be normal in 5- to 7-day blastocysts, though it was not a constant finding. Usually, mucin was present either in all the members of a litter, or in none. Isolated globules of mucin were sometimes seen in the trophoblast.

**Effect of hormones**

The experiments with hormones were largely confined to the blastocyst stage and the period of implantation.

*Oestradiol benzoate*

When given in doses of 0.5–1.0 mg. per animal, on days 4–5 after mating, this hormone induced marked uterine hypertrophy; often there was pyometra, or if this was absent, there was abundant uterine secretion, and the endometrium was oedematous and haemorrhagic. Blastocyst recovery was impeded by pus, and the embryos showed signs of degeneration and infection. However, in pus-free uteri, well-developed 6½-day blastocysts were usually recovered, and some appeared to be embedding prematurely. In two experiments the primitive streak had formed in 40–50 per cent. of the embryos.

Following treatment of rabbits with oestradiol benzoate, on days 4 to 5 of gestation, implantation seldom succeeded, presumably owing to the grossly altered uterine environment, and not because of any primary damage to the embryos themselves.

*Diethylstilboestrol*

This was given in doses of 0.5–5.0 mg. per animal, on days 4–5 after mating. Unlike with oestradiol, pyometra was infrequent, but the uteri were enlarged and
contained an excessive amount of uterine secretion. In one experiment over 50 per cent. of the 6½-day blastocysts showed signs of primitive-streak formation, and were already attached to the oedematous endometrium; in another, blastocysts of the same embryonic age were small (diameter 2.7×2.3 mm.) and the disks in an early stage of development, none being more advanced than stage B-C.

Stilboestrol given at 4–5 days, did not prevent blastocyst implantation. However, foetal development did not continue beyond 12–14 days, presumably owing to the profound changes in the uterine tissues.

**Dimethylstilboestrol**

Unlike diethylstilboestrol, this stilboestrol derivative given to rabbits in doses of 0.1–5.0 mg. per animal, on days 4–5, caused little hypertrophy in either the myometrium or the endometrium. The 6½-day blastocysts appeared normal on microscopical examination. Nevertheless, pregnancy terminated a few days after implantation.

**Progesterone**

There was no stimulating or other discernible effect upon the 6½-day blastocysts, when progesterone (1 mg. per animal) was given daily on days 1–4.

**Desoxycorticosterone acetate**

This hormone probably had no effect upon 6½-day blastocysts following treatment of rabbits with less than 5 mg. per animal daily, starting on days 1, 2, or 3; with 5-mg. doses some embryos showed signs of localized degeneration in the trophoblast. Implantation was normal when treatment did not exceed 1–2 mg. daily.

**Cortisone acetate**

Normal 6½-day blastocysts were recovered from rabbits injected daily with 12 mg. per animal, beginning on day 0 or 1, and continuing until day 5. The treatment did not interfere with the progress of pregnancy.

**Effect of colchicine derivatives**

**Colcemid**

Rabbits given single injections of Colcemid ranging from 0.3 to 8.0 mg. per kg. body-weight developed diarrhoea and lost weight. These symptoms were transient with doses up to 6 mg. per kg.

**Ovulation and fertilization.** There was no interference with either function within the above dose-range. Similarly, does that had been treated with small amounts of Colcemid on alternate days for 3 weeks were capable of mating and conception.
Cleavage. The administration of 2–5 mg. per kg. caused arrest of ovum cleavage within a few hours of the injection.

Blastocyst stage. Blastocysts were examined at 5½, 6½, and 6¾ days of age, after long- and short-term experiments. In the former, a single dose of Colcemid was given on days 1, 2, 4, or 5, and the blastocysts were examined at 6½ days. In the latter, the animals were injected either on day 5 or on day 6, and autopsied 1, 5, 9, or 14 hours later.

The effect of this drug on the histology of the blastocysts was related both to the time of administration and the dose level. In long-term experiments, 1 mg. per kg. or less, given on day 1 or 2, had no visible effect at 6½ days. The same dose, given on day 4 or 5, had an inhibitory effect on the discs, none of which had reached a stage beyond B–C, by 6½ days. The injection of 2 mg. per kg. on day 5, 36 hours before autopsy, had a marked deleterious effect. The blastocysts were small, some had collapsed, and in all the discs were irregular and degenerating, while the trophoblasts also showed signs of damage. In some instances there were numerous lymphocytes on the outer surface of the blastocysts.

**Table 3**

*Mitotic activity in the abembryonic area of the trophoblast in blastocysts from normal and Colcemid-treated rabbits*

<table>
<thead>
<tr>
<th>Dose of Colcemid (mg./ kg. body-wt.)</th>
<th>Interval between injection and autopsy in hours</th>
<th>Age of blastocysts at autopsy (days after mating)</th>
<th>No. of blastocysts on which counts were made</th>
<th>Total no. of cells counted</th>
<th>Cells in mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>4</td>
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<td>2,493</td>
<td>194</td>
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</tbody>
</table>

When Colcemid was given on day 6, at 1, 5, or 9 hours before autopsy, the characteristic antimitotic activity of the drug was clearly revealed: cell-division in both disc and trophoblast was arrested in metaphase (Plate 2, figs. G, H), and as a result no anaphases were seen. Cells continued to attempt division, however, and prophases were present in all experiments. It can be seen from Table 3, in
which each experiment refers to one litter, that in the majority of 5½- to 6½-day blastocysts from untreated control rabbits less than 5 per cent. of the cells in the abembryonic area of the trophoblast, in which cell counts were made, were dividing. In blastocysts examined 1 hour after injection of 2 mg. per kg. Colcemid, 7–8 per cent. of the trophoblastic cells were in division, the majority in metaphase, but late telophases were seen in some blastocysts, presumably representing the end of the last cycle before the mitotic block had become effective. With a 5-hour interval between injection and autopsy, the percentage of dividing cells was 18–35, and when the interval was 9 hours, the percentage was 64–69. In the last two groups of experiments, again the majority of dividing cells was in metaphase but, in contrast to the embryos examined 1 hour after injection, the only other stage seen was prophase.

Blastocysts examined on day 6, 14 hours after injection, showed no signs of metaphase arrest; 6 per cent. of the abembryonic cells were in division, and some multinucleate cells were present, probably resulting from the earlier disturbance in mitotic activity. The block to spindle formation and anaphase appeared to wear off during the 9–14 hours’ interval after treatment, allowing the cells to complete their division. Results were essentially similar when short-term experiments were started on day 5; 47 per cent. of the cells in the abembryonic area of the trophoblast were in prophase or metaphase 5 hours after Colcemid injection.

Treatment with Colcemid also resulted in marked cellular degeneration apparent in the discs 5 hours after injection (Plate 2, fig. G). In the trophoblasts, this was not obvious until 14 hours after administration of Colcemid. The degeneration was still visible 36 hours after treatment, long after normal mitotic activity had presumably been resumed. Growth and differentiation of the discs was suppressed; the mean diameters of discs at 6½–6¾ days, 5 and 9 hours after treatment, were 0·60 x 0·57 and 0·71 x 0·63 mm., respectively; none had developed beyond stage B. The size of the entire blastocysts varied considerably but was within normal limits in the majority of the embryos.

Implantation and later stages of pregnancy. In rabbits that had received 2 mg. per kg. Colcemid on day 6 and were autopsied 1–2 days later, about 50 per cent. of the embryos were implanted, but the rest were lying free; the implantation domes and the unattached blastocysts were smaller than normal. There were no obvious changes in the uterine tissues. Chemical analysis of the blastocyst fluid from the 7½-day embryos which failed to implant showed that they were retarded in their development; this was indicated by a high content of bicarbonate (150 ml. CO₂/100 ml.), and low values for glucose (15 mg./100 ml.) and lactate (38 mg./100 ml.), such as are typical of 6-day blastocysts (Lutwak-Mann, 1960).

Rabbits given 0·5 mg. per kg. on alternate days for 3 weeks before mating carried their litters to term. At birth the young were rather small but there were no obvious malformations. In spite of apparent good maternal health, the litters did not survive; this was thought to be due to failure of lactation. The
does were then mated again; they conceived and this time successfully reared their offspring.

Thiolcolciran

Rabbits were given injections of 2–12 mg. per kg. body-weight which were followed by transient diarrhoea and a fall in weight. The impression was gained that this colchicine derivative was less toxic to rabbits than Colcemid.

Ovulation and fertilization. These were not affected by the doses used.

Cleavage. Cell-division was rapidly arrested after the administration of 5–10 mg. per kg.; smaller amounts were not tested.

Blastocyst stage. The response elicited in blastocysts depended on the time of treatment. A small dose given on day 4 allowed the formation of some apparently normal blastocysts, but numerically the litter was reduced in comparison with previous pregnancies. Treatment with 8–10 mg. per kg. on day 5 resulted in blastocysts which at 6½ days were normal in size (diameter of 3·5 × 3·2 mm.) but had very small discs (0·63 × 0·53 mm.), none of which had progressed beyond stage B. Injection of 4–6 mg. per kg. on day 6, followed by autopsy 9 hours later, caused the appearance of numerous small degeneration granules in the disc, and some degeneration in the trophoblast. In contrast to experiments with Colcemid, no upset in mitotic activity was observed.

Implantation. Blastocyst nidation was largely prevented by 8–12 mg. per kg. given on days 5½–6. The effect on later stages of gestation was not studied.

Effect of polysfunctional alkylating agents

Thiotepa

This was given parenterally in doses of 3–10 mg. per kg. body-weight. The larger amounts caused a marked loss of weight, but there were no other overt symptoms in experiments of limited duration.

Ovulation and fertilization. These were not affected by doses up to 6 mg. per kg.; with higher doses some 25 per cent. of the ova shed were unfertilized.

Cleavage. Early arrest of cleavage invariably occurred, even in response to small doses.

Blastocyst stage. Thiotepa given on days 3, 4, 5, or 6 affected all blastocysts adversely, though there were differences in the susceptibility of the litters. The histological appearance of the 6½-day embryos depended on the timing of the treatment, the earlier it was given, the more drastic the effect. When the interval between injection and autopsy was only 9 hours, the disc was full of degeneration granules but its stage of development could still be recognized, the majority of embryos being in stage C. Following treatment on day 5, the 6½-day disc had lost its general organization, and degeneration granules were still present (Plate 2, fig. I). After treatment on day 3 or 4, only a very small thin disc containing a few degeneration granules and sometimes mucin, remained. The effect of Thiotepa seemed to be exerted mainly on the disc, but the trophoblast was
probably also affected, as blastocysts were small (diameter 2–3 mm.) at 6½ days, following treatment on days 3 and 4. No changes in mitotic activity were visible in the embryos at the time of autopsy.

**Implantation and later stages of gestation.** No experiments were done to examine the effect on nidation as such. However, even low doses of Thiotepa prevented gestation in rabbits, none of which produced living offspring following treatment on days 6–8 with 3 mg. per kg.

**Triethylenemelamine (TEM)**

This substance caused a marked fall in weight, even in doses of 0·5 mg. per kg. body-weight; no other symptoms were seen in experiments of short duration.

**Ovulation and fertilization.** Single doses not exceeding 2 mg. per kg. given on day 0, or a series of 12 injections of 0·5 mg. per kg. given over a period of 24 days prior to mating, had no effect on ovulation. Following 2 mg. per kg. given immediately after mating, 25 per cent. of the ova shed were unfertilized.

**Cleavage.** Doses of 1-0–2-0 mg. per kg. rapidly inhibited ovum cleavage.

**Blastocyst stage.** A single dose of 0·5–2-0 mg. per kg. given on days 2, 4, 5, or 6 had a drastic effect on the blastocysts, the response generally being the more marked the earlier the drug was given.

Usually only debris of cleaving ova or morulae were found at 6 days in the uteri of animals treated soon after mating. In one experiment, following a small dose on day 0, one apparently normal blastocyst was recovered, while the rest of the embryos were completely degenerated. The rate of growth of the embryos recoverable at 6 days was slowed down: 6½-day blastocysts from does treated on day 4 were smaller (3·0×2·9 mm.) than those from an animal injected on day 5 (4·0×3·7 mm.). Histological changes, dependant upon the time of treatment, were seen in the discs of 6½-day embryos. When the experimental interval was limited to 9 hours, marked signs of degeneration were present. Following treatment on day 5, the 6½-day discs were small and thin, and the cells contained distinct, heavily stained granules (Plate 2, fig. J)—probably chromatin, a picture resembling that produced by Thiotepa given on day 5. After TEM injection on day 4, the 6½-day discs were very small and contained mucin and large extracellular granules (Plate 2, fig. K). Although there was some degeneration in the trophoblast, there were many dividing cells there, and mitosis was not obviously affected.

**Implantation and later stages of pregnancy.** A low dose of TEM on day 6 was incapable of preventing the attachment of blastocysts, but the further development of such embryos usually terminated within a few days. By treating rabbits with not more than 0·5–1·0 mg. per kg. on day 8, i.e. after implantation, it was possible to maintain pregnancy till term, but the young were born dead; malformations were not seen. The does were again mated and conceived normally. However, by about day 20 of gestation massive vaginal haemorrhage abruptly terminated the pregnancies. Inspection of the reproductive tract did not reveal
abnormalities, apart from the haemorrhagic residue. In this respect, that is in being apparently able to induce discrete yet long-term changes in the reproductive system of rabbits, TEM differed from the other agents used in this study.

**Degranol**

This nitrogen mustard derivative was given intravenously in doses of 12–45 mg. per kg. body-weight. The lower dose was well tolerated; the larger amount caused some loss of weight, but the rabbits survived for another 10 days, when the experiments were terminated.

*Ovulation and fertilization.* Neither was affected at the 12-mg. per-kg. dose level; higher doses were not tested.

*Cleavage.* No experiments *in vivo* were done; *in vitro*, the development of 2-to 4-cell rabbit ova was arrested after a few hours’ incubation in serum-Ringer solution containing Degranol 1:10,000.

*Blastocyst stage.* Normal blastocysts were found at 6 1/2 days in one rabbit given 12 mg. per kg. on days 0 and 1; some of the embryos of another animal given 45 mg. per kg. on day 5 were so fragile that they burst before they could be fixed in methanol. This did not occur with any other treatment and was probably the result of an adverse effect upon the trophoblast or the zona pellucida. The remainder of these blastocysts all showed degenerative changes in various degrees, in both the discs and trophoblasts.

*Implantation and later stages of pregnancy.* Blastocyst nidation was not prevented by 12 mg. per kg. given on day 6; a normal litter was born to a doe injected with this dose on day 8. In a rabbit given 38 mg. per kg. on day 8, small implants were found on day 12; several of these were degenerating, while some were still alive. The foetal placentae were strikingly pale and poorly developed, and the exocoelomic fluid was scarce and contained stringy masses.

**E 39 soluble**

This was given as a single subcutaneous injection of 2.0–2.8 mg. per kg. body-weight on day 4 or 5. The dose was well tolerated and there was no local reaction. So far, the effect of this drug has been studied on blastocysts only, on which it had a severe destructive action. In some animals 50 per cent. of the embryos were almost completely destroyed. The blastocysts which survived to day 6 1/2 were small (mean diameters less than 3 mm.); there was much degeneration in the discs, and rather less in the trophoblasts, the embryos from mothers treated earlier being more seriously affected (Plate 3, figs. L, M). There appeared to be some effect on mitosis as some abnormal mitotic figures were seen.

**Myleran**

Doses of 4.8–12 mg. per kg. of body-weight given parenterally or orally, on days 1 or 5, or 4 and 5, had no effect on 6 1/2-day blastocysts.
Effect of purine and pyrimidine analogues

6-Mercaptopurine

This was injected subcutaneously in doses ranging from 25 to 250 mg. per kg. of body-weight. Rabbits were remarkably resistant to mercaptopurine and withstood even large amounts well. In most experiments on the pre-implantation blastocysts the injections were made 24–48 hours before autopsy, but in others they were given on day 0 or 1.

Ovulation and fertilization. There was no interference with either within the dose-range.

Cleavage. Tubal ova examined on days 1, 2, or 3, following administration of mercaptopurine 20–24 hours earlier, showed no detectable abnormalities on microscopical examination. Moreover, such ova, when recovered on day 1 or 2 and subsequently cultured in vitro in serum-Ringer solution, continued to divide normally for another 24 hours. Nevertheless, as will be demonstrated below (p. 481), the majority of such ova would have been incapable of normal full-term development.

Blastocyst stage. Blastocysts were examined at 6–7½ days of age. Their size and gross appearance at 6–6½ days was not perceptibly altered following treatment of rabbits shortly after mating or on days 4–5. However, at 7–7½ days, when normally rabbit blastocysts are already attached to the uterus, most embryos from mercaptopurine-treated rabbits had failed to implant although they continued to expand in size. Such 'giant' free-lying blastocysts often reached a diameter of 5 mm., the maximum size recorded being 7·6×6·1 mm., and weighed up to 100 mg. Occasionally at that stage the uterine horns of the treated animals were filled with a water-clear, alkaline fluid, in which, on microscopical examination, blastocyst debris could be seen. Chemical analysis showed that this fluid contained bicarbonate, glucose, and lactic acid in amounts corresponding to those established for 6- to 6½-day blastocyst fluid.

Histological study of these blastocysts indicated that mercaptopurine exerted a strongly deleterious effect on the embryos, whether administered to the mother shortly after mating, or on days 4–5. The embryonic disc had almost disappeared in some blastocysts treated on day 0 or 1, while in others it consisted of a few cells with darkly staining cytoplasm (Plate 3, fig. N) surrounding a mass of reddish globular material, believed to be mucin. In blastocysts from animals treated on days 4–5 (Plate 3, fig. O) there were more degeneration granules, but less mucin, than in those injected soon after mating (Plate 3, fig. N). The trophoblasts were relatively less affected than the disks and appeared almost normal in some embryos. In many but not all of the 7- to 7½-day blastocysts, the development of the trophoblastic knobs was either reduced or prevented.

Later stages of pregnancy. In rabbits treated as indicated above a few blastocysts implanted, but foetal development usually terminated between days 12 and 16; on autopsy, the foetuses were undersized and in various stages of
degeneration. The maternal and foetal placentae were regressing. No living young were born to rabbits that at any time after mating had received more than 75 mg. per kg. mercaptopurine.

8-Azaguanine

This was injected in doses 25–165 mg. per kg. on 1 or 2 of the first 5 days after mating. The health of the animals remained good.

Ovulation and fertilization. There was no interference with ovulation when azaguanine was given immediately after mating, but 10–15 per cent. of the ova shed were unfertilized when the dose was large.

Cleavage. Following treatment on days 0, 1, or 2 it was not possible to detect any morphological changes in cleaving ova recovered 24 hours after the injection. Yet these ova must have incurred discrete damage, as can be inferred from the egg-transfer experiments.

Blastocyst stage. Following treatment of rabbits on days 0, 1, or 2 and sometimes 3, blastocysts were seldom found at 6½ days, but on flushing the uterine horns with saline it was possible to recover remnants of degenerating morulae or even early blastocysts. In one experiment, with injection on day 3, small blastocysts (diameter less than 1·7 mm.) were recovered on day 6§. These embryos were severely damaged: the embryonic disc had almost disappeared and consisted of a few degenerating cells, but there was less mucin than in similar experiments with mercaptopurine. On the other hand, apparently normal blastocysts were observed following treatment on day 4 or 5. The impression was gained that with azaguanine the lesions in the embryos developed slowly, so that unless enough time was allowed to elapse between the time of administration and autopsy, the extent of damage appeared relatively slight. Possibly also, rabbit embryos were more sensitive to this purine analogue in the early, rather than in the slightly more advanced, stages of development.

When 25 mg. per kg. azaguanine was injected with an equal amount of mercaptopurine on days 4 and 5, very small blastocysts (mean diameters 2·2 × 2·1 mm.) were recovered at 6½ days. All embryos showed marked signs of degeneration in the disc while the trophoblast had suffered less damage. The combined effect of the two purine analogues seemed more severe than that of a similarly timed dose of 50 mg. per kg. mercaptopurine alone. There were no changes in the uterine tissues, upon visual examination, with either of these substances.

5-Bromouracil

A limited number of experiments was done with this pyrimidine analogue. Doses of 50–80 mg. per kg. administered on days 2 and 3 had no deleterious effect on 6½-day blastocysts as regards size and microscopical appearance. Rabbits similarly treated but allowed to go to term produced litters of normal
size which, however, failed to survive although maternal health was not noticeably affected.

*Embyronic mortality following temporary transfer of cleaving eggs to the oviducts of rabbits treated with purine analogues*

**Egg-transfer technique**

Eggs were recovered from the oviducts of 12 donor rabbits which had been mated 24–26 hours earlier with 2 fertile males. Some of the eggs were incubated *in vitro* in Krebs’s Ringer solution+homologous blood plasma (1:1) at 37°C. for 4½–6 hours. These eggs are referred to as ‘controls’. Other eggs were transferred for 4½–6 hours into the oviducts of rabbits serving as ‘temporary recipients’; these animals were given one injection of either mercaptopurine or azaguanine (110–180 mg. per kg.) on the evening preceding, and another on the day of, the experiment. Such eggs, referred to as ‘treated’, were next recovered for transfer into the oviducts of untreated permanent recipients; the luteal stage of these animals (in terms of hours after injection of luteinizing hormone) was arranged to correspond with the age of the transferred ova. The ‘treated’ eggs were introduced on one, the ‘control’ eggs on the other side of the reproductive tract of the permanent recipients. The further development of the transplanted embryos was examined by laparotomy or autopsy between days 9–14 of gestation; 3 does were allowed to go almost to term.

**Results**

The 40 ‘control’ eggs underwent one cleavage division, reaching the 4-cell stage during incubation *in vitro*. Out of the 70 eggs placed in the oviducts of the ‘temporary recipients’, 3 of which were under treatment with mercaptopurine and 3 with azaguanine, 57 were recovered. With few exceptions, all had undergone one cleavage division. Morphologically, the ‘treated’ eggs appeared indistinguishable from the ‘controls’.

Following temporary exposure of 21 eggs to the tubal environment of rabbits treated with mercaptopurine, the implantation rate was 23-8 per cent. and that for 17 ‘control’ eggs 70-6 per cent. A high proportion of foetuses from the ‘treated’ eggs showed retarded growth at autopsy on day 12; these probably would not have survived to term. In one recipient which had received 9 ‘treated’ eggs a single, apparently normal, foetus was found at 28 days.

In analogous experiments with azaguanine, some 60 per cent. of the 20 ‘treated’ eggs were found implanted at 10–14 days, but several implantation sites were poorly developed, and in some the embryos were dead. One doe which went to term had 2 apparently normal foetuses from 6 ‘treated’ eggs. In contrast, all foetuses from the 11 ‘control’ eggs in these experiments were normally developed.

Though restricted in number, the results indicate that the deleterious effect of mercaptopurine and azaguanine can be transmitted to the cleaving egg during
its stay in the oviduct, and that a relatively brief exposure in vivo suffices to induce a high percentage of embryonic loss. It is noteworthy, and in line with observations recorded earlier (pp. 479 and 480), that the in vivo effect of the purine analogues was not immediately apparent, but that it became manifest at about the time of, or after, ovum implantation.

**Effect of excess vitamin A**

The vitamin A preparation was fed or injected between day 0 and 5; the total varied from 60,000–300,000 i.u. per animal. The treatment had no effect upon the health of the rabbits.

*Blastocyst stage.* There was no evidence of any deleterious effect of excess vitamin A upon 6½-day blastocysts; indeed, some embryos were larger than normal, and in one litter all showed signs of primitive-streak formation (stage D or beyond), a well-formed streak being present in 57 per cent.

*Implantation and later stages of pregnancy.* There was no interference with either nidation or subsequent foetal development, when treatment with excessive amounts of vitamin A was restricted to days 0–5. Normal litters born to treated does were reared and in turn produced normal offspring.

**Effect of vitamin antagonists**

*Analogues of vitamin B₁₂*

B₁₂ anilide alone, or a mixture of B₁₂ methylamide and ethylamide (Smith, Parker, & Gant, 1956) were injected daily in doses of 100–500 μg. per animal, starting 10 days before mating and continuing throughout pregnancy and lactation. The animals' health remained excellent.

*Blastocyst stage.* There was no demonstrable effect upon 6½-day blastocysts.

*Implantation and later stages of pregnancy.* Normal litters were born to all treated rabbits.

*Analogues of folic acid*

Aminopterin and amethopterin were the folic acid antagonists used. Only the effect upon blastocysts was investigated.

*Aminopterin.* Given parenterally during the pre-implantation period, 1–2 mg. per kg. had no marked deleterious effect upon rabbits. Blastocysts recovered at 6½–6¾ days from rabbits given 1 mg. per kg. on day 4 were small (diameter 2·6 × 2·4 mm.) and the discs were immature: the most advanced stage was B–C, in which were 43 per cent. of the discs. However, after similar treatment on day 5, 6½-day blastocysts appeared normal. When 2 mg. per kg. was given on day 6 and followed by autopsy 9 hours later, the embryos were normal; they showed no disturbance of mitotic activity.

*Amethopterin.* This was administered parenterally or orally. Injections of up to 6·5 mg. per kg. on days 0, 2, or 3, or oral doses of 5 mg. per kg. on days 0–5, did not visibly impair the health of the rabbits. There were no visible effects upon 6½-day embryos with either type of treatment.
EMBRYOTOXIC AGENTS

Miscellaneous agents

**Trypan blue**

A 10 per cent. solution of the dye in saline was injected either on days 0–5, or 7–9, in doses of 100–200 mg. per kg. It exerted no ill-effects on the rabbits.

*Blastocyst stage.* Trypan blue administered within the first 5 days after mating failed to penetrate unimplanted blastocysts, which remained colourless, although the uterine secretion and the endometrium were strongly stained. Microscopically these embryos were normal. Attempts were made to increase the permeability of pre-nidation blastocysts by combining trypan blue treatment with a single small dose of TEM or Colcemid given on day 5. Even then, however, the dye did not enter the blastocysts.

*Implantation and later stages of pregnancy.* The negative results in the pre-implantation embryos contrasted with the effect of trypan blue when it was injected during the implantation period, i.e. on days 7–9. At that stage the dye freely entered the blastocyst cavity; a large proportion of young born to rabbits treated in this way showed malformations; none survived beyond a few days.

**Neptazane**

This potent sulphonamide inhibitor of carbonic anhydrase was used to study the structure and chemical composition of blastocysts developing under conditions in which the activity of endometrial carbonic anhydrase was greatly curtailed. Neptazane was given intravenously, 100–120 mg. per kg. body-weight, in subdivided doses, on days 4, 5, and 6 without causing any toxic symptoms. Following such treatment the activity of blood carbonic anhydrase was almost abolished; however, some residual enzyme activity invariably remained in the endometrium (20–25 e.u. per g.).

*Blastocyst stage.* No abnormal structural changes were observed in 6½-day embryos following exhaustive treatment with Neptazane. The content of bicarbonate, glucose, and lactate in the blastocyst fluid at 6–8 days did not differ from values established for normal blastocysts.

*Implantation and later stages of pregnancy.* There was no interference with nidation. Later foetal development was normal.

**Carbon tetrachloride**

This hepatotoxic substance was fed to pregnant rabbits in arachis oil, 0·6 ml. per kg. body-weight on day 5 or 1·0 ml. per kg. on days 4 and 5. The treatment caused no overt symptoms in these short term experiments. Blastocysts recovered at 6½ days were well developed and appeared quite normal after treatment with the smaller dose. With the larger amount there was some cellular degeneration in the embryonic discs, and the trophoblasts of some embryos contained very large nuclei with prominent nucleoli.
Carbutamide

This hypoglycaemia-inducing agent was administered parenterally, in doses 100 mg. per kg. body-weight and was well tolerated.

Blastocyst stage. Following treatment on days 4, 5, and 6 no abnormalities were seen in 6½-day blastocysts.

Implantation and later stages of pregnancy. There was no interference with foetal development; a normal litter was born to a treated doe.

Cysteamine hydrochloride

Doses of this radiation-protective agent ranging from 70 to 160 mg. per kg. body-weight were well tolerated.

Blastocyst stage. Following injection on days 2 and 3, 3 and 4, or 4½ and 5, blastocysts recovered at 6½–6⅔ days appeared unaffected by the treatment.

Implantation and later stages of pregnancy. There was no interference with nidation or further foetal development.

Total body exposure to X-rays

Pregnant rabbits were exposed to X-radiation ranging from 450–650 r., given at a dose-rate of 150 r. per minute. The Maximar X-ray machine was run at 220 kV., 15 Ma. and an added 1 mm. aluminium filter was used. The unanaesthetized animals were confined in a lidless wooden box, in such a way as to receive the radiation beam mainly in the lumbar region, the distance from the animal’s body to the source of radiation being 20 cm. There were no overt symptoms following this type of exposure within the limited experimental period.

Two rabbits were treated on day 5 with 450 r. and 650 r., respectively, and the blastocysts were fixed 36–40 hours later; two other animals were similarly irradiated on day 6 and autopsied 5 and 9 hours later. Following irradiation on day 5 with 450 r., the development of the disc appeared retarded, the most advanced stage at 6⅔ days being C, but the mean diameter of the blastocysts was normal (4·2 × 3·9 mm.) and 70 per cent. of them had trophoblastic knobs. There was some nuclear degeneration but this was probably not excessive. Similarly timed treatment with 650 r. resulted in small blastocysts (3·1 × 2·9 mm.); at 6⅔ days none was more advanced than stage B. In this experiment degeneration granules were rather numerous. The blastocysts recovered 9 hours after irradiation of the rabbit with 650 r. on day 6 were well developed, 90 per cent. having primitive streaks, and they showed no sign of abnormal degeneration. The embryos from the animal autopsied 5 hours after exposure to 450 r. on day 6 contained many degeneration granules; there were globules of mucin in all the embryonic discs, 50 per cent. of which were in Stage C and the rest in more advanced stages (Plate 3, fig. P). These few experiments indicate that pre-implantation embryos can be affected by irradiation of the mother. However,
further study would be required to determine in detail the extent of damage and the ability of the embryos to recover from such treatment.

Activity of endometrial carbonic anhydrase

With the exception of the above described results obtained with the specific carbonic anhydrase inhibitor, Neptazane, none of the agents used in this study exerted any inhibitory influence upon the activity of this enzyme in the uterine endometrium.

DISCUSSION

The flat mount preparation was originally devised to provide a simple and rapid method for evaluating the effect upon rabbit blastocysts of culture media, such as might conceivably be used in biochemical in vitro experiments. It was, however, envisaged even then that the procedure could be equally useful in studies where experimental factors are made to act upon embryos not directly, but via the maternal organism. The present study attests the applicability in this respect of the flat mount preparation. It has enabled us to observe certain morphological features in normal blastocysts and to classify 5- to 7-day normal blastocysts into 6 developmental stages, based mainly on the degree of maturity shown by the embryonic discs. These data, together with the dimensions of the entire blastocysts, served as a basis for assessing the condition of embryos recovered from animals which had been subjected to various treatments. Results obtained with normal blastocysts have brought out the existence of fairly wide limits of morphological variability, within individual members of a litter as well as between different, but chronologically identical, litters. These are findings of some interest and significance: possibly, they explain at least one of the causes underlying the differences in drug susceptibility frequently encountered in the young embryos. Moreover, they indicate that results of histological, chemical, or other analyses, undertaken with single blastocysts, can yield misleading information, and that it is advisable, when studying embryos of polytocous laboratory animals, to use truly representative samples.

We regard the use of animals which have had previous litters as another prerequisite for experiments involving factors likely to exert embryotoxic effects; a fertility record often helps to estimate the effect of a treatment upon the number of embryos.

Of considerable importance to this kind of study is a carefully adjusted schedule as regards the timing of treatment and drug dosage; in most cases these appeared more significant than the route of administration. We recognize that some of the results presented above could be modified by alterations in either timing or dose level: thus, consecutive small amounts of a drug might have induced in blastocysts a condition markedly different from that due to a single large dose.

The substances used for our study belong to categories of agents of which
some could be expected to act predominantly upon the embryo itself, viz. the
cytostatic compounds or the metabolic analogues, and of which others were
likely to affect primarily the uterine environment, such as the ovarian and other
hormones or Neptazane, the sulphonamide inhibitor of endometrial carbonic
anhydrase. Some of the embryotoxic materials with which we have treated our
experimental animals have also been used by other investigators in studies
chiefly concerned with more advanced pregnancy, for the most part in rats or
mice. However, we have, in addition, examined the effect of some newer anti-
neoplastic drugs not hitherto studied in this respect, namely, Degranol (Vargha,
Toldy, Feher, & Lendvai, 1957), and E 39 soluble (Domagk, Petersen, & Gauss,
1954); the former we have found to be relatively non-toxic to the rabbit embryos,
but the latter harmful, even in low doses.

The emphasis in this work was on the blastocyst stage, but in several instances
observations were also made covering the period from ovulation and fertilization
up to and beyond implantation. It was interesting to note that no damage to
ovarian function ensued, in so far as ovulation was concerned, in consequence of
treatments which proved effective at other stages. This resistance may, of course,
be species-specific. On the other hand, fertilization of the ova shed was some-
times affected when large amounts of Thiotepa, TEM, or azaguanine, were
administered immediately after mating.

The inclusion in our study of the cleaving egg has permitted us to note how
vulnerable the zygote is, during its tubal passage, to the action of agents trans-
mitted from the mother. This fact, amply illustrated by the experiments in-
volving egg transfer and the results obtained with the various cytostatic drugs,
dispose of the view occasionally expressed that, because the tubal egg when
cultivated in vitro shows divisions which fall within physiological time limits, it
is largely independent of its environment.

With regard to implantation, when the interval allowed for drug action was
brief, as was mostly the case in our experiments, it was seldom possible entirely
to prevent blastocyst attachment, even with relatively large doses of potent
agents. A rather effective short-term inhibitor of implantation in the rabbit was
oestradiol benzoate. In this it differed from diethyl- and dimethylstilboestrol,
neither of which prevented nidation, though, like the oestrogenic hormone, both
compounds interrupted further foetal development, presumably owing to
changes induced in the uterine tissues. Other instances of interference with
implantation were the experiments with mercaptopurine, in which a large
percentage of blastocysts failed to implant on day 7 if a suitable dose of the
purine analogue was injected only some 36 hours earlier. On the other hand,
the depression of endometrial carbonic anhydrase activity by Neptazane was
ineffective in preventing ovum attachment, perhaps because it proved impossible
to achieve complete and lasting inhibition of the uterine enzyme, even with
near-toxic doses of this sulphonamide.

Some of the substances which we have administered to pregnant rabbits, for
instance the colchicine derivatives and some alkylating agents, acted equally promptly on all of the investigated stages of pregnancy. But with others, notably the purine analogues mercaptopurine and azaguanine, the effect upon tubal eggs was not discernible, and only became manifest about the time of implantation. This is presumably due to the fact that these antimitobolites must first be incorporated and metabolized to the 'active' derivatives, which become biologically potent and ultimately interfere with embryonic development. Our results suggest that, like tumour and haemopoietic tissue, the early embryo is prominent in its capacity to anabolize purine analogues to such active derivatives. Those few embryos which in spite of exposure survive the treatment, thus appearing resistant to the action of the purine analogues, may be incapable of the 'lethal synthesis', i.e. the conversion of mercaptopurine or azaguanine to the corresponding nucleotides. It would be most interesting to obtain direct proof of this phenomenon experimentally.

However, we have also dealt with metabolic antagonists which, under our experimental conditions, produced relatively little or no effect on rabbit blastocysts. These include the antagonists of folic acid, aminopterin, and amethopterin, and also the analogues of vitamin B_{12}. The vitamin B_{12} analogues, the antagonistic action of which has so far only been demonstrated in certain specialized systems (Cuthbertson, Gregory, O'Sullivan, & Pegler, 1956), obviously lack the ability to interfere with pregnancy in the rabbit, even when administered for extended periods and in large amounts. Although negative, these findings are interesting in view of the fact that the endometrial secretion and blastocyst fluid are exceptionally rich sources of vitamin B_{12} (Jacobson & Lutwak-Mann, 1956), the successful 'replacement' of which by a truly competitive antagonist might have been expected to suppress embryonic development.

Similarly, no adverse effect upon rabbit blastocysts was achieved with excessive doses of vitamin A during the first 5 days of gestation. This contrasts with the well-established adverse effect of hypervitaminosis A in rodents when it occurs in the post-implantation phase (Giroud & Martinet, 1954; Millen & Woollam, 1960).

Another finding worthy of comment was the differential sensitivity within the blastocyst encountered in response to certain agents, as between the embryonic disc and the trophoblast, the former being on the whole more conspicuously affected. We assume that this distinctive susceptibility of the disc and trophoblast, respectively, is the expression of differences in both type and rate of metabolic processes, whereby the more active and rapidly proliferating tissue of the embryo proper suffers adverse effects more readily. Good examples of such differential behaviour were the experiments with Thiotepa, TEM, and E 39 soluble; and also those with mercaptopurine, in which, as late as day 7\textsuperscript{a}, it was possible to recover free-lying, giant vesicles, practically anembryonic yet obviously capable of taking up and retaining water and other constituents characteristic of blastocyst fluid.
On the other hand, with Colcemid, cell-division was affected in both trophoblast and disc within 60 minutes after injection; this was 2–3 hours before the animals developed signs of diarrhoea. In this respect Colcemid differed from Thiolcolciran, another closely related colchicine derivative, which did not induce such striking changes in the rate of cell-division.

The prompt response of the rabbit blastocyst to a variety of extraneous agents, coupled with the straightforwardness of the flat mount preparation, whereby it is equally easy to observe the condition of the embryonic disc and cells within the trophoblast area, combine to suggest a technique suitable for the screening of chemotherapeutic drugs used in malignant diseases, possibly also of cytopathogenic factors such as the viruses. For preference, these should be administered to the pregnant animals in amounts which, in experiments of short duration, do not seriously upset maternal health, but act on the blastocysts either generally or selectively by attacking mainly the embryo proper.

SUMMARY

1. A study was made of the action upon the rabbit embryo of various agents administered to pregnant animals in doses which did not seriously upset maternal health. The pre-implantation blastocyst, examined as a flat mount, was the principal object of study, but observations were also made on ovulation, fertilization, cleavage, nidation, and subsequent phases of pregnancy.

2. To facilitate comparison between embryos from normal and treated animals 5- to 7-day-old blastocysts from untreated animals, grouped according to size, were classified into 6 developmental stages, depending upon the degree of maturity of the embryonic disc. Additional procedures which served to evaluate the condition of blastocysts and their uterine environment were: mitotic counts made in the abembryonic part of the trophoblast, chemical determinations of certain characteristic constituents of blastocyst fluid, and measurement of carbonic anhydrase activity in the endometrium.

3. The experimental agents comprised hormones, colchicine derivatives, several polyfunctional alkylating compounds, metabolic analogues, a sulphonamide inhibitor of carbonic anhydrase, and substances endowed with teratogenic, hepatotoxic, radiation-protective, and hypoglycaemia-inducing properties; total body exposure to X-rays was also investigated.

4. Results obtained at various stages of pregnancy with the different treatments are described and discussed; several results relating to blastocysts are illustrated by photomicrographs.

5. Among significant general findings emerging from the study are: the speed with which exogenous agents are transmitted to embryos even before uterine attachment; differential drug sensitivity, frequently evident in the pre-implantation blastocysts, as between the embryonic disc and trophoblast, the former usually being more susceptible; and variability in response, both individual and between litters, of the embryos to parenterally administered agents.
6. It is suggested that the blastocyst flat mount preparation could be adapted as a conveniently rapid and simple procedure for the screening of cytostatic and other growth-modifying agents.

**RéSUMÉ**

*L’action des agents divers sur l’embryon de Lapin*

1. On a étudié l’action sur l’embryon de Lapin de divers agents administrés à des femelles gravides à des doses qui ne troublent pas sérieusement la santé de la mère. Le blastocyste non encore implanté et examiné par montage *in toto* de sa paroi étalée a été l’objet principal de cette étude, mais des observations ont été également faites sur l’ovulation, la fécondation, le clivage, la nidation et les phases ultérieures de la gravidité.

2. Pour faciliter la comparaison entre les embryons provenant d’animaux normaux ou traités, des blastocystes de 5 à 7 jours provenant d’animaux non traités ont été groupés d’après leur taille et classés en six stades de développement, établis d’après le degré de maturité du disque embryonnaire. D’autres techniques ont été également utilisées pour apprécier l’état des blastocystes et celui du milieu utérin. Ce furent: des numérotations de mitoses effectuées sur la partie antiembryonnaire du trophoblaste; des déterminations chimiques de certains constituants caractéristiques du liquide blastocœlien; et des mesures d’activité de l’anhydrase carbonique dans l’endomètre.

3. Les substances expérimentées ont été des hormones, des dérivés de la colchicine, divers composés polyfonctionnels complexants, des analogues métaboliques, un inhibiteur sulfamidé de l’anhydrase carbonique, ainsi que des corps possédant des propriétés soit tératogènes, hépatotoxiques, radioprotectrices, ou produisant de l’hypoglycémie; l’exposition totale du corps aux rayons X a été également comprise parmi les situations examinées.

4. Les résultats obtenus aux divers stades de la gravidité par les différents traitements sont décrits; divers effets se rapportant aux blastocystes sont reproduits dans des illustrations microphotographiques.

5. Parmi les constatations de signification générale qui se sont dégagées de cette étude, on peut citer: la vitesse avec laquelle des agents exogènes sont transmis aux embryons même avant la fixation de ceux-ci à l’utérus; la sensibilité différentielle aux toxiques fréquemment évidente dans les blastocystes non encore implantés, entre le disque embryonnaire et le trophoblaste, le premier étant en général le plus sensible; et aussi la variabilité, à la fois entre individus et entre nichées, quant à la réponse des embryons à des substances administrées par voie parentérale.

6. Il est suggéré que la méthode de préparation des blastocystes étalés par montage *in toto* paraîtrait bien adaptée pour fournir une technique pratique, rapide et simple pour la sélection des substances cytostatiques ou susceptibles de modifier la croissance.
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REFERENCES


EXPLANATION OF PLATES

All preparations shown in these plates were stained with Delafield's haematoxylin.

PLATE 1

The figures shown in this plate illustrate 5 stages (A, B, C, D, & F) in the normal development of the embryonic disk in 5- to 7-day blastocysts from untreated rabbits. Figs. A–E are all at the same magnification, as indicated in fig. A.

FIG. A. Stage A; 5-day embryo. The disk is 1–2 layers of cells thick and slightly irregular in outline.

FIG. B. Stage B; 64-day embryo. The disk is about 3 layers of cells thick and has a smooth outline.
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Plate 1
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Plate 2
Plate 3
Fig. C. Stage C; 6-day embryo. The disk has elongated and is beginning to grow out in a posterior direction.

Fig. D. Stage D; 6½-day embryo. Cells are beginning to condense in the midline of the zone of outgrowth to form the primitive streak.

Fig. E. Stage F; 7-day embryo. The primitive streak is present and stellate mesodermal cells can be seen growing out from the disk in a fan-shaped area round its posterior end. The trophoblast was removed from the anterior end of the disk during preparation.

Plate 2

Fig. F. Trophoblastic knobs in the equatorial region of the trophoblast of a 7-day embryo. Between the knobs the large nuclei of the trophoderm and the smaller endodermal nuclei can be seen.

Fig. G. Part of the embryonic disk of a 6½-day blastocyst from a rabbit treated 5 hours before autopsy with 2 mg. Colcemid per kg. of body-weight. Cell-division was arrested in metaphase; marked cellular degeneration can be seen.

Fig. H. Part of the abembryonic region of the trophoblast of a 6½-day blastocyst of a rabbit treated 9 hours previously with 2 mg. Colcemid per kg. body-weight. The trophoblast in this region consists of only one layer of cells and 8 metaphases together with 2 prophase (one shown incompletely) and 2 interphases can be seen; anaphases and telophases were absent from this specimen.

Fig. I. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 2 mg. Thiotepa per kg. body-weight. The disk is small and irregular in shape; degeneration granules and a nodule of mucin (M) can be seen. The magnification of this specimen is greater than that of the controls shown in Plate 1.

Fig. J. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 2 mg. TEM per kg. body-weight. The disk is only 1–2 layers of cells thick and the cells contain heavily stained granules, probably chromatin; some cells are dividing and an anaphase can be seen close to the edge of the disk. The magnification of this specimen is about twice that of the controls shown in Plate 1.

Fig. K. The embryonic disk of a 6½-day blastocyst from a rabbit that had been treated on day 4 with 0.7 mg. TEM per kg. body-weight. The disk is very small (magnification is the same as in fig. J) and contains numerous degeneration granules. Several mitotic figures can be seen in the trophoblast.

Plate 3

Fig. L. Very degenerate embryonic disk of a 6½-day blastocyst from a rabbit treated on day 4 with 2.9 mg. E 19 soluble per kg. body-weight. The disk consists mainly of nodules of mucin (M). Degeneration granules and some dividing cells can be seen in the surrounding trophoblast.

Fig. M. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 5 with 2.5 mg. E 19 soluble per kg. body-weight. The disk is larger than that shown in fig. L (it is shown here at the same magnification) but it is degenerating.

Fig. N. Embryonic disk of a 6½-day blastocyst from a rabbit treated on day 1 with 60 mg. 6-mercaptopurine per kg. body-weight. The disk is small and its cells have darkly stained cytoplasm, but there are only a few degeneration granules; a nodule of mucin (M) can be seen.

Fig. O. Embryonic disk of a 6½-day blastocyst from a rabbit treated on days 4 and 5 with 50 mg. 6-mercaptopurine per kg. body-weight. There are more degeneration granules but less mucin than in fig. N. A few mitotic figures can be seen in the surrounding trophoblast.

Fig. P. Part of the embryonic disk of a 6½-day blastocyst from a rabbit that had received 450 r. of X-rays 5 hours before autopsy. Some globules of mucin (M) and severe cellular degeneration are shown.

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