Studies on the hormonal basis of egg implantation

in mice.

Peter Sutherland Grant

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University of Edinburgh

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CONTENTS

GENERAL INTRODUCTION

Eggs in the uteri of ovariectomised pregnant mice only implant following treatment with progesterone and oestrogen (Bloch, 1958; Smithberg and Runner, 1960; Yoshinaga and Adams, 1966), whereas trophoblastic invasion and occasionally embryonic development proceeds in various extra-uterine sites irrespective of the endocrine status of the host (Fawcett, Wislocki and Waldo, 1947; Jones, 1952; Kirby, 1963a & b). The uterine selectivity and control of implantation appears to be the consequence of the sequential action of oestrogen and progesterone. In the rat, studies have shown that proestrus oestrogen primes the uterus for subsequent progesterone action on which is superimposed a release of oestrogen that sensitizes the uterus and induces egg-implantation (see reviews by Psychoyos, 1966; De Feo, 1967; Nalbandov, 1971; Shelesnyak and Marcus, 1971). Experimental conditions identical to these (except that events take place a day sooner) allow the induction of deciduomata or implantation in ovariectomised nonpregnant mice (Finn, 1966; Humphrey, 1967a; 1969).

Morphological changes occuring in the uterus and blastocyst at the time of implantation in the mouse have been studied with the light microscope (Boyd and Hamilton, 1952; Snell and Stevens,1966; Finn and McLaren, 1967) and by electron microscopy (Reinius, 1967; Potts, 1968; 1969; Bergström, 1971). Progesterone treatment of ovariectomised mice causes closure of the uterine lumen with close complementarity between apposing luminal walls (Finn and McLaren, 1967; Martin, Finn and Carter, 1970) and the action of oestrogen superimposed on that of progesterone causes an even tighter closure

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of the lumen that leads to fusion between apposing luminal walls
(Nilsson, 1966; 1967; 1970; Ljungk̄v̄ist, 1971).   Following loss
of the zona pellucida, perhaps due to the action of uterine enzymes
(McLaren, 1969; 1970) trophoblastic microvilli are exposed
(Bergström, 1971).   In progesterone treated ovariectomised mice
microvilli from both the trophoblast and epithelium interdigitate
(Potts and Psychoyos, 1967).   Both sets of microvilli are lost
however, following treatment with oestrogen, leading to what Nilsson
and his co-workers call the 'attachment reaction' of early implan-
tation (Nilsson, 1970; Bergström, 1971; Ljungkvist, 1971).   The
embryonic tissue is always situated at the mesometrial pole of the
blastocyst (see Kirby, Wilson and Potts, 1967) and the trophoblast
in the region of the abembryonic pole is usually the first to
invade the maternal epithelium.   The epithelium is removed rapidly
and the trophoblast appears to invade by disruption and phagocytosis
of the epithelium (Finn and Lawn, 1968; Potts, 1969).

The first two sections of this study (together with the
Appendices) describe the development of an organ culture system
with which attempts were made to try and duplicate some of the
above changes in vitro.   The first section deals with hormone-
induced changes in mouse uteri cultured alone and the second section
deals with the behaviour of eggs cultured within the lumina of
uteri.   Changes were followed with the light microscope only.
Organ culture allows the maintenance of the structural organization
of tissues under experimental conditions and has been found useful
for studies on the effects of hormones on various tissues, including
the mammary gland, vagina and male accessory organs (see reviews
by Lasnitzki, 1965; Waymouth, 1966).   Very few studies have been

concerned with effects of sex hormones on cultured mice uteri (see

Gaillard, 1942; Waymouth, 1966) although the technical problems have

been largely solved (Trowell, 1959). Most work has been concerned

with uterine tissue from larger species, e.g. the rat (Lagova, 1967),

guinea pig (Everett, 1962; 1963), hamster (Lenahan, 1968), rabbit

(Glenister, 1961; Kornman, 1967) and human (Kohorn and Tchao, 1969;

Hughs, Demers, Csermely and Jones, 1969). Potentially in vitro

studies on the control of egg-implantation would permit closer

analysis of certain crucial stages than is possible in vitro and

would have relevance to post-ovulatory fertility control. Further-

more, opportunities would be provided for study of the control of

embryogenesis and embryonic-uterine interactions during implantation

and also to test for the effects of possible teratogens (see Ferm,

1971). Currently, the rabbit is the only species in which

implantation has been studied in vitro (see Glenister, 1971).

The third and fourth sections of this study examine the use of

the potent synthetic progestins, medroxyprogesterone acetate (MAP)

and melengestrol acetate (MGA). These compounds have the advantage

of prolonged activity after a single injection relative to progesterone

(Revesz and Chappel, 1966; Duncan, Lyster, Hendrix, Clark and Webster,

1964). The third section is concerned with the use of these com-

pounds in virgin cyclic mice to control uterine receptivity for trans-

ferred eggs, such that a subsequent dose of oestrogen would induce

uterine sensitivity and implantation as in ovariectomised mice treated

with progesterone. The aim was to take advantage of the gonadotro-

phin suppressive action of these compounds (Logethetopoulos, Sharma

and Kraicer, 1961; Miyake, 1961; Duncan et al. 1964) to override the

mouse's own oestrous cyclic activity while at the same time

supplying progestin to promote uterine receptivity and maintain

pregnancy.   The fourth section investigates the side-effects of

these compounds on the development of the external genitalia and on

the subsequent reproductive performance of daughters from mothers

treated during pregnancy.   Masculinization of daughters from MAP-

treated mothers has been observed in rats (Revesz, Chappel and Gaudry,

1960) and guinea pigs (Foote, Foote and Foote, 1968).   The useful-

ness of the compounds as progestins to support pregnancy would be

inversely related to the severity of their side-effects.

Finally, the fifth section is concerned with the qualitative

and quantitative requirements of oestrogen for implantation

in vivo.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON IMMATURE UTERI

MAINTAINED AS ORGAN CULTURES

ABSTRACT

Uterine horns from immature mice were maintained as organ cultures for two days. Either ethanol (0.125%), oestradiol-17β (5μg/ml), progesterone (5μg/ml) or progesterone plus oestradiol (5μg + 5μg/ml) were added to Trowell's T_8 medium containing 20% foetal calf serum. The CO_2 concentration in the atmosphere was approximately 5% and the O_2 content varied from 20-29%. The degree of closure of the uterine lumen and the sharpness in outline of the intraluminal projections were assessed by an independent observer.

Uteri treated with oestradiol alone usually did not differ from those treated with ethanol, but progesterone treatment either alone or in combination with oestradiol lead to luminal closure and the sharpening of the corners. Treatment with both hormones or with oestradiol occasionally caused the fusion of the apposing epithelial luminal walls. Progesterone may have acted to close the lumina by increasing the tone of the circular uterine muscles.

INTRODUCTION

Progesterone and oestrogen act together to prepare the uterus to receive and support the implantation of fertilized eggs. Each uterine tissue responds in a characteristic morphological way to these hormones (for references see Martin, Finn and Carter, 1970; Nilsson, 1970 and Ljungkvist, 1971). Understanding of the causal basis of these phenomena would be facilitated if the characteristic morphological response of each uterine tissue could be duplicated in vitro. However the results obtained in an in vitro system would only be relevant to the in vivo situation if normal tissue relationships were maintained (see review by Lasnitski, 1965) as occurs in organ cultures. Trowell (1959) has shown that entire uterine horns from 20g rats can be maintained in good condition for up to nine days as organ cultures. Lostroh (1963) has used a similar technique with uterine horns from young or ovariectomised mice to investigate hormonal effects on protein synthesis.

There have been few in vitro studies in which ovarian hormones have caused their characteristic morphological changes in uterine tissues. Endometrial glands have responded to hormones by an increase in size and elaboration of secretion in explanted pieces of guinea pig (Everett, 1962) and human (Kohorn and Tchao, 1969) endometrium. Coujard (1943) and Everett (1963) have also shown that progesterone depressed the mitotic rate of epithelial cells on explanted endometrium. Martin and Finn (1968) have since demonstrated that progesterone has this action in vivo in the mouse.

This paper presents evidence for another characteristic progesterone induced response in immature uterine horns maintained as organ cultures.

MATERIALS AND METHODS

Uterine horns from prepubertal Q strain mice (body weights from 7 to 10g) were cultured after the methods of Trowell (1954; 1959) and Baker and Neal (1969). Difficulties encountered in the development of the culture technique are described in Appendix No.2. Mice were killed by decapitation and their uteri removed and dissected free from attendant tissues. Oviducts were left attached and used for subsequent handling.

Trowell's T_8 culture medium (Flow Laboratories) with 20% foetal calf serum (Flow Laboratories) and with 100 I.U. of penicillin plus $2\mu g$ of amphotericin B ('Fungizone'; Squibb) per ml was used. Crystalline oestradiol-17β and progesterone (Koch-Light) were dissolved in 100% ethanol. This was then diluted to a 50% solution by adding distilled water. Each millilitre of the four treatments used contained either

1) 0.00125ml of ethanol alone, or

2) $5\mu g$ of progesterone + 0.00063ml of ethanol, or

3) $5\mu g$ of oestradiol + 0.00063ml of ethanol, or

4) $5\mu g$ of progesterone plus $5\mu g$ of oestradiol + 0.00125ml of ethanol.

The level of culture medium added to plastic petri dishes (50 x 15mm, A/S Nunc.) was adjusted to reach the underside of the level platform of a stainless steel grid. Lens tissue (Green's C105) was drapped over the grid to make contact with the media.

Uterine horns were placed on the lens tissue and a stainless steel pin (disposable 26G needle tip, Becton, Dickinson and Co. Ltd) driven through the horn near its cervical end down through the mesh

of the grid.   The horn was then stretched taut by pulling on the

oviduct and the tension was maintained while a second pin was driven

through the uterotubal junction to anchor the top end of the horn to

the grid.

Four culture dishes, each containing one treatment medium, were

then gassed in 2lb 'Kilner' jars modified by the method of Baker and

Neal (1969).   The culture dishes were stacked in a stainless steel

or glass holder which was placed on a level wet cotton wool pad

situated on the bottom of the jar.   The lids of the jars were

adapted to contain inlet and outlet gasing tubes and a silicone

rubber washer (Silescol SR300, Esco Rubber Ltd.).   Jars were gased

with 5% CO_2 and from 20-29% O_2 with N_2 as the remainder.   Oxygen

concentrations were measured by the method of Fainstat (1968) and

the gas pressure in the jars varied from 0.5 to 3lbs per sq. in.

The jars were then maintained for two days at 35°C.   After which

the horns were fixed in Bouin's fluid, serially sectioned at 6μ

and stained with haematoxylin (Harris's) and eosin.

An independent observer, without knowing the treatment which

each uterine horn had received assessed sections for the degree of

complementarity in the patterning of the apposing epithelial

surfaces.   Although fixation shrinkage usually opens the uterine

lumen the degree of closure during culture can be estimated by

noting the degree of complementarity between apposing surfaces

(McLaren, 1968; Martin et al., 1970).   Scores were given from one

(close complementarity or tight closing, Pl.1, Fig. 1) to five (no

complementarity or open lumina, Pl. 1, Fig. 3).

The sharpness in outline of intraluminal projections and crypt

bases varied and was assessed along with luminal closure.   Scores

were given from one (very sharp corners, Pl. 1, Fig. 1) to five (rounded corners, Pl. 1, Fig. 4). Both characteristics were scored twice and means calculated.

The presence of a surface wave pattern (the corrugations of Finn and McLaren, 1967) were noted. Statistical tests for luminal closure and for corner sharpness compared the number of uteri with mean scores of two or less, between treatments either by 2 x 2 χ^2 contingency tables (Snedecor, 1956) or by Fisher's exact method (Fisher, 1958). Comparisons made were orthogonal. Concordance between first and second scores was checked by partitioning the variance as in an analysis of variance and estimating the variance due to discordant scoring as a percentage of between treatment variance.

RESULTS

Summarised numerical results are shown in Table 1 and the statistical analyses are given in Table 2.

The three orthogonal comparisons tested the number of uteri with mean scores of $\ll 2$ according to treatment group. Comparison between the pooled results of progesterone with nonprogesterone treatments (P + POe vs C + Oe) demonstrated that progesterone treatments were associated with tighter luminal closure (P < 0.001) (Pl. 1, Figs. 1 & 2) and sharper corners than nonprogesterone treatments (P < 0.001) (Pl. 1, Figs. 3 & 4). The within progesterone treatments comparison (P vs P + Oe) for both luminal closure and corner sharpness were both not significantly different which meant that the addition of oestradiol did not significantly modify

TABLE 1.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON UTERI MAINTAINED AS ORGAN CULTURES

Treatment	Number of uteri	Luminal closure		Corner sharpness	
		Mean score	No. of uteri with mean score $\leqslant$ 2	Mean score	No. of uteri with mean score $\leqslant$ 2
0.125% ethanol	8	8.875	3	8.250	1
5µg/ml progesterone	8	2.375	8	3.125	6
5µg/ml oestradiol	8	7.875	1	9.000	0
5µg/ml progesterone + 5µg/ml oestradiol	8	3.750	6	4.125	6

TABLE 2.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON UTERI MAINTAINED AS ORGAN CULTURES. CHI-SQUARE TESTS AND THE PARTITIONING OF THE SCORING VARIANCE.

[*]Comparison	χ^2	P
1. [**]P + POe vs C + Oe		
a) Luminal closure	10.286	< 0.001
b) Corner sharpness	12.955	< 0.001
2. P vs POe		
a)	–	< 0.233
b)	–	N.S. (by observation)
3. C vs Oe		
a)	–	< 0.246
b)	–	N.S. (by observation)

Variance partitioning

	Luminal closure		Corner sharpness	
Source	df	MS	df	MS
Between scores	1	2.641	1	0
Between treatments	3	39.557	3	34.375
Interaction	3	0.682	3	1.458
Residual	56	1.257	56	1.290

Variance due to discordant scoring as % of the between treatment variance

$$\frac{2.641 + 0.682}{39.557} = 8.40\% \qquad\qquad 4.24\%$$

[*] Between uteri with a score of $\not< 2$.

[**] Abbreviations; P = progesterone, POe = progesterone plus oestradiol, C = ethanol and Oe = oestradiol.

the action of progesterone (Pl. 1, Fig. 1 cf Fig. 2). Oestradiol
treatment alone had no effect on either of the criteria when com-
pared with the ethanol controls (C vs Oe, NS; Pl. 1, Fig. 3 cf Fig.4).

Two uteri with corrugations were observed, one from the group
that received progesterone alone and the other from the group that
received both progesterone and oestradiol (Pl. 1, Fig. 5).
Persistant fusion between apposing luminal walls was seen in two
uteri. Extensive fusion occurred in the presence of progesterone
and oestradiol (Pl. 2, Fig. 7) and intermitant fusion was observed
in the horn treated with oestradiol alone (Pl. 2, Fig. 8).

The estimate of the variance for discordance between first and
second scorings was calculated as a percentage of the variance due
to treatment differences. There was good agreement between scores.
Discordant variance was 8.4% and 4.2% for luminal closure and corner
sharpness, respectively.

DISCUSSION

Validation of the subjective scoring system chosen depended on
a person other than the experimenter being repeatedly able to divide
uteri into the same classes when given only the criteria of separation.
The five point score system was chosen to allow for variation within
and between uteri. The methods chosen were repeatable as the
variance generated by discordant scoring was small relative to that
arising from treatment differences (less than 10% for both luminal
closure and corner sharpness).

Progesterone treatment caused changes in the morphology of the
luminal epithelium consistent with luminal closure. Although

fixation shrinkage usually caused all lumina to open, the high

degree of opposite luminal wall complementarity and the occurrence

of sharp corners suggest that these lumina were closed during

culture.   Oestradiol alone, usually did not cause luminal closure.

Lumen closure in vivo has been observed following progesterone

treatment of adult ovariectomised mice (Nilsson, 1966; 1967; Finn

and Martin, 1969a; Martin, Finn and Carter, 1970; McLaren, 1971).

The epithelial surface from closed uteri often shows a wave pattern,

or corrugations (Finn and McLaren, 1967; Martin, et al., 1970).

In the present study two progesterone treated uteri (one treated

with both progesterone and oestradiol) had this corrugated pattern.

Luminal closure is probably an important uterine requisite for

implantation and has been described in normal (Nilsson, 1967;

Reinius, 1967; Potts, 1968) and lactationally delayed implantation

(McLaren, 1968).   Lumina remain closed when ovariectomised mice

were given both progesterone and small doses of oestrogen (Martin,

et al., 1970).   It was likewise observed that progesterone and

oestradiol treatment in vitro was associated with luminal closure.

Electron micrographs of uteri from progesterone and oestrogen

treated ovariectomised mice and rats show that although there was

close apposition between apposing luminal walls, after progesterone

treatment apposition was even closer and more regular following

concurrent oestrogen administration (Nilssen, 1966; Ljungkvist, 1971).

High doses of oestrogen were associated with a changed ultrastructure

of the epithelium and an increase in intraluminal space.   Martin

et al. (1970) also observed that progressively higher doses of

oestradiol to progesterone-treated ovariectomised mice progressively

reduced the incidence of progesterone-induced corrugations.

The persistant 'fusion' between the apposing luminal walls that was extensive in the uterine horn treated with both progesterone and oestradiol and intermittant in the horn treated with oestradiol alone may have been a specific response to oestrogen. The failure to observe this 'fusion' regularly could have been due to the large amounts of oestradiol used. Oestradiol was usually not associated with closure of the uterine lumina. These were often open and the intraluminal projections usually had rounded corners. The pleted surface pattern of the epithelium (Pl. 2, Fig. 9) sometimes observed after treatment with oestrogen is similar to that described by Ljungkvist (1971) after treatment of ovariectomised rats with oestrogens. Explanted pieces of uteri from the rabbit (Coujard, 1943) and the rat (Lagova, 1967) showed no characteristic oestrogenic response. In contrast Gaillard. (1942) claimed that oestrogen caused an increase in the amount of myometrium in cultured immature uteri, and Everett (1962) found that oestriol stimulated the gland growth in explants of guinea pig endometrium.

Although attempted, treatment prediction, based on epithelial cell height variation or based on the degree of epithelial cell crowding, was not possible; the height of the epithelium in ovariectomised mice has been increased with oestrogen (Nilsson, 1958; 1959) and progesterone treatment (Martin et al., 1970). These workers also reported that nuclei were larger and more irregularly arrayed after oestrogen treatment than in either ovariectomised or ovariectomised-progesterone treated mice. Epithelium height was reported by Everett (1962) and Lenahan (1968) to be maximal for guinea pig and hamster uterine explants, respectively, when both oestrogen and progesterone were in the culture medium. However,

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as these workers also reported that tissue survival was much improved under these conditions it is not possible to know if an increase in epithelial height was a specific response to the hormones present or whether it reflected general tissue viability.

The luminal closure associated with progesterone treatment was a tight closure. Persistance of complementary epithelial surface patterning between apposing luminal walls despite fixation shrinkage implied that the stromal tissue itself was moulded to conform to the epithelial contours. Obliteration of intraluminal space with the concomitant increase in corner sharpness accompanied by moulding of the stroma tissue could be effected by an increase in stromal volume (cellular or fluid) or an increase in epithelial cell height given a constant myometrial response, or by an increase in centri-petal pressure effected by the myometrium. Neither stromal oedema nor an increase in the number or size of stromal or epithelial cells were observed after progesterone treatment. And as the uterine horns were pinned out taut prior to culture closure of the lumen due to a general shortening of the horns was prevented. Thus the progesterone was interpreted to have acted by allowing the circular uterine muscle to exert centripetal pressure, perhaps by an increase in tonus. Genell (1940) has observed that in the cyclic rat, uterine tonus (as defined by Evans 'a resistance of its substance to extension') was highest at metoestrus and dioestrus and lowest at proestrus and oestrus. Furthermore, the tonus of the uteri of castrate rats was significantly lower after treatment with 'oestrin'. Genell did not test the effect of progesterone, though in the cyclic rat plasma progesterone levels are highest during dioestrus (Telegdy and Endröczi, 1963).

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PLATE 1

Figs. 1-5 are longitudinal sections of uterine horns maintained as organ cultures for two days.   x 253 .

Fig. 1.   Cultured in media containing progesterone.   The complementary patterning of the opposite luminal walls and the sharp pointed intraluminal projections suggest that the lumen was tightly closed during culture and has only openned during subsequent histological procedures.

Fig. 2.   Cultured in media containing progesterone and oestradiol. The luminal morphology is similar to that shown in Fig. 1.

Fig. 3.   Cultured in media containing oestradiol.   The patterning of the epithelium on one side of the lumen bears no consistent relationship to that of the opposite side and the intraluminal projections are low with rounded outlines. This lumen was probably not closed during culture.   The stromal nuclei are larger and more regularly shaped than those in the uteri cultured with progesterone (c.f. Fig. 1).

Fig. 4.   Cultured in media containing ethanol alone.   The luminal morphology is similar to that shown in Fig. 3.

Fig. 5.   Cultured in media containing progesterone and oestradiol. In addition to the complementarity in shape between opposite luminal walls, there are small 'corrugations' present on the epithelial surface.

Fig. 6.   Cross-section of a uterine horn from a mature virgin mouse autopsied during metoestrus-1.   Note the prominent 'corrugations' present on the epithelial surface.   x 506 .

PLATE 2

Figs. 7-10 are longitudinal sections of uterine horns maintained as organ cultures for two days.

Fig. 7. Cultured in media containing progesterone and oestradiol. There was extensive fusion between opposite luminal walls in this horn. The area illustrated shows both persistant fusion and regions where the luminal walls have separated. x342 .

Fig. 8. Cultured in media containing oestradiol. Persistant fusion between opposite luminal walls was intermittant in this horn and one of these areas is illustrated. x342 .

Fig. 9. Cultured in media containing oestradiol. A high-power view of the luminal epithelium and underlying stromal tissue. Note the 'pleted' pattern of the epithelial surface. This pattern was most often seen in areas of varying size in horns cultured in the presence of oestradiol. An epithelial surface pattern that approximated this was sometimes confined to local areas of horns cultured in the presence of ethanol alone (see Fig. 4). x684 .

Fig. 10. Cultured in media containing progesterone and oestradiol. A high power view of the luminal epithelium and underlying stroma. The jagged, complementary epithelial surfaces again resemble the corrugations seen in Fig. 6. This type of epithelial surface was never seen in the absence of progesterone. x684 .

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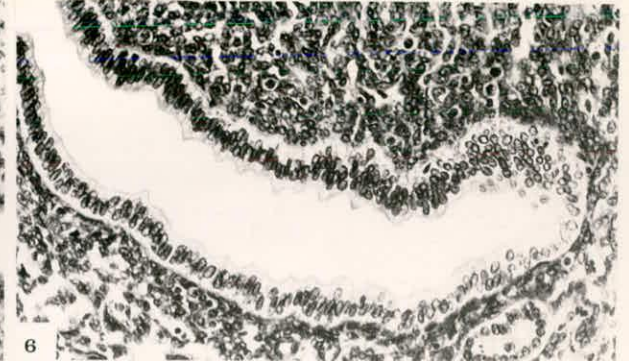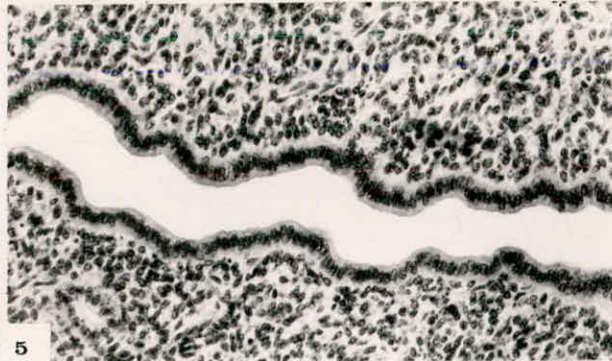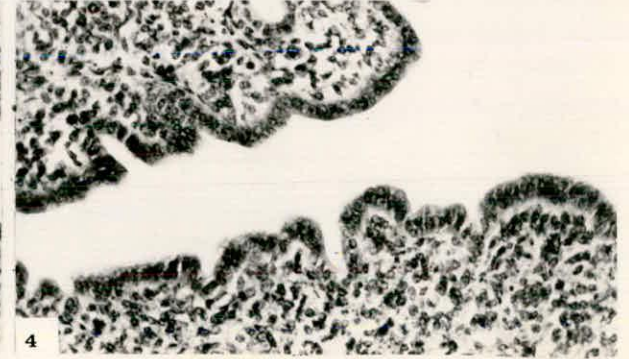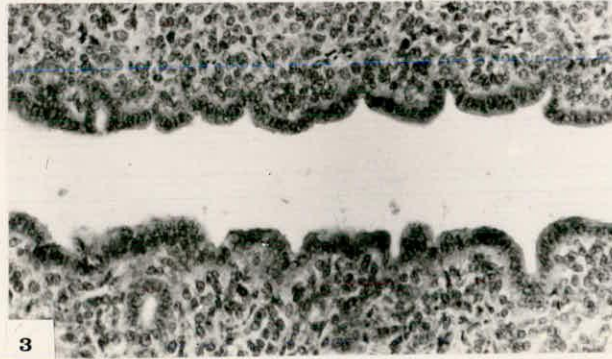
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THE EFFECT OF PROGESTERONE, OESTRADIOL AND THE CONCENTRATION OF

OXYGEN ON THE FATE OF BLASTOCYSTS CULTURED WITHIN THE LUMINA

OF IMMATURE UTERI

ABSTRACT

Ovariectomy-delayed blastocysts were flushed from mice that had been treated with medroxyprogesterone acetate and injected into the lumina of immature uterine horns.   The uterine horns were then maintained as organ cultures for two or three days in media that contained either ethanol or ethanol plus progesterone, oestradiol or progesterone and oestradiol.

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 decidualize.

Embryonic development in eggs became grossly abnormal around the time of the differentiation of the distal endoderm.   Although further development occurred, it was impeded by the progressive collapse of the yolk cavity.   This allowed giant trophoblast cells to intermingle with proximal endodermal cells.   Reichert's membrane did not develop.

The behaviour of eggs cultured in media containing both progesterone and oestradiol was assessed when the supplied oxygen concentration varied from 22 to 95%.   Development was optimal between 26 and 30% oxygen when 36% of injected eggs invaded into the uterine stromal tissue.

These results are compared with the behaviour of eggs undergoing implantation in vivo.

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 (Boyd and Hamilton, 1952). 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 precise maternal conditions prevail.   In order to reduce and control some of the maternal variables involved, an in vitro implantation system has been developed.   This approach provides an alternative basis for analyzing normal implantation and has been used by Glenister (1961) to study implantation in the rabbit.   In this method blastocysts invaded into strips of endometrium and commenced embryonic development in an organ culture system.

In the present study, the mouse has been used as an experimental animal.   Entire uterine horns can be maintained in organ culture (Trowell, 1959) with the attendant advantage that normal tissue relationships are maintained (Lasnitzki, 1965).   Also adequate numbers of blastocysts of varying ages can be obtained by flushing uteri from mice in which implantation has been delayed by ovariectomy (Smithberg and Runner, 1956; Yoshinaga and Adams, 1966).   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 has been studied both with the light (Boyd and Hamilton, 1952; Wilson, 1963; Snell and Stevens,1966; Finn and McLaren, 1967) and the electron microscope (see Potts, 1969;

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Nilsson, 1970) so as to provide adequate basis for comparison. The expected 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 and 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 and Wilson (1970) have more regularly obtained 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 7-10g) were maintained as organ cultures. For details of the method see Grant

(1972a).   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 the 12th day) from mice that had been ovariectomised and given 2.5mg of 6α-methyl-17α-acetoxyprogesterone (as 'Depoprovera'; Upjohn) subcutaneously on the afternoon of the 3rd day of pregnancy. A small number of blastocysts were flushed from both intact and ovariectomised mice on the 4th day of pregnancy.

Trowell's T_8 medium (with 0-20% foetal serum) was used both to flush donor uteri and to inject blastocysts into the horns.   Blasto-cysts were injected from a fine glass pipette controlled by a micromanipulater (Singer) and activated by a micrometer syringe (Alga; Burroughs and 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 6μ.   Sections were stained with haematoxylin (Harris's) and eosin and selected treatments were restained with Mallory's Trichrome (Hughesdon's modification) or with methylgreen and toluidine blue, stains.   Mallory's Trichrome stain was used after

17.

fixation with formol-saline to identify pieces of remaining zona
pellucidae (Finn and McLaren, 1967) and after either fixative to
show the distribution of uterine connective tissue.   The methyl-
green and toluidine blue stain was used to demonstrate W-Bodies
(Finn and McLaren, 1967).   Egg size (in microns) was estimated by
multiplying the number of serial sections on which any part of an
egg (invading trophoblast included) appeared, by the thickness of
the sections (approx. 6μ).   Egg and uterine survival were subjec-
tively estimated from the percentage of cells alive after culture.
Uterine survival is given as a percentage and eggs are designated
as 'alive' (greater than 50% of their cells alive) and 'dead' (less
than 50% of their cells alive), and 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 T_8 culture
medium (Flow Laboratories) that contained 20% foetal calf serum (Flow
Laboratories), 100 I.U. of penicillin and $2\mu g$ of 'Fungizone' (Ampho-
tericin B, Squibb) per ml.   There were four treatment groups; viz.
progesterone ($5\mu g/ml$ of medium), oestradiol ($5\mu g/ml$), progesterone
plus oestradiol ($5\mu g/ + 5\mu g/ml$) 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 experiments, for each of which each treatment was
represented once.   For each experiment the donor uteri, usually from
sibs (or from mice with similar bodyweights) and the number and type
of eggs injected in each treatment were usually identical.   The

18.

culture times were 1.3 days (one experiment), two days (11 experiments) and three days (three 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.) 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

Fifty six experiments were performed during which a total of 534 blastocysts were injected into 59 immature uterine horns (one experiment utilized three horns). The culture medium contained 5µg/ml of both progesterone and oestradiol and was the same as that used in the equivalent hormonal treatment group. Fifteen of the 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) two days (49 experiments) and three days (six experiments). The oxygen concentration varied from 19-95% and the carbon dioxide concentration varied from around 3-5% with the remainder nitrogen. The gas pressure within the culture jars varied from 0.0 - 5.0 lb/sq.in. (usually 0.5 - 2.0). The oxygen concentration was measured by the pyrogallol absorption technique with the method of Fainstat (1968). At the conclusion of most experiments three separate samples of gas from the culture jars had their oxygen concentration estimated. The concentration was taken as that of the sample with the highest consistent reading.

Statistical methods

Discrete data were anlyzed after their arrangement into contingency tables. When there were observed values of 5 or less in 2 x 2 tables, Yate'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 analyzed by 2 x 2 tables using three orthogonal comparisons. These were 1) progesterone treated groups (i.e. progesterone and progesterone plus oestradiol groups) versus nonprogesterone 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 results from 'The effect of the oxygen concentration on eggs cultured within the lumina of uteri' section, 2 x 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 x 2 contingency 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 (see McLaren, 1952) and comparisons were made between measures of intensity.

Data that showed continuous variation were analyzed by analyses of variance, correlation and regression techniques. Results as percentages were transformed by the arc sin $\sqrt{\text{%age}}$ method (Snedecor, 1956) before analysis. Results were considered to be significant

if the probability of their occurrence due to chance, was equal to or less than 5%.

<div align="center">RESULTS</div>

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

1. **The number and viability of eggs after culture (Tables 1 & 2)**

Forty three percent of the eggs injected at the beginning of culture were found on histological sections. A smaller proportion of the injected eggs were found in the progesterone and in the pro-gesterone plus oestradiol treated groups (i.e. progesterone-treated groups) than in the ethanol and oestradiol treated groups (i.e. non-progesterone-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 ethanol and oestradiol than from the progesterone-treated groups ($P < 0.01$ and < 0.001, respectively).

2. **Trophoblast activity**

a). Giant cells.

Trophoblast giant cell transformation occurred during culture in all treatment groups (Tables 1 & 2). Transformation occurred first near the abembryonic pole or in the equatorial regions of the eggs. Giant cells were arbitrarily divided into small and large types according to the type and size of their nuclei. Small giant cells had nuclei about $1\frac{1}{2} - 2$ times the size of the nuclei of uterine epithelial cells (Pls. 1 & 6, Figs. 2 & 39, respectively) and large giant cells usually had larger nuclei with a different

TABLE 1.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON EGGS CULTURED IN THE LUMINA OF UTERI.

THE NUMBERS AND VIABILITY OF EGGS, AND THEIR STATE OF DEVELOPMENT AFTER CULTURE.

Eggs Found	Ethanol	Progesterone	Oestradiol	Progesterone + Oestradiol	Totals
No. of eggs injected	112	113	113	114	452
No. of eggs found	57	35	63	40	195
Embryonic Viability					
No. of 'dead' eggs	7	0	10	1	18
No. of eggs with 'dead' trophoblast tissue	7	2	15	2	26
No. of eggs with 'dead' embryonic tissue	9	0	17	2	28
Trophoblast Activity					
No. of eggs without giant cells	28	5	20	4	60
No. of eggs with giant cells	29	30	43	36	85
No. of eggs free	42	5	34	10	91
No. of eggs attached	15	6	18	15	54
No. of eggs invading the epithelium	0	9	4*	4	17
No. of eggs invading the stroma	0	15	7	11	33
Embryonic Development					
No. of eggs in delay	46	6	40	13	105
No. of eggs with proximal endoderm	7	5	5	15	32
No. of eggs with distal endoderm	4	6	9	1	20
No. of eggs beyond distal endoderm	0	18	8	11	37

* Includes 2 eggs invading on the serosal (external) surface of a uterine horn.

TABLE 2.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON EGGS CULTURED IN THE LUMINA OF UTERI.

CHI SQUARE VALUES FOR 2 X 2 CONTINGENCY TABLES

Comparisons	Progesterone + progesterone & oestradiol (a) vs oestradiol & ethanol (b)	Progesterone (a) vs progesterone & oestradiol (b)	Oestradiol (a) vs ethanol (b)
Proportion of injected eggs found	18.971, P < 0.001, b > a	0.434, NS	0.534, NS
Viability			
Proportion of eggs that were 'alive'	8.924, P < 0.01 , a > b	By inspection, NS	0.032, NS
Proportion of eggs with 'live' trophoblast tissue	0.094, NS	By inspection, NS	2.657, NS
Proportion of eggs with 'live' embryonic tissue	12.056, P < 0.001, a > b	0.534, NS	2.210, NS
Trophoblastic Activity			
Proportion of eggs with giant cells	17.492, P < 0.001, a > b	By inspection, NS	3.766, NS
Proportion of eggs both free and attached	44.412, P < 0.001, b > a	7.220, P<0.01, b>a	8.960, P<0.01, b>a
Proportion of eggs invading the epithelium	By inspection significantly different a > b	2.214, NS	2.057, NS
Proportion of eggs invading the stroma	27.292, P < 0.001, a > b	1.944, NS	4.855, P<0.05, a>b
Embryonic Development			
Proportion of eggs in delay	39.869, P < 0.001, b > a	2.327, NS	4.365, P<0.05, b>a
Proportion of eggs with proximal endoderm	9.346, P < 0.01 , a > b	5.144, P<0.05, b>a	0.627, NS
Proportion of eggs with distal endoderm	By inspection NS	3.158, NS	0.971, NS
Proportion of eggs beyond distal endoderm	30.741, P < 0.001, a > b	4.507, P<0.05, a>b	5.849, P<0.02, a>b

chromatin pattern (Pl. 3, Fig. 16).   When stained with haematoxylin

and eosin nontransformed trophoblast cells had dense black coloured

nuclei, little cytoplasm and darkly staining cell walls (Pl. 1,

Fig. 1 & 2).   While 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 (Pl. 1, Fig. 4).   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.

The proportions of eggs with giant cells was higher for the

progesterone than for the ethanol and oestradiol 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). Free and attached eggs.

Eggs that were not attached and did not look as if they had

been attached to the uterine epithelium were called free eggs

(Pl. 1, Fig. 1).   Eggs that attached during culture sometimes

remained so after histological procedures and the persistance of

connecting strands between the egg and the epithelium (Pl. 1, Fig. 3)

or the slightly frayed appearance of the epithelium or trophoblast

suggested prior attachment.   However it is probable that some eggs

that were attached during culture showed no signs of this after

histological procedures as 40% of all free eggs had small giant

cells and only 4% of all attached eggs had no small giant cells.

Therefore both free and attached eggs are considered together as an

early stage of trophoblast activity for comparisons between treatment

groups.

The proportion of eggs that were both free and attached was lower for the progesterone than for the ethanol and oestradiol treated groups (P < 0.01) (Tables 1 & 2).   Also this proportion was lower for the progesterone than for the progesterone and oestradiol treated group (P < 0.01).   Again this proportion was lower for the oestradiol than in 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 (Pl. 1, Fig. 4).   Evidence of the rupture of the epithelial basement membrane by the trophoblast was interpreted as invasion of the uterine stroma (Pl. 3, Fig. 14).   It was usual however for the trophoblast tissue of eggs invading the stroma to have penetrated well beyond the epithelial basement membrane (Pls. 3 & 7, Figs. 16 & 49, respectively).   The trophoblast of eggs that were invading the epithelium was often phagocytosing epithelial and stromal cells (Pls. 1 & 2, Figs. 5 & 11, respectively). Occasionally sections were found on which the trophoblast appeared to have encircled healthy epithelial cells (Pls. 2 & 6, Figs. 8, 9, 10 & 40, respectively).   Two eggs that escaped from the uterine lumen were invading the serosal surface of a uterine horn (Pl. 3, Figs. 17 & 18).

Although some giant cells were seen in the trophoblast tissue overlaying the inner cell mass in a few eggs (Pl. 5, Fig. 30), this embryonic pole trophoblast was never seen to be actively invasive. The most advanced embryos showed no evidence of ectoplacental cone development (Pl. 7, Fig. 51 & 52).

The proportions of eggs invading the epithelium and the stroma

were higher (Table 1) for progesterone than for ethanol and oestradiol treated groups (P, significant by inspection and <0.001, respectively) (Table 2). No eggs invaded either the epithelium or the stroma in the ethanol treated group and treatment with oestradiol allowed four and seven eggs to invade the epithelial and stromal tissues, respectively.

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 (Pl. 1, 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 and Stevens, 1966) (Pl. 1, Fig. 4). 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 and Stevens,1966) (Pl. 3, Fig.13). When the mass of embryonic tissue had increased beyond that associated with the appearance of the distal endoderm eggs were termed beyond the distal endoderm stage eggs (Pl. 3, Fig. 15). In the eggs with more advanced embryonic development it was usually not possible to identify distal endoderm cells. The yolk cavity had usually collapsed (Pls. 3 & 6, Figs. 14 & 42, respectively) which allowed trophoblast giant cells to intermingle with cells in the region of the proximal endoderm (Pl. 7, Fig. 48).

In some aspects embryonic development was equivalent to _in vivo_ 5-day old egg cylinders (Pl. 4, Figs. 19 & 20). The embryo shown in Pl. 7, Fig. 49 has a small proamniotic cavity bordered by almost normal shaped embryonic ectoderm although proximal endoderm cells

can not be recognized.   Embryonic development was usually disorgan-
ized and it was not possible to distinguish extra- from embryonic
ectoderm and usually difficult to identify endoderm cells (see Pl. 7).

Stages of embryonic development after culture

The proportion of eggs that were in delay was lower (Table 1)
in the progesterone than in the ethanol and oestradiol treated
groups (P < 0.001) (Table 2), and higher in the ethanol than in the
oestradiol treated group (P < 0.05).

The proportion of eggs whose embryonic development had progres-
sed beyond the distal endoderm stage was higher in the progesterone
than in the ethanol and oestradiol treated groups (P < 0.001).   This
proportion was also higher in the progesterone than the progesterone
and oestradiol treated group (P < 0.05) and higher in the oestradiol
than in the ethanol treated group (P < 0.02).   There were no eggs
in the ethanol treated group that had developed beyond the distal
endoderm stage and only four eggs that had distal endoderm.

The majority of dead eggs were free and had not developed
beyond the delay stage.   There were however exceptions in the
oestradiol treated group, where three dead eggs were attached, one
of which had differentiated distal endoderm and another had
embryonic development beyond the distal endoderm stage.

4. The correlation between trophoblast activity and embryonic
   development

It was of interest to know if trophoblast activity progressed
in phase with embryonic development.   The null hypothesis holds that
any stage of trophoblast activity would be equally likely to occur
with any one stage of embryonic development.

Eggs were both free (in the lumina of uteri) 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) and nonsignificant for the ethanol treated group (Table 3). The intensity of this association was stronger for the progesterone and progesterone and oestradiol 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 nonsignificant for the ethanol treated group. When pooled the association was significant for the progesterone treated groups (P < 0.001) and nonsignificant for the nonprogesterone treated groups.

Epithelial invasion was protracted in the oestradiol treated group (Pl. 4, Figs. 31 & 36) and only in this group was the association between the invasion of the epithelium and possession of distal endoderm significant (P < 0.02). When the results were pooled this association was significant for the two nonprogesterone treated groups (P < 0.01) and nonsignificant for the progesterone treated groups.

The association between invasion of the stroma and possession of embryonic tissue beyond the distal endoderm stage was significant for all groups treated with hormones (P, < 0.01 - < 0.00). The association was complete for the group given progesterone and oestradiol.

5. The form and size of 'live' eggs according to their trophoblast activity after culture (Table 4)

Expanded eggs are those with an ovoid shape and a relatively

26.

TABLE 3.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON EGGS CULTURED WITHIN THE LUMINA OF UTERI.

THE ASSOCIATION BETWEEN TROPHOBLAST ACTIVITY AND EMBRYONIC DEVELOPMENT.

Comparisons	Progesterone + progesterone & oestradiol vs oestradiol & ethanol		Progesterone vs progesterone & oestradiol		Oestradiol vs ethanol	
Eggs that were both free and in delay						
χ^2 values	31.358,P<0.001	10.215,P<0.01	11.474,P<0.001	15.176,P<0.001	14.964,P<0.001	0.102,NS
Log Odds values	2.435,P<0.02		0.041,NS		-	
Eggs that were both attached and with proximal endoderm						
χ^2 values	20.638,P<0.001	1.438,NS	11.474,P<0.001	6.371,P<0.02	4.464,P<0.05	By in-spection NS
Log Odds values	-		1.306,NS		-	
Eggs that were both invading the epithelium and with distal endoderm						
χ^2 values	1.758,NS	8.641,P<0.01	0.965,NS	By inspection NS	6.600,P<0.02	Not tested*
Log Odds values	-		-		-	
Eggs that were both invading the stroma and beyond distal endoderm						
χ^2 values	44.084,P<0.001	45.821,P<0.001	10.697,P<0.01	Complete association	21.560,P<0.001	Not tested*
Log Odds values	0.521,NS		-		-	

* In the ethanol treated group no eggs invaded the epithelium or the stroma.

TABLE 4.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON EGGS CULTURED IN
THE LUMINA OF UTERI.   THE NUMBER, FORM AND SIZE (MICRONS) OF
'LIVE' EGGS ACCORDING TO TROPHOBLAST ACTIVITY.

Treatment and form	Trophoblast activity			
Progesterone	Free	Attached	Invading epithelium	Invading stroma
Expanded	3	5	5	2
Collapsed (or contracted)	2	0	0	0
Solid	0	1	4	11
Mean size ± S.E. (n)*	64.29±5.81(7)		75.00±12.98(8)	75.43±10.10(14)
Progesterone + progesterone and Oestradiol				
Expanded	6	11	4	0
Collapsed (or contracted)	4	3	0	3
Solid	0	0	0	8
Mean size ± S.E. (n)	83.20±10.67(15)		96.00±15.87(3)	99.00±7.71(6)
Oestradiol				
Expanded	23	14	4	2
Collapsed (or contracted)	2	0	0	0
Solid	2	1	0	5
Mean size ± S.E. (n)	85.74±4.26(38)		96.00±9.49(4)	75.00±9.26(6)
Ethanol				
Expanded	25	11	0	0
Collapsed (or contracted)	8	3	0	0
Solid	3	0	0	0
Mean size ± S.E. (n)	82.04±4.51(44)			

*   The number of eggs from which the mean was calculated.

large blastocoel or yolk cavity (Pl. 1, Figs. 1-3).   Most free and
attached eggs were of this form.   Eggs in which the size of the
blastocoel 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) (Pl. 4, Fig. 23) or con-
tracted (i.e. although the eggs were ovoid in shape the circum-
ferential distance was shortened (Pl. 4, Fig. 24).   The trophoblast
in the contracted eggs sometimes showed degenerative changes.   The
nuclear membranes were then crinkled in outline and the internuclear
distance was shortened while in the cytoplasm vacuoles were apparent
(Pl. 7, Fig. 47).   Occasionally local proliferation of the tropho-
blast filled in part of the blastocoel (or yolk) cavity (Pl. 2, Fig.
12).   Collapsed eggs were more common among early stages and con-
tracted 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 were
of this type (see Pl. 7).   Also many dead eggs were solid in form
(Pl. 4, Fig. 26).

The mean size of the eggs according to their trophoblast
activity for each of the treatment groups is given in Table 4.
There were no significant differences between treatment groups
indicated by analyses of variance.

6. Trophoblast activity according to the age of the eggs at the
beginning of culture

The ages and the numbers of eggs injected and found are shown
in Table 5 for each age, together with their trophoblast activity.

Comparisons (with 2 x 2 contingency tables) between 4th and
5th plus 6th day eggs, and between 5th and 6th day eggs for the

TABLE 5.

THE EFFECT OF PROGESTERONE AND OESTRADIOL ON EGGS CULTURED IN THE LUMINA OF UTERI.

TROPHOBLAST ACTIVITY ACCORDING TO THE AGE OF THE EGGS AT THE BEGINNING OF CULTURE.

Treatment and egg age[+]	No. of eggs injected	No. found	No. mostly dead	No. free	No. attached	No. invading the epithelium	No. invading the stroma
Progesterone							
* 4th day	17	4	0	2	0	2	0
5th day	64	24	0	2	5	7	10
6th day	29	7	0	1	1	0	5
7th day	3	0					
Progesterone + progesterone and Oestradiol							
* 4th day	5	0					
5th day	62	20	1	7	1	2	10
6th day	24	14	0	3	10	0	1
7th day	3	0					
12th day	20	6	0	0	4	2	0
Oestradiol							
* 4th day	17	9	0	7	2	0	0
5th day	72	33	9	18	10	1	4
6th day	21	19	1	9	6	1	3
7th day	3	2	0	0	0	2[++]	0
Ethanol							
* 4th day	12	7	1	5	2	0	0
‡ 4th day	5	1	1	1	0	0	0
5th day	68	38	2	24	12	0	0
6th day	24	10	2	8	2	0	0
7th day	3	1	1	1	0	0	0

+ Eggs flushed on the 5th, 6th, 7th and 12th day of pregnancy were from donor mice that were ovariectomised and given 2.5mg of MAP on the 3rd day of pregnancy.   The vaginal plug was seen on the 1st day.

* Eggs flushed from intact donor mice.

‡ Eggs flushed from donor mice that were ovariectomised on the 3rd day of pregnancy and not given MAP.

++ These two eggs were invading the serosa on the outside of the uterine horn.

proportions found after culture were nonsignificant ($\chi^2 = 0.668$ and 1.752, respectively). The same comparisons were nonsignificant for the proportions of eggs that invaded both the epithelium and the stroma ($\chi^2 = 1.835$ and 1.630, respectively). However the only eggs that did invade the stroma or have embryonic development beyond that of the distal endoderm stage were flushed on the 5th or 6th day of pregnancy from ovariectomised progestin-treated mice.

7. <u>Loss of zona pellucidae in culture</u>

All eggs flushed on the 4th day of pregnancy and about half of those flushed on the 5th day of pregnancy from ovariectomised-progestin treated mice were in their zona pellucidae. Eggs flushed at later stages of pregnancy had usually lost their zonae. Fixation with formolsaline was unsatisfactory but was used occasionally because it does not dissolve the zona, whereas Bouins, a much better fixative, does dissolve zonae.

In the ethanol treated group one of eleven 5th day eggs found had retained pieces of zona after 1.3 days in culture. Three of thirty two 5th day eggs retained pieces of zona after culture for two days.

In the progesterone treated group pieces of zona were seen near four eggs after two days in culture (of seven 5th day eggs found) (Pl. 6, Fig. 46). No pieces of zona were seen in uteri in which one 4th day egg and three 5th day eggs were cultured for two and 1.3 days respectively.

In the oestradiol treated group traces of zona were seen around one egg (of eight 5th day eggs) after 1.3 days culture and in two of seven 5th day eggs after two days culture.

In the progesterone and oestradiol treated group traces of zona were seen around two eggs (of five 5th day eggs found) cultured for 1.3 days and were not seen around any of four 5th day eggs cultured for two days. Remnants of zona were clearly visible near six (of 15 4th day eggs found) after culture for two days (Pl. 6, Figs. 44 & 45). In all eggs around which pieces of zonae were seen it appeared to have been partly dissolved in situ without prior hatching.

The eggs around which pieces of zona were identified were all free in the uterine lumen with the exception of the eggs in the progesterone treated group which were invading the stroma.

8. The survival of the uteri and their reaction to invading eggs

The mean percentages of uterine cells viable after culture were 62, 68, 70 and 69 for the ethanol, oestradiol, progesterone and the progesterone and oestradiol treated groups, respectively.

When stained with Mallory's Trichrome an increased concentration of uterine stromal collagen could be seen in front of the invading trophoblast tissue (Pl. 7, Figs. 48, 49 & 50). The epithelial basement membrane sometimes appeared as continuous with the concentrated collagen (Pl. 7, Fig. 50).

Uterine stromal decidual cells were not seen in any experiment.

Fusion between the apposing luminal walls was occasionally observed in uteri treated with oestradiol and progesterone plus oestradiol.

The effect of the oxygen concentration on eggs cultured within the lumina of uteri

1. The number of eggs after culture (see Table 6 and Text-fig.1).

Forty-eight percent of all eggs injected prior to culture were

TABLE 6.

THE EFFECT OF THE OXYGEN CONCENTRATION ON EGGS CULTURED IN THE LUMINA OF UTERI.

NUMBERS OF EGGS CLASSED ACCORDING TO THEIR TROPHOBLAST ACTIVITY AND EMBRYONIC DEVELOPMENT

AND CHI-SQUARE VALUES FOR THE PROPORTIONS OF EGGS FOUND ACCORDING TO THEIR CLASSIFICATION.

	22-25	26-30	31-40	41-50	51-70	71-95	χ^{2*}	P
Oxygen concentration (%)	22-25	26-30	31-40	41-50	51-70	71-95		
Uterine viability after culture (%)	72	74	80	79	84	80		
No. of eggs injected	123	139	46	58	41	55		
No. of eggs found	41	89	31	9	23	30		
Trophoblast activity								
No. of eggs with giant cells	41	70	26	4	15	19	21.329	< 0.001
No. of eggs without giant cells	0	8	2	1	5	0		
No. of eggs of unknown status	0	11	3	4	3	11	26.507	"
No. of eggs that were free	1	12	6	5	9	20	54.290	"
No. of eggs that were attached	28	16	8	4**	6	6	37.120	"
No. of eggs that were invading the epithelium	8	7	2	0	4	3	6.778	NS
No. of eggs that were invading the stroma	4	54	15	0	4	1	60.929	< 0.001
Embryonic development ***								
No. of eggs in delay	16(5)	19(5)	5(3)	3(1)	10(3)	9(5)	52.773	"
No. of eggs with proximal endoderm	21(2)	18(7)	9(6)	3(1)	6(3)	21(21)		
No. of eggs with distal endoderm	3(2)	14(9)	2(1)	3(2)	3	0	52.773	"
No. of eggs beyond distal endoderm	1	38	15(2)	0	4	0		

*   Chi-square values from 2 x 6 contingency tables.

**   Two of these four eggs were attached to the external surface of a uterine horn.

*** The numbers in bracketts are the numbers of eggs about whom classification was uncertain.
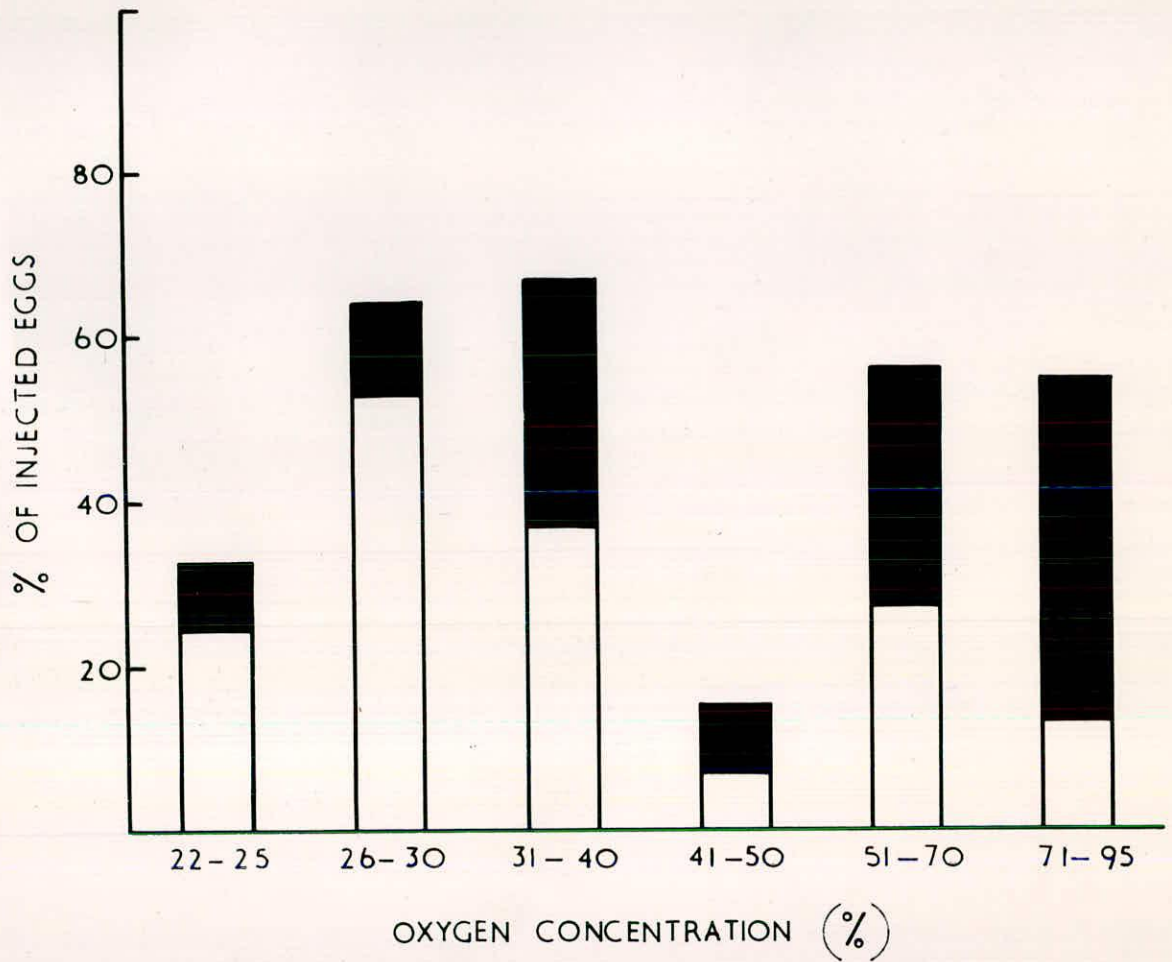
The percent of eggs that were dead

The total height of the column represents the percent of eggs found

Text-figure 1. The effect of the oxygen concentration on the percent of injected eggs recovered and their viability.

found on histological sections.   No consistent effect of oxygen concentration was observed.

2. Egg viability after culture

The proportions of the eggs found that were 'alive' varied significantly over the 22-95% O_2 concentration range (P < 0.001) (Table 7).   This was mainly due to disproportionately large numbers of 'dead' eggs in the 22-25% and 71-95% O_2 groups (Text-fig. 1).

There were more eggs with their trophoblast 'dead' than there were 'dead' eggs (χ^2 = 4.769, 1df, P < 0.05).

In the 71-95% O_2 group some eggs had their embryonic tissue selectively killed (Pls. 5 & 6, Figs. 37 & 41, respectively).

Most of the 'dead' eggs were free in the uterine lumen (P < 0.001) and the stromal invasion stage of trophoblast activity had the lowest number of 'dead' eggs (P < 0.001).

3. Egg orientation after culture

Data available for the positioning of 64 eggs relative to the position of the mesometrium in 17 cultured uteri suggested that orientation was random.   There was no significant tendency (P < 0.2) for eggs in the same uteri to be orientated in the same way.   The embryonic tissue was however positioned at the opposite pole to that at which invasion began.

4. Trophoblast activity

    a). Giant cells.

It was not possible to determine whether or not some eggs had giant cells.   The proportions of eggs not known to have giant cells were heterogeneous over the 22-95% O_2 concentration range (P < 0.001) and highest in the 71-95% O_2 group in which there were many dead eggs.

TABLE 7.

THE EFFECT OF OXYGEN CONCENTRATION ON EGGS CULTURED IN THE LUMINA OF UTERI.

VIABILITY AFTER CULTURE, STAGE OF TROPHOBLAST ACTIVITY REACHED BY EGGS CLASSIFIED

AS 'DEAD' AND CHI-SQUARE VALUES FOR THESE PARAMETERS.

A. Viability

Oxygen concentration (%)	22-25	26-30	31-40	41-50	51-70	71-95	χ^{2*}	P
The % of eggs that were 'alive'	73	83	55	45	48	23	42.474	< 0.001
The % of eggs that had live trophoblast tissue	68	72	55	33	48	37	17.670	< 0.01
The % of eggs that had live embryonic tissue	71	81	48	55	56	13	47.794	< 0.001

B. Stage of trophoblast activity reached by eggs that were dead after culture

Trophoblast activity	Free	Other stages	Free & attached	Invading epithelium & stroma	Invading stroma	Other stages
No. of 'dead' eggs	12	129	53	88	75	66
No. of live eggs	41	39	68	12	4	76
χ^2 for 2 x 2 contingency tables	51.141		46.311		49.532	
P	< 0.001		< 0.001		< 0.001	

* From 2 x 6 contingency tables.

There were significant differences in the proportion of eggs found that had giant cells for the groups over the 22-95% O_2 range (P < 0.001). All the eggs found in the 22-25% O_2 group had giant cells whereas the lowest proportion with giant cells was in the 71-95% O_2 group (viz. 75%).
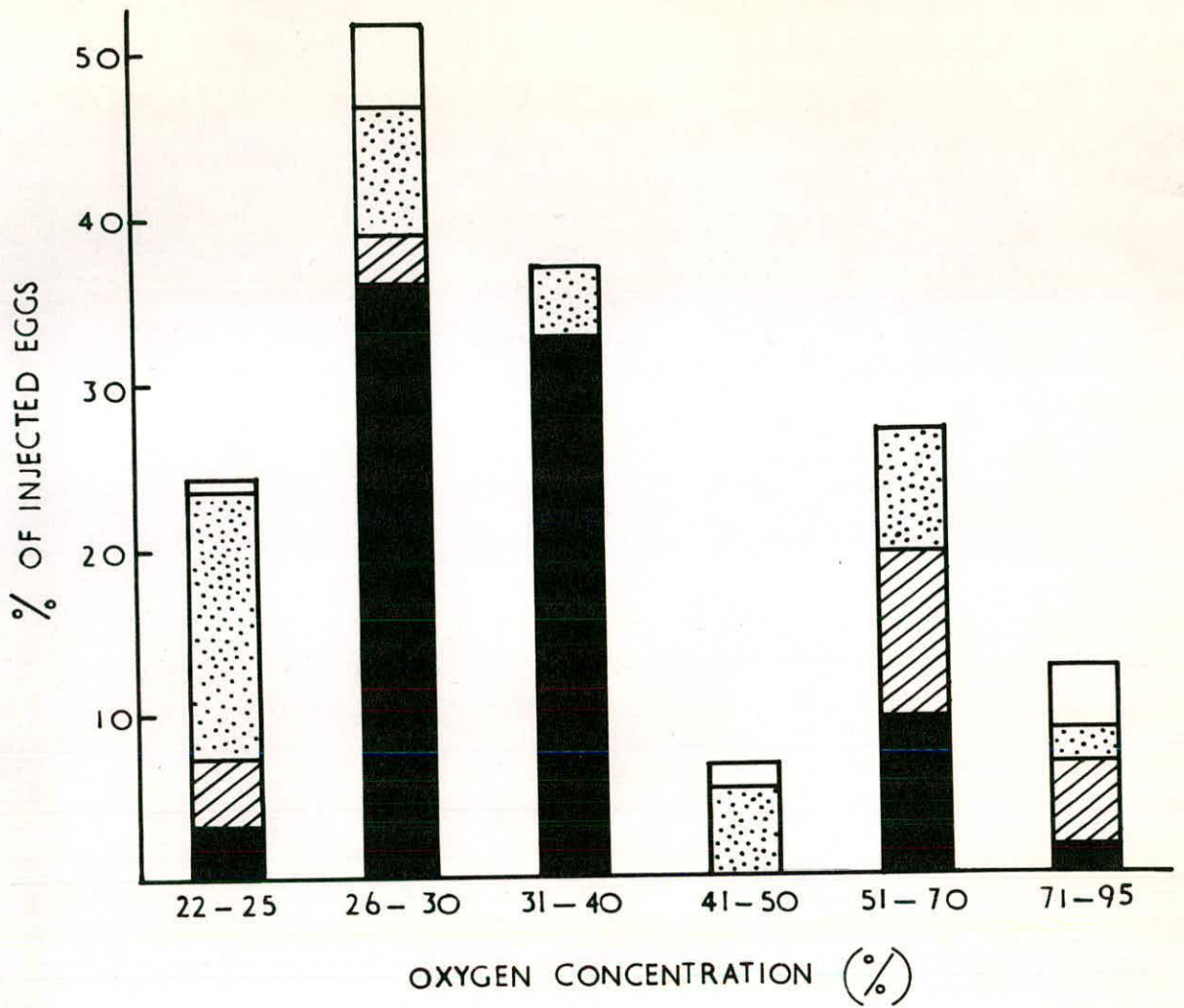
Invasive trophoblast usually contained large giant cells.

b). The stage of trophoblast activity after culture (see

Table 6 and Text-fig. 2).

The proportions of eggs found that were either free in the lumina or attached to the epithelium varied significantly over the 22-95% O_2 range (P, < 0.001 in each case).

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 and McLaren, 1967) (Pls. 1, 2 & 6, Figs. 4, 8 & 39, respectively). At O_2 concentrations of 38% or higher, damaged epithelium was found next to some eggs that contained dead or dying trophoblast cells (Pl. 7, Fig. 47). These eggs were sometimes classified as attached although the trophoblast may earlier have been attempting to invade the epithelium. Further, the area of stromal invasion in the presence of high oxygen concentrations was usually small (Pls. 3 & 6, Figs. 15 & 43, respectively) and occasionally the trophoblast died after invasion (Pl. 5, Fig. 35).

The proportions of eggs found that were invading the stroma was heterogeneous (P < 0.001). Sixty percent of the eggs in the 26-30% O_2 group invaded the stroma while none did in the 41-50% O_2 group.

Text-figure 2. The effect of the oxygen concentration on trophoblast activity.

5. Embryonic development (Table 6 and Text-fig. 3)

Because of the doubtful status of many eggs, eggs 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 found that were in the early and in the late stages of development were significant ($P < 0.001$). Fifty-eight percent 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.

6. The correlation between trophoblast activity and embryonic development

In the 22-25% O_2 concentration group there was no significant association between equivalent successive stages of trophoblast activity and embryonic development. For the 26-30% O_2 range there were significant associations for 'live' eggs that were both free and in delay, both attached and with proximal endoderm and both invading the stroma and with embryonic development beyond the distal endoderm stage ($P < 0.001$). None of the four live eggs that were invading the epithelium had distal endoderm.

The number of live eggs in the 31-40% O_2 range was too small for the estimation of these associations. The numbers of eggs in the high O_2 groups (i.e. 41-50%, 51-70%, 71-95%) were pooled for analysis. There were significant associations for live eggs that were both free and in delay and those that were both invading the stroma and with embryonic development beyond the distal endoderm stage ($P < 0.05$ and < 0.001, respectively).

32.
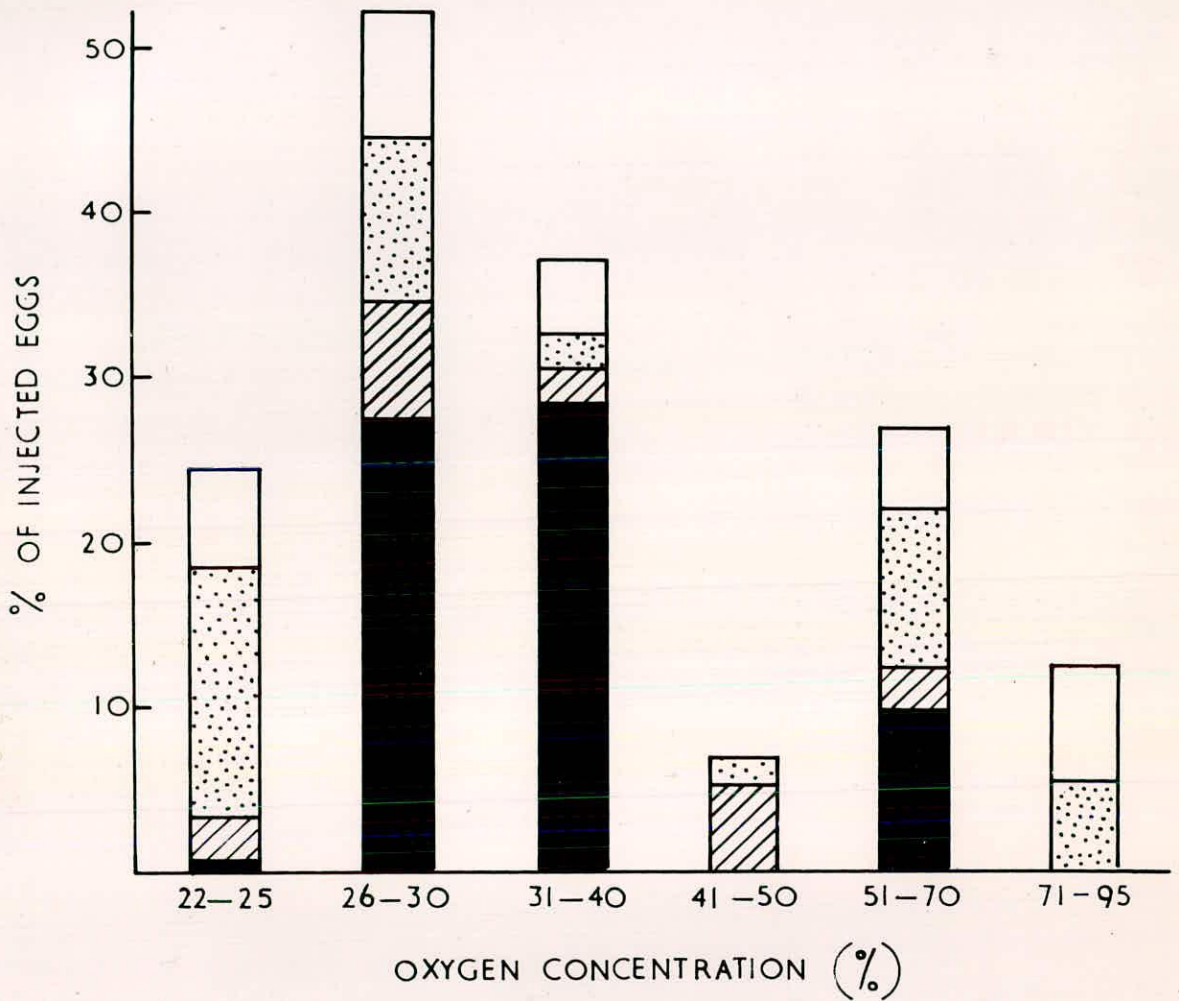
The percent of injected eggs that were alive and in delay

"   "   "   "   "   "   "   "   with proximal endoderm

"   "   "   "   "   "   "   "   "   distal   "

"   "   "   "   "   "   "   "   and beyond the distal endoderm stage

Text-figure 3. The effect of the oxygen concentration on embryonic development.

7. The form and size of eggs after culture

Eggs were grouped into three oxygen concentration ranges as shown in Table 8. Free eggs in the two low oxygen ranges (i.e. 19-25% and 26-30%) were either expanded or collapsed in form. While eggs free in the highest O_2 range (viz. 31-95%) were usually solid in form.

Attached eggs and eggs invading the epithelium were usually expanded in form at all O_2 levels. While eggs that had invaded the stroma were usually solid iñ form at all O_2 levels.

Eggs found in contact with one another in the lumina were often fused (this was also observed in the experiment to test the effect of hormones) (Pls. 4 & 5, Figs. 27 & 28, respectively). Occasionally eggs were seen with what appeared to be two masses of embryonic tissue (Pl. 4, Fig. 21).

The pooled size estimations for eggs that were both free and attached and for eggs that were both invading the epithelium and the stroma were significantly larger for the 19-25% O_2 group than for other O_2 groups (P,< 0.01 and < 0.05, respectively).

8. Uterine survival after culture (Table 6)

The mean uterine survival estimate over the 22-95% O_2 range was 76%. The regression of uterine survival on the O_2 concentration was significant (r = +0.29, 51df, P < 0.05).

The nuclei of epithelial cells from uteri cultured at O_2 concentrations of around 56% and higher were positioned apically and there was a prominent subnuclear epithelial band (Pl. 5, Fig. 32). Whereas for lower O_2 levels nuclei were positioned centrally (Pl. 5, Fig. 33) or in the basal region of the epithelial cells (Pl. 5, Fig. 34).

TABLE 8.

THE EFFECT OF THE OXYGEN CONCENTRATION ON EGGS CULTURED IN THE LUMINA

OF UTERI.   (A) THE NUMBERS, FORM AND SIZE (MICRONS) OF LIVE EGGS[*]

ACCORDING TO THEIR TROPHOBLAST ACTIVITY.

(B) SUMMARY OF STATISTICAL TESTS.

O_2 concentration and form	Trophoblast activity			
19-25%[**]	Free	Attached	Invaded the epithelium	Invaded the stroma
Expanded	2	22	11	2
Collapsed (or contracted)	0	3	1	2
Solid	0	3	0	8
Mean size ± S.E. (n)	105.56±5.86(27)		95.06±6.44(16)	
26-30%				
Expanded	3	6	2	3
Collapsed (or contracted)	4	2	0	4
Solid	0	2	2	42
Mean size ± S.E. (n)	80.31±7.73(13)		79.32±4.35(22)	
31-95%				
Expanded	0	5	5	0
Collapsed (or contracted)	1	2	0	0
Solid	4	1	0	20
Mean size ± S.E. (n)	77.40±6.29(10)		79.71±2.72(21)	

(B)   SUMMARY OF ANALYSES OF VARIANCE FOR SIZE DIFFERENCES

Eggs that were both free and attached	Source	d.f.	MS	F	P
	Between O_2 conc. ranges.	2	4388.84	5.571	<0.01
	Within O_2 conc. ranges	47	787.78		
Eggs that were invading both the epithelium and the stroma					
	Between O_2 conc. ranges	2	1410.83	3.624	<0.05
	Within O_2 conc. ranges	56	389.21		

*   Size estimates were not made for all the eggs included here.

**   The initial O_2 concentration for some eggs in this group ranged from
     31-36% but decreased during culture due to the leakage of the culture
     jars.

DISCUSSION

In the presence of hormones some blastocysts cultured for 2-3 days within the lumina of immature uteri invaded through the epithelium and into the stroma. Embryonic development tended to proceed in parallel with the degree of trophoblast activity and reached the 'egg cylinder' stage in uteri treated with progesterone (with or without oestradiol) when the oxygen concentration was around 30%.

The proportion of eggs injected into the lumina of uteri that were found on serial sections after culture depended both on the hormonal treatment and on the oxygen concentration. It is 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 nonprogesterone (ethanol and oestradiol) treated groups. Most dead eggs appeared free in the lumen and had undergone little or no embryonic development beyond the delay stage. However most of the free eggs classified as alive (i.e. with more than 50% of their cells alive) did have some dead cells. It seems likely that, had the culture time been longer, these eggs would have died and disappeared (as around 50% of all eggs injected did) and would not have developed further. Therefore one explanation of the above paradox is that uteri were metabolically most active under the influence of progesterone and that dead or partially dead eggs would undergo cytolysis more rapidly in these uteri. This proposed cytolytic effect coupled with the fact that progesterone treatment provided the strongest stimulus (or created the most suitable conditions) for egg development would mean that healthy eggs would

progress and most others would disappear.

Variations in the oxygen concentration, in the presence of progesterone and oestradiol, had a pronounced effect on the proportions of injected eggs that were found after culture and their viability. The best recovery rate was obtained when the oxygen concentration was 26-40% and survival was best when it was 26-30% (see Text-fig. 1). It is not known why only 15% of injected eggs remained in the 41-50% oxygen group. The healthiest egg in this group possessed distal endoderm and was invading into the longitudinal muscle layer from the external surface of the uterine horn (Pl. 3, Fig. 18). The only egg with a comparable degree of development within the lumina was solid in form and although it contained both embryonic and trophoblast tissue the latter was unhealthy (Pl. 4, Fig. 22). The highest levels of oxygen killed most eggs and often selectively killed the inner cell mass tissue (Pl. 5, Fig. 37). Embryonic development probably did not progress beyond the proximal endoderm stage for this range. Glenister (1970) has also found that high oxygen levels were selectively lethal to embryonic tissue in rabbit embryos that were invading endometrial tissue in culture.

Giant cell transformation of the trophoblast occurred more frequently in the progesterone than in the nonprogesterone 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 and Waldo, 1949; Bryson, 1964; Kirby and Cowell, 1968),

transformation of eggs within uteri may require hormones. Blasto-cysts in mice where implantation has been delayed by ovariectomy or by lactation do not transform (Dickson, 1963; 1966a; McLaren, 1968) unless both progesterone and oestradiol are given to the ovariecto-mised mice (Yoshinaga and Adams, 1966) or oestrogen (or removal of the litter) is given to lactating mice (Whitten, 1955). Transfor-mation is then induced experimentally by oestrogen and occurs as an early stage of normal implantation and is probably associated with proliferation of the abembryonic pole trophoblast (Dickson, 1966b) and early invasion (Snell and Stevens, 1966).

The present findings may differ from the in vivo situation in several respects. Transformation in the uterus 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 nontreated ovariectomised 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. Gwatkin suggested that activation of delayed blastocysts could be regulated by the supply of amino acids. The uterine epithelium seems capable of selective resorption and absorption that may be hormone controlled (Nilsson, 1970). Perhaps the direct supply of media to 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 dependant 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 findings would indicate _in vitro_. Proestrus oestrogen primes the uterus for the secretion from the corpora lutea which leads to a state of progesterone dominance that is interrupted briefly on the beginning of the 4th day of pregnancy by a period of oestrogenic activity. This is followed by a period of uterine receptivity during which eggs present can implant (see Grant, 1972c & e for references). Implantation with either progesterone or oestradiol alone and implantation with both progesterone and oestradiol in uteri which had not received correct sequential treatment would be unexpected _in vivo_, but occurred _in vitro_.

In evaluating the relevance of _in vitro_ to _in vivo_ implantation, account must be taken of the experimental situation. 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 cannot operate _in vitro_. For example, high doses of oestradiol (as used in the present work) administered to pregnant mice cause expulsion of the eggs via the cervix, presumably by activating the myometrium. 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 some eggs in the oestradiol treated group (and in the 38-95% O_2 group) failed to invade the epithelium although transformation had occurred (Pl. 5, Figs. 31, 36 & 38).   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.   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.

Treatment with progesterone alone allowed the highest percentage of injected eggs to 'implant' although this proportion was not significantly different from that obtained with both progesterone and oestradiol.   As discussed, progesterone treatment may have been associated with conditions most favourable for egg development and least favourable for the survival and persistance of defective eggs.

Implantation regularly occurs in ovariectomised mice treated with certain brands of progesterone and with 'pure' progesterone plus testosterone (Grant, 1972e).   In mice ovariectomised after the morning of the 4th day of pregnancy (but not before the 4th day) a full implantation response is usually obtained with progesterone treatment alone (Humphrey, 1967b; Grant, 1968; 1972e).   The time differential effect of ovariectomy is attributed either to an oestrogen release early on the 4th day of pregnancy (see Bindon and Lamond, 1969) or to sufficient oestrogen accumulation by noon on the

4th day to allow implantation (Bloch, 1968; Smith, 1966) in progesterone-treated mice.   It is possible that in vitro as in vivo small amounts of oestrogenic or androgenic impurities, contained in the foetal calf serum used in the media, could have combined with progesterone to allow implantation.   Further the immaturity of the uteri used may have been associated with the lack of specificity shown in response to the hormones.

The levels of oestradiol and progesterone used in these experiments are high (5µg/ml of either or both hormones).   The rate and size of trophoblast outgrowth in plastic Petri dishes was not influenced by the addition of 5µg/ml of media of both progesterone and oestradiol (see Appendix 2).   Although 25µg of oestradiol (about the total amount present in the media with each uterine horn) would be an enormous dose administered in vivo, it was not demonstrably toxic in the present experiments.   The addition of proteins to the media, in the form of foetal calf serum, may however have bound some of the added steroids (see Heap, 1969).

Morphology of implantation

Loss of the zona pellucida from eggs in cultured uteri appeared to be by dissolution of the zonae in situ irrespective of the presence of hormones.   Eggs cultured in vitro lose their zonae by hatching, while the method of loss in vivo depends upon the endocrine state of the mother, the age and viability of the eggs (McLaren, 1969; Dickmann, 1969) and perhaps genetic factors (Mintz, 1971). Unfertilized and dead eggs retain their zonae for longer than their fertilized contemporaries (Orsini and McLaren, 1967).   The only complete zona seen in the present work surrounded a dead egg.

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

Progesterone treatment of uterine horns cultured in vitro
induced luminal closure (Grant, 1972a) and eggs in such horns were
often situated in well formed crypts. Crypt formation usually
involved the enlargement of existing crypts or depressions
(occasionally they were entirely egg-made). Eggs in horns treated
with oestradiol or ethanol were often found in the centre of large
lumina with little evidence of crypt formation.

Fusions between eggs that were in contact in the lumina of
uteri were 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 ovariectomised nontreated mouse. Fusion in this study
was usually between blastocysts that had developed beyond the delay
stage.

In mice, fusion between apposing luminal walls occurs in normal
pregnancy at about 100 hours post coitum (Reinius, 1967; Potts, 1968)
and can be induced in progesterone-treated mice and rats by an
injection of oestrogen (Nilsson, 1966; Ljungkvist, 1971). This
fusion was noted in one of the two oestradiol-treated uterine horns
that contained invading trophoblast and was extensive in two

progesterone and oestradiol treated uteri.

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 and McLaren, 1967) were first noted in these regions. In these respects the in vitro situation parallels that seen in vivo (Snell and Stevens, 1966; Finn and McLaren, 1967). W-bodies (the primary invasive cells of Wilson (1963) are probably dying cells (Finn and Lawn, 1968; Wilson and Smith, 1970) of maternal origin. Trophoblast processes were observed to encircle apparently viable epithelial cells (see Pl. 2). Fixation shrinkage sometimes caused encircled cells to be plucked off the epithelial basement membrane (Pl. 1, Fig. 5). Finn and Lawn (1968) and Wilson and 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 is protracted in vitro and may have been impeded when the oxygen concentration was above 38%.

Progressively fewer eggs were invasive as the oxygen concentration increased from 31-95%. Eighty-three percent was the highest level at which the stroma was invaded and the amount of trophoblast beyond the epithelium for the one egg concerned was very small. The extent of two-dimensional trophoblast outgrowth observed when blastocysts were cultured in plastic petri dishes was progressively lessened as the oxygen concentration was increased from 20 to 90% (Grant, unpublished data). The highest oxygen levels probably did inhibit trophoblast activity but the possibility that intermediate

levels may have induced more resistant epithelium cannot be excluded. The trophoblast of eggs cultured in 38-70% oxygen often damaged the epithelium and subsequently showed degenerative changes (Pl. 7, Fig. 47).

In the uteri treated with oestradiol alone, the epithelium seemed more resistant to invasion. Oestradiol did not limit the extent of blastocyst outgrowth in vitro (Grant, Appendix 2), and in vivo it stimulates epithelial mitosis, and the epithelium in the oestrogen treated uteri may have been in a growth phase (see Martin and Finn, 1971).

The concentration of collagen in the uterine stroma immediately in front of the invading trophoblast (Pl. 7, Figs, 48, 49 & 50) has also been described 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. Where stromal cells were in direct contact with the trophoblast they contained fibrillar material below their plasma membranes.

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 that were not in well formed crypts were clearly misshapen because of luminal closure

(see Pl. 5, Fig. 29).

The solid form of many of the embryos that had developed beyond the distal endoderm stage was probably due to the fusion of the abembryonic trophoblast with the advancing embryonic tissues. Intermediate stages with persistant yolk cavities were relatively uncommon (see Pl. 3, Fig. 14).   Compared with normal embryos $5\frac{1}{2}$ days post coitum (Pl. 4, Figs 18) advanced in vitro embryos (Pl. 7, Figs. 51 & 52) have no yolk cavity and more trophoblast tissue in the region of the abembryonic pole.   The prominent yolk cavity character-istic of in vivo embryos is considered by Potts (1969) to represent a shrinkage artifact.   However normal $5\frac{1}{2}$ day post coitum egg cylinders flushed directly from uteri also have a prominent yolk cavity (Pl. 4, Fig. 19).   During this stage of development Reichert's membrane is positioned so that there is a definite yolk cavity. There was no obvious Reichert's membrane between the trophoblast and the endoderm in the in vitro embryos.   Earlier development in vitro was accompanied by trophoblast proliferation with concomittant collapse of the yolk cavity (Pl. 2, Fig. 12).   Even an egg invading on the external surface of a uterine horn showed proliferation of trophoblast cells at the borders of invasion (Pl. 3, Fig. 17).   To what extent such trophoblast is abnormal is not known, but degenerative changes were seen in trophoblast tissue at all stages of activity (Pl. 4 & 7, Figs. 20 & 49, respectively) and it is probable that the present culture conditions did not allow optimal trophoblast development.

PLATE 1

Figs. 1-6 show blastocysts after culture (two days for nos. 2-6 and three days for no. 1) within the lumina of uteri. Staining was with haematoxylin and eosin for nos. 1-3 and with toluidine blue and methyl green for nos. 4-6. x 661 .

Fig. 1.   A blastocyst that was free in the lumen. The nucleus (N) of the trophoblast (T) cell is from a nontransformed cell and the inner cell mass (ICM) is nondifferentiated. Cultured in media containing progesterone and oestradiol in an oxygen concentration of 20% (20% O_2).

Fig. 2.   A blastocyst that was apparently free in the lumen. The nuclei indicated are from small giant cells. Cultured in media containing ethanol in 29% O_2.

Fig. 3.   A blastocyst that was attached to the uterine epithelium. The nuclei indicated (N) are from nontransformed trophoblast cells (other sections contained transformed cells). Indications of attachment are the roughened surfaces of the trophoblast and its adjacent epithelium (on the right hand side of the photograph) and the occurrence of vacuolated epithelial cells (V). Although proximal endoderm (PE) cells are present other cells of the inner cell mass are vacuolated and look unhealthy. Cultured in media containing progesterone and oestradiol in 48% O_2.

Fig. 4.   This blastocyst was invading the epithelium. The epithelial surface is notched and elsewhere cells were displaced. The trophoblast shown is transformed and has the characteristic pale-staining cytoplasm with vague irregular borders. There is a W-body (WB) between the epithelium and the stroma. Cultured in media containing progesterone and oestradiol in 25% O_2.   PE = proximal endoderm.

Fig. 5.   The dead cells indicated (arrow) are probably epithelial cells that have been phagocytosed by the trophoblast and then plucked from the gap in the epithelium due to tissue shrinkage. Cultured in the presence of progesterone and oestradiol in 20% O_2.

Fig. 6.   A probable epithelial cell from the uterus shown in Figure 5 after phagocytosis by the trophoblast.
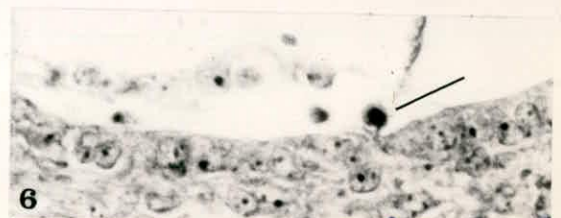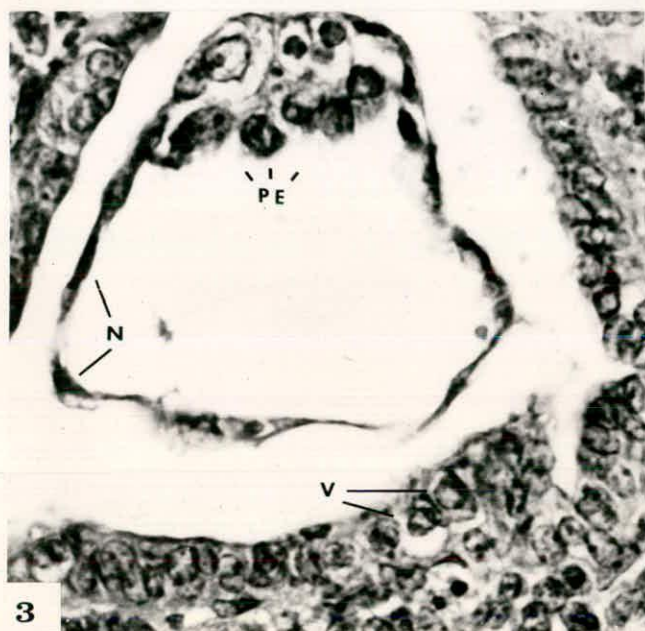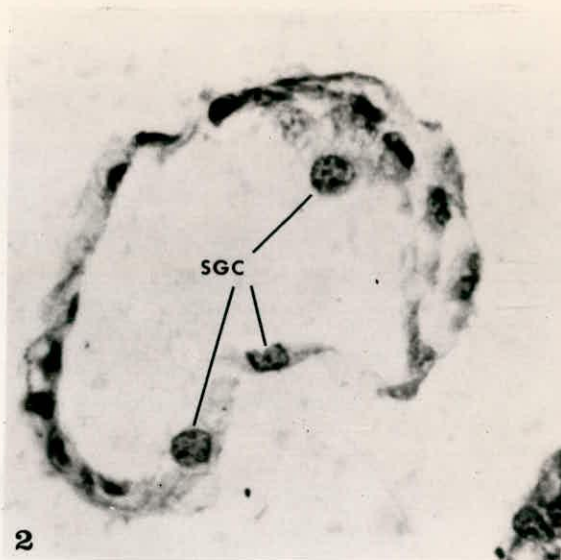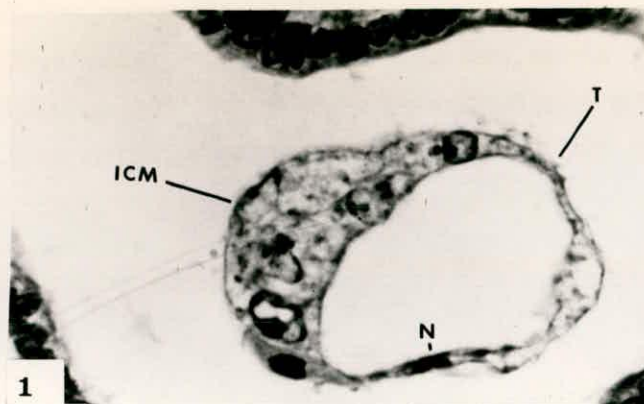
PLATE 2

The blastocysts shown on this plate were cultured for two days in media containing progesterone (Fig. 10) or progesterone and oestradiol (the remainder).

Fig. 7.   A vacuolated epithelial cell in the region of trophoblastic contact.   From the blastocyst shown in Pl. 1, Fig. 6. x 661 .

Fig. 8.   Invasion of the epithelium with trophoblastic encirclement of an epithelial cell (arrow).   This blastocyst was also shown in Pl. 1, Fig. 6.   WB = W-body.   x 661 .

Fig. 9.   A vacuolated region of epithelium associated with contact of the trophoblast (T).   The vacuolated region appears to have been encircled by the trophoblast.   The concentration of oxygen is unknown (probably 20-30%).   Mallory's Trichrome stain.   x 661 .

Fig. 10.   Encirclement of two epithelial cells (arrow) by transformed trophoblast tissue (T).   This blastocyst is also shown in Pl. 1, Fig. 4. x 1322 .

Fig. 11.   The two nuclei (arrows) resemble those of stromal cells. As shown they are near the surface of the epithelium (E) and appear to have been phagocytosed by the trophoblast (T).   Elsewhere on this section areas of epithelium were removed and the trophoblast was invading the stromal tissue. 26% O_2.   Stained with haematoxylin and eosin (H & E). x 1322 .

Fig. 12.   Epithelial invasion with disruption (nuclear-free gaps, swollen nuclei, irregular alignment of nuclei) of the epithelium. Giant cells (GC) are present adjacent to the inner cell mass (ICM) and at the abembryonic pole where local proliferation of the trophoblast is occurring. 56% O_2.   Stained with H & E.   x 661 .
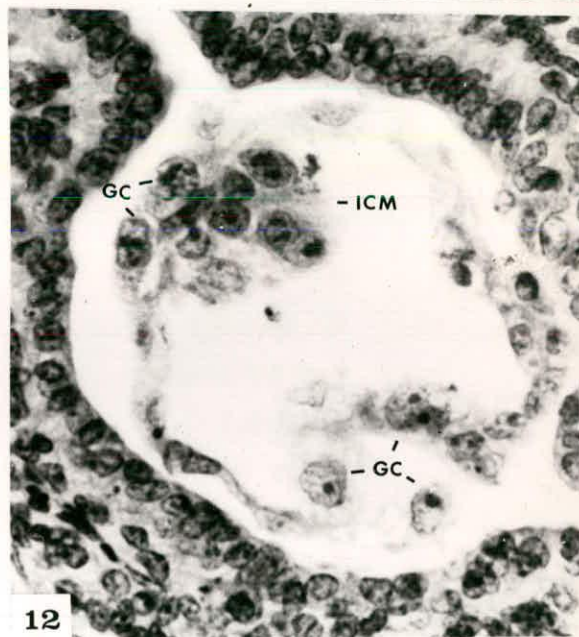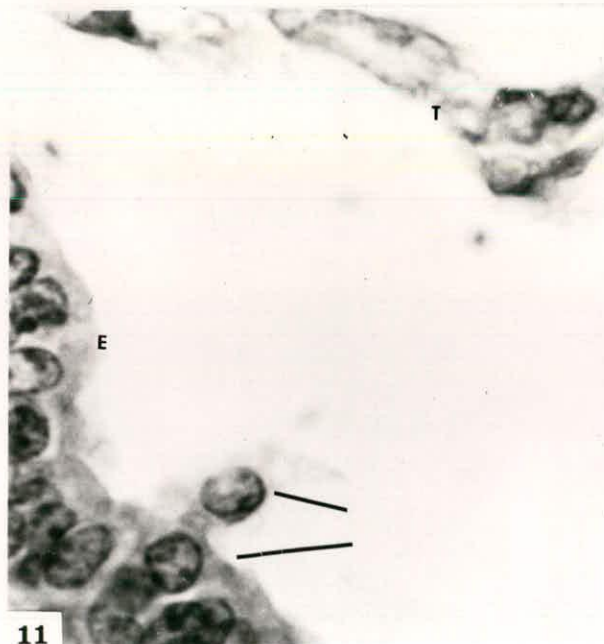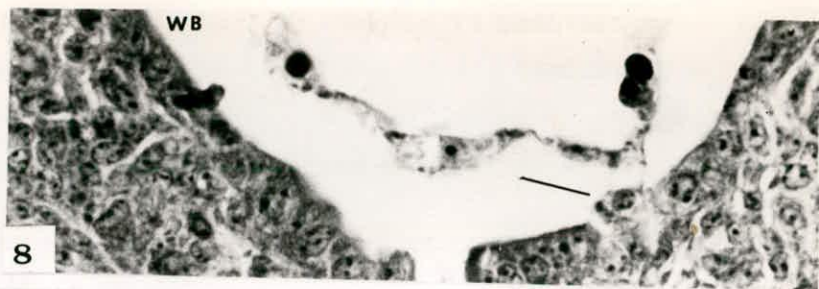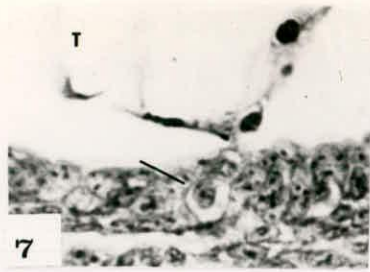
7

8

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10

11

12

PLATE 3

The embryos shown on this plate were all cultured for two days in media containing progesterone (Figs. 14 & 16) and progesterone and oestradiol (Figs. 13, 15, 17 & 18).   All figures were stained with H & E.

Fig. 13.   The distal endoderm (DE) has grown around the inside of the trophoblast (T) forming a bilaminar omphalopleure.   The oxygen concentration was unknown (probably 20-30%).   x 657 .

Fig. 14.   An embryo invading the stroma.   This is a relatively rare intermediate form between an expanded and a solid embryo. The yolk cavity (YC) is still present and is lined with endodermal cells except at the abembryonic region.   Note the proliferating trophoblast near the areas of invasion. 28% O_2.   x 380 .

Fig. 15.   A solid embryo with trophoblast tissue (T) invading the stroma.   The yolk cavity has collapsed allowing the trophoblast giant cells to make contact with probable embryonic ectodermal cells.   Endodermal cells cannot be seen.   E = embryonic pole.   31% O_2.   x 380 .

Fig. 16.   A large giant cell from the embryo shown in Fig. 14. that has invaded into the stromal tissue and is positioned directly above a gland (G).   Note the large central nucleolus (c.f. Pl. 1, Fig. 2). x 657.

Fig. 17.   An embryo invading the myometrium from the exterior surface of a uterine horn.   Note the thickening of the trophoblast tissue on the left side of the photograph. 42% O_2.   x 657 .

Fig. 18.   A different view of the embryo shown in Fig. 17.   The cells indicated (arrows) are from the distal endoderm. x 657 .
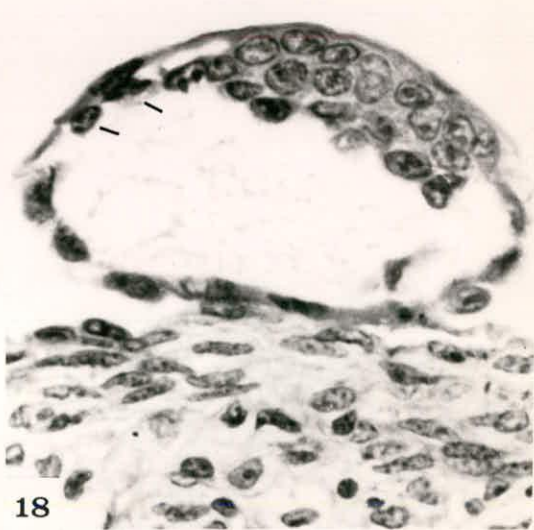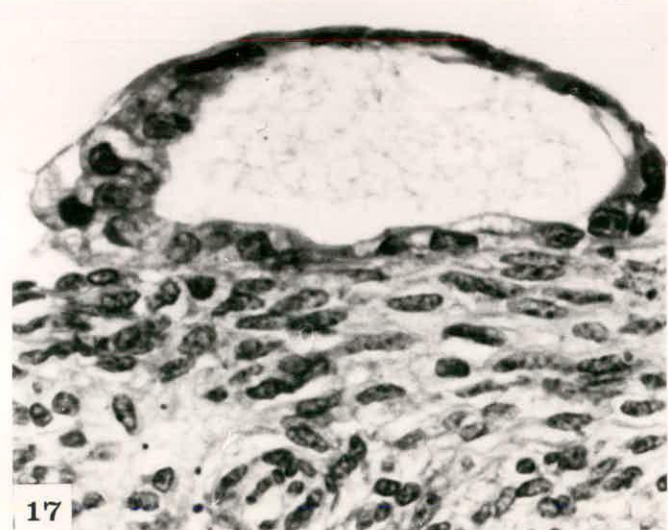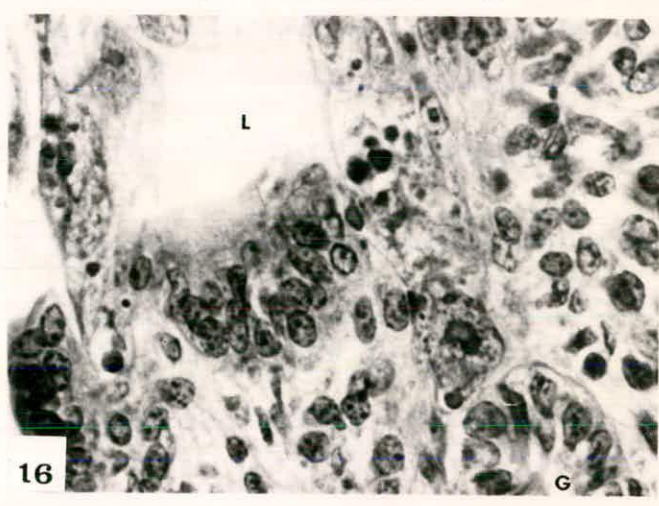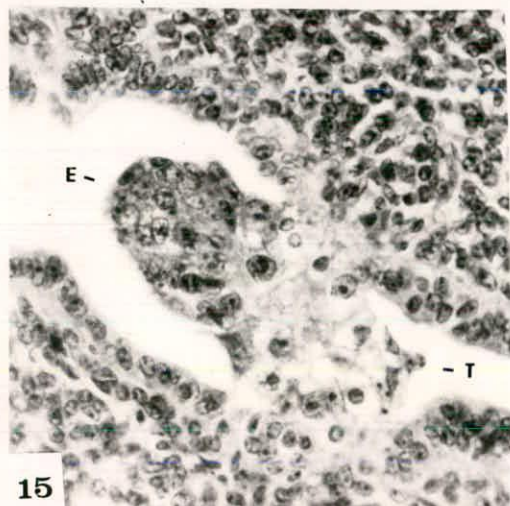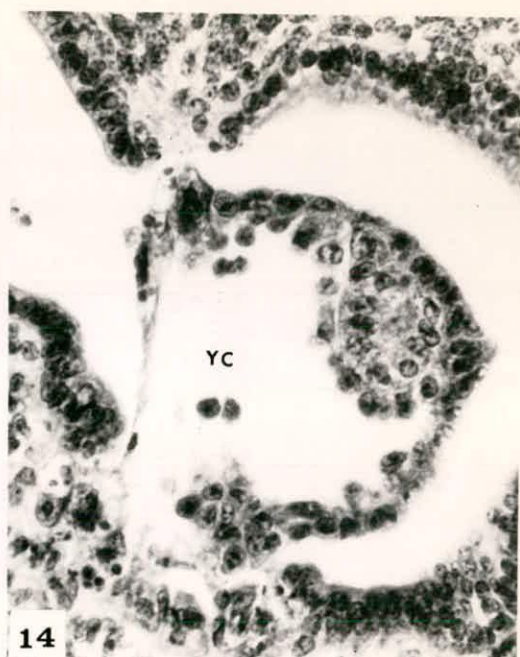
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PLATE 4

Fig. 19.   A normal $5\frac{1}{2}$ day post coitum (pc) embryo.   Reichert's
membrane (R) separates giant cells of the trophoblast from cells
of the distal endoderm.   YC = yolk cavity; PE = proximal endo-
derm.   Stained with H & E.   x 505 .

Fig. 20.   A normal 5 day pc embryo, photographed immediately after being
flushed from the uterus with saline.   The embryonic ectoderm
(EE) surrounds a small proamniotic cavity.   Otherwise labelled
as in Fig. 19. x 486 .

      Figs. 21-27 show embryos that were cultured for two days in media con-
taining progesterone and oestradiol.   The sections were stained with H & E.

Fig. 21.   An embryo that appears to have two inner cell masses.   The lower
'mass' was probably formed by proliferation of abembryonic tro-
phoblast and/or incorporation of tissue after fusion with the
unhealthy egg shown in the bottom of the photograph.   56% O_2.
x 505 . .

Fig. 22.   The healthiest embryo within the lumina of all uteri cultured in
41-50% oxygen.   The healthiest embryo after culture in this
range was invading the external surface of a uterine horn (Pl.3,
Fig. 19).   The trophoblast (T) of this embryo has not invaded
into or otherwise damaged the uterine epithelium.   E = the
embryonic pole.   42% O_2.   x 505 .

Fig. 23.   A collapsed blastocyst.   The blastocoel cavity (B) has been
reduced in size by the fusion of the opposite trophoblastic
walls.   22% O_2.   x 505 .

Fig. 24.   A contracted blastocyst.   The blastocoel cavity is small rela-
tive to the size of the inner cell mass.   Note the asymetrical
thickening of the trophoblast wall (arrows). 20% O_2. x   .

Fig. 25.   A 'dead' contracted egg within the uterine lumen.   Compare
the distances between the swollen abembryonic trophoblast nuclei
with the distances between nuclei on the lateral trophoblast
walls.   38% O_2.   x 505 .

Fig. 26.   Two solid 'dead' eggs that were fused together (F).   In addition
to nuclear gaps in the epithelium some epithelial cells have been
phagocytosed by the trophoblast (EP).   Thus it is probable that
earlier invasive attempts were made by this egg.   These eggs
were in the same uterus as the egg shown in Fig. 22 and in Pl. 3,
Figs. 18 & 19.   x 505 .

Fig. 27.   Two solid embryos that have fused together at their abembryonic
poles (F).   These eggs were in the same uterus as the egg shown
in Pl. 3, Fig. 15.   x 505 .

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PLATE 5

Cultures were maintained for three days (Fig. 35) and two days (the remainder). Media contained oestradiol (Figs. 28, 31 & 36) and progesterone and oestradiol (the remainder). Sections were stained with toluidine blue and methyl green (Fig. 37) and H & E (the remainder).
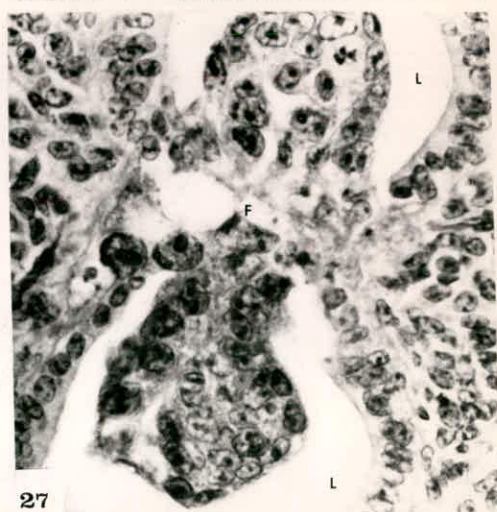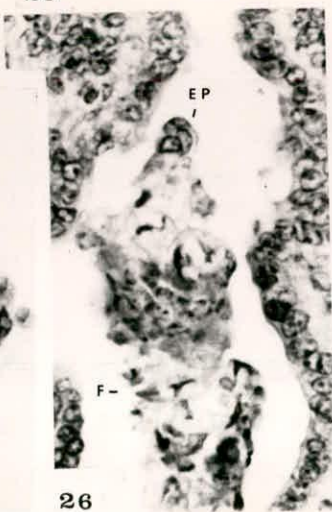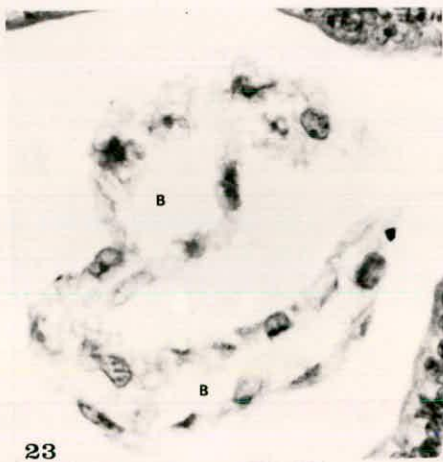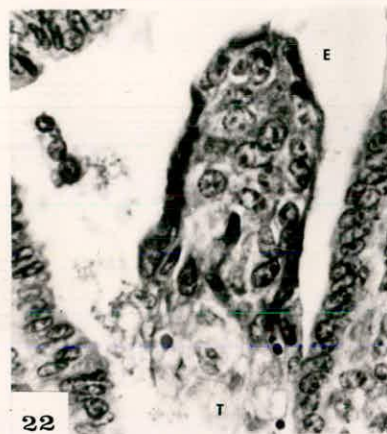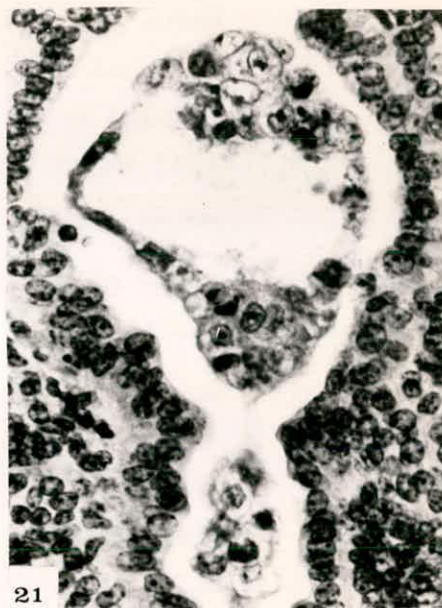
Fig. 28.   Fused blastocysts within the lumen of a uterine horn.   One blastocyst has invaded into the stroma and although giant cells have proliferated at the abembryonic pole (arrow) of a second blastocyst the adjacent epithelium is not damaged.   22% O_2. x 275 .

Fig. 29.   A misshapened 'dead' embryo with a solid form.   E = embryonic tissue and T = trophoblast tissue.   26% O_2.   x 275 .

Fig. 30.   A blastocyst that was probably attached during culture.   Observe the small giant cells adjacent to the inner cell mass.   25% O_2. x 275 .

Fig. 31.   An advanced embryo with well developed embryonic ectoderm (EE) and distal endoderm (DE).   The invasion of the epithelium is proceeding slowly relative to the state of embryonic development. Although the epithelium is damaged, there are dead giant cells in the trophoblast (arrows).   25% O_2.   x 550 .

Fig. 32.   The epithelial nuclei are rod shaped and positioned apically 95% O_2.   x 550 .

Fig. 33.   The epithelial nuclei are rod shaped and positioned in the centre of the cells.   33% O_2.   x 550 .

Fig. 34.   The epithelial nuclei are ovoid in shape and positioned near the base of the cells.   26% O_2.   x 550 .

Fig. 35.   The embryo illustrated has invaded the stroma in an atmosphere of 54% O_2.   The trophoblast tissue (T) was less healthy than the embryonic tissue.   x 550 .

Fig. 36.   Another blastocyst in the same uterine horn as shown in Fig. 31. The giant cells (arrowed) appear unhealthy and have been unable to invade through the epithelium.   x 550 .

Fig. 37.   The outer trophoblast cells are viable and inner embryonic tissue is dead.   83% O_2.   x 550 .

Fig. 38.   A solid embryo with transformed trophoblast cells.   The adjacent epithelium is however undamaged.   38% O_2.   x 550 .
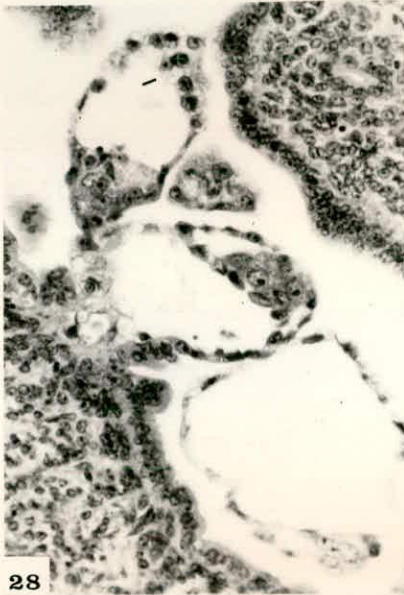
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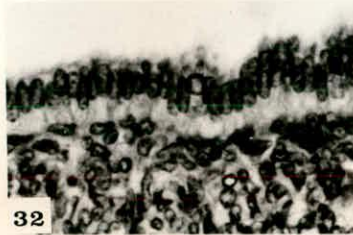
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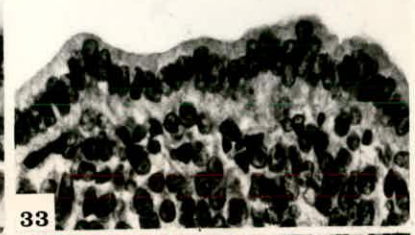
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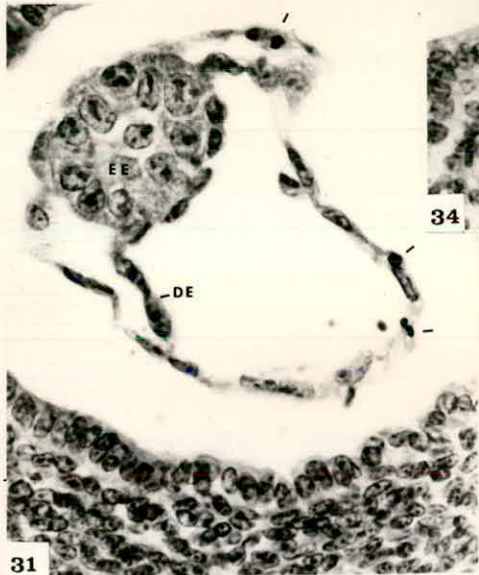
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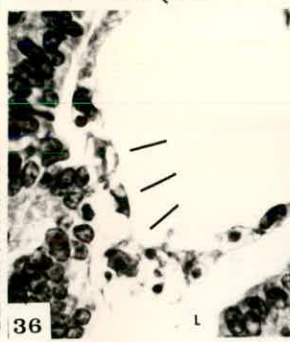
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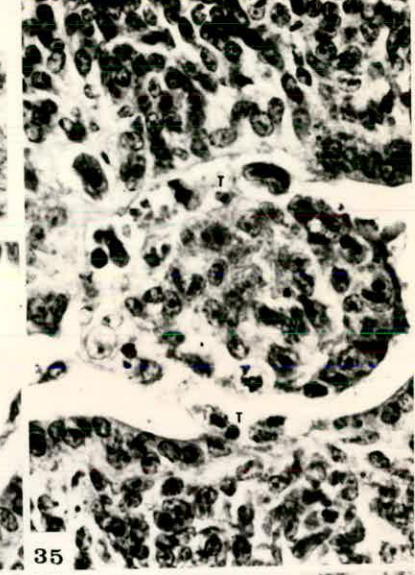
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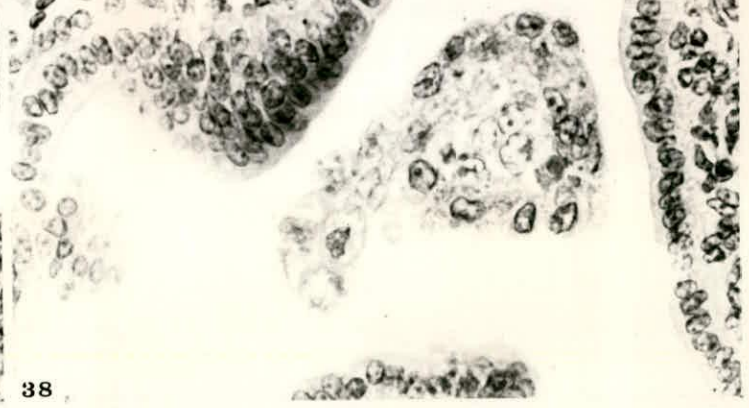
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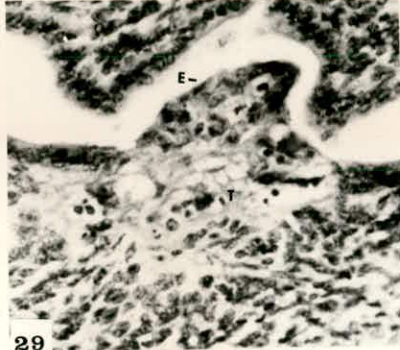
PLATE 6

Cultures were maintained for three days (Fig. 43) and two days (the remainder). Media contained progesterone (Fig. 46) or progesterone and oestradiol (the remainder). Sections were stained with H & E (Figs. 39-43) and Mallory's Trichrome (Figs. 44-46).

Fig. 39. Note the eosinophilic W-body attached to the trophoblast and situated at the surface of the epithelium. 77% O_2. x 961.

Fig. 40. Encirclement of two epithelial cells (arrow) by trophoblastic tissue (this section is shown at higher magnification on Pl. 2, Fig. 10). x 267.

Fig. 41. Selective death of the embryonic tissue (arrow) after culture in 83% O_2. x 267.

Fig. 42. Persistance of a small yolk cavity (aroow) after trophoblast invasion through the epithelium. The trophoblast tissue is disorganized. Cells have proliferated and allowed the collapse of the yolk cavity. Further, normal endodermal cells are not distinguishable. The initial O_2 concentration was 33% but owing to leakage of the culture jar, fell and was 20% after culture. x 267.

Fig. 43. Solid embryo with healthy invasive and unhealthy noninvasive trophoblast (T) tissue in an O_2 concentration of 54%. (This is the same uteri, though a different embryo, from that shown on Pl. 5, Fig. 35). x 267.

Fig. 44. This blastocyst was injected into the uterine horn on the 4th day of pregnancy while it was still in its zona pellucida. Most of the zona has been lost though a large piece (Z) is positioned above the blastocyst. ICM = inner cell mass and T = trophoblast. 27% O_2. x 267.

Fig. 45. Another two blastocysts in the lumen of the uteri shown in Fig. 44. Again the zona material in the lumen stains blue. x 267.

Fig. 46. There are at least four embryos (1-4) in the lumen of the uterine section shown. Note the blue coloured zona material that surrounds the embryos numbered 3 and 4. 29% O_2. x 267.

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43 E T

44 Z ICM T

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46 1 2 3 4
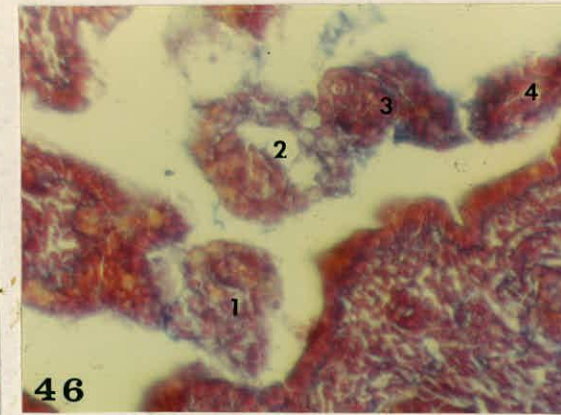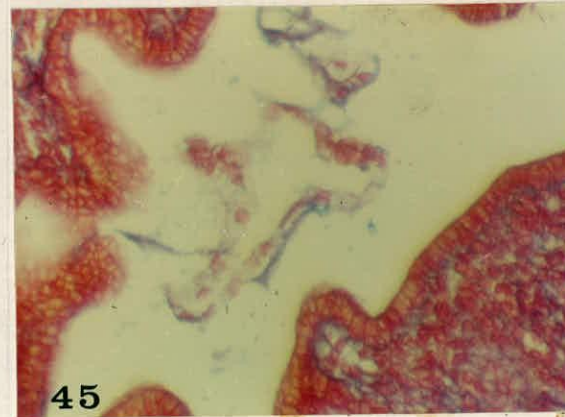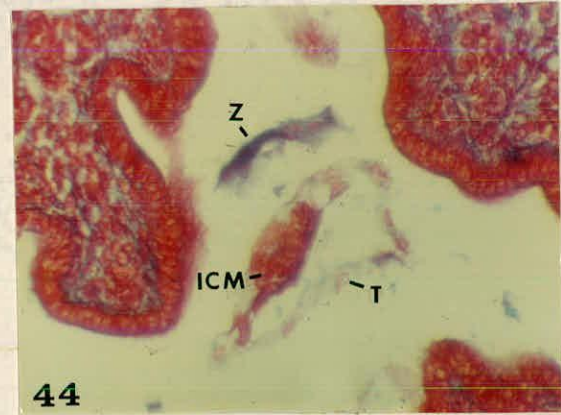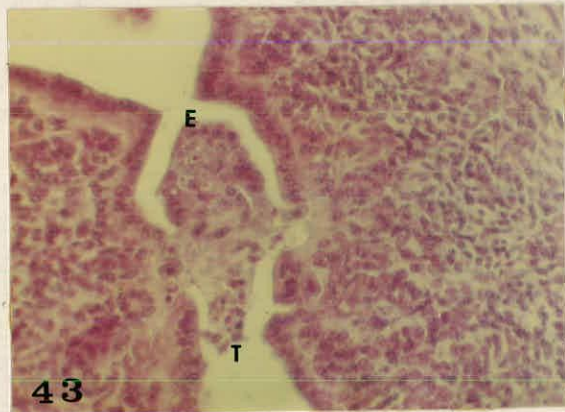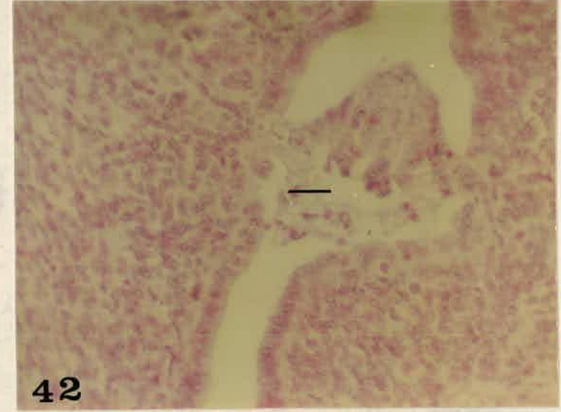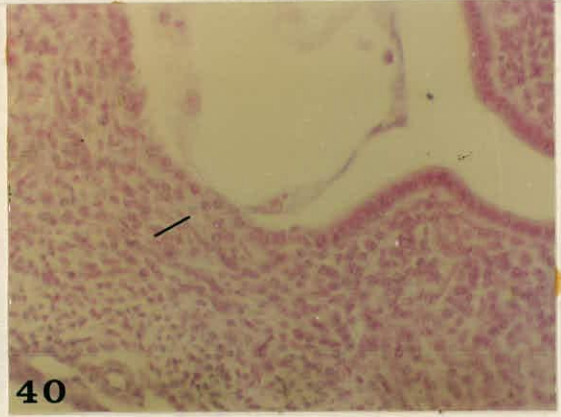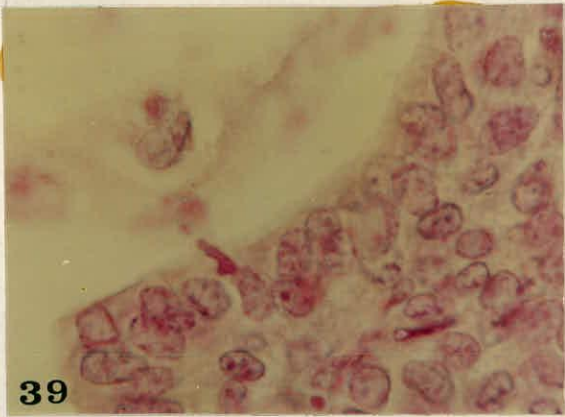
PLATE 7

Cultures were maintained for three days in media containing proges-
terone and oestradiol at an oxygen concentration of 54% (Fig. 47) or
32% (the remainder).   Figs. 47-50 x 667, and Figs. 51 & 52 x 267.

Fig. 47.   This embryo is contracted with 'dead' trophoblastic tissue.
            Pyknotic giant cell nuclei are positioned close together and
            the size of the yolk cavity is reduced.   Vacuolation of some
            of the epithelial cells and the irregular alignment of others
            suggests that early invasive attempts were made.   Note the
            position of the vacuolated cells beneath the surface of the
            epithelium (see also Pl. 2, Fig. 7).   The embryonic tissue
            is mostly viable although endoderm cells are not obvious.
            Other embryos in the same uterine horn are shown on Pl. 5,
            Fig. 35 and Pl. 6, Fig. 43.   Stained with H & E.

Figs. 48, 50 & 52.   This is one of the most advanced embryos (judged
            by the amount of embryonic tissue present) obtained after
            the culture of blastocysts within the lumina of uteri.
            Fig. 52 was stained with H & E (T = trophoblast) and Figs.
            48 & 50 were stained with Mallory's Trichrome.   This stain
            colours collagen dark blue and the blue patches on the right
            hand side of the photographs were interpreted as uterine
            collagen situated immediately ahead of the invading tropho-
            blast.   The collagen concentration appears continuous with
            the epithelial basement membrane.   The embryonic tissue is
   clearly disorganized (c.f. Pl. 4, Figs. 19 & 20).

Figs. 49 & 51.   Another embryo from the same uterus as that shown in
            Figs. 48, 50 & 52.   The concentration of collagen in Fig. 49
            (Mallory's Trichrome stain) is again situated immediately
            ahead of the invading trophoblast (c.f. with Fig. 51).
            The embryonic tissue is organized into a circular ring of
            probable embryonic ectodermal tissue enclosing a cavity
            (proamniotic - c.f. Pl. 4, Fig. 20)   (T = trophoblast in
            Fig. 51 which was stained with H & E).
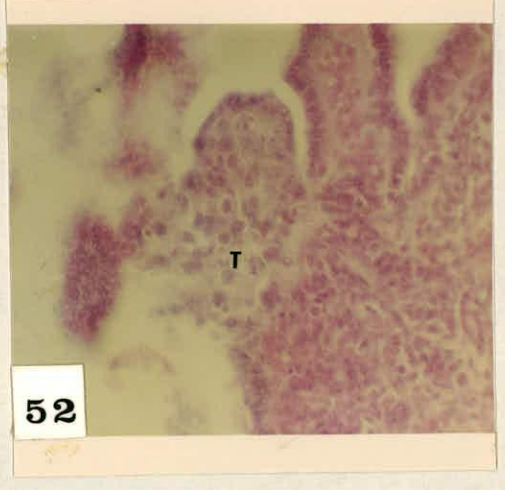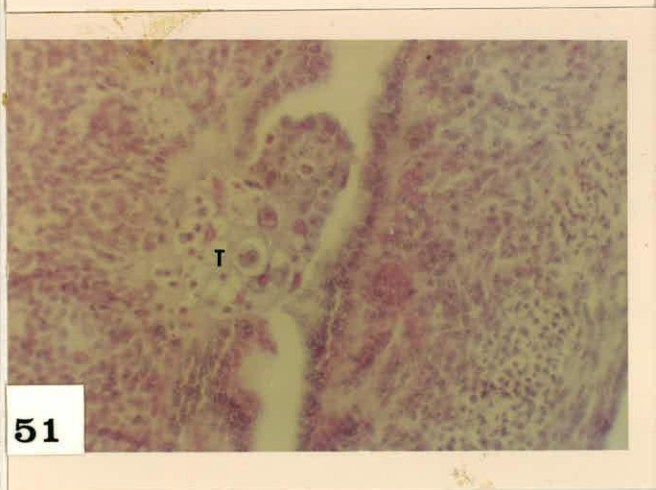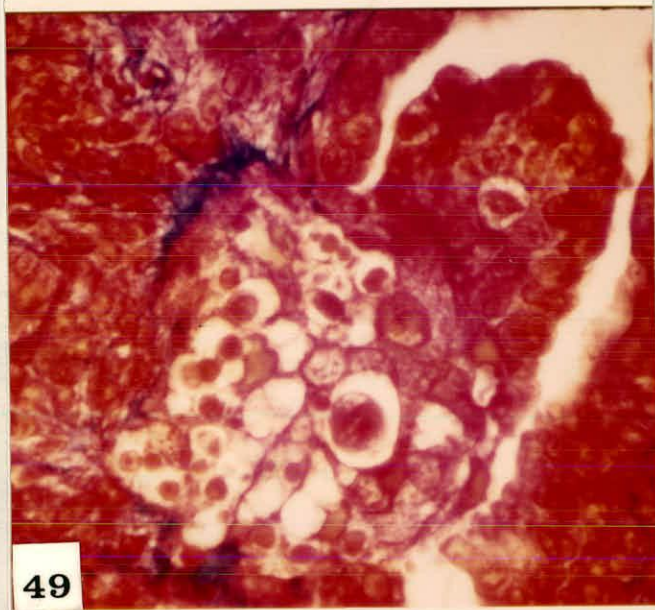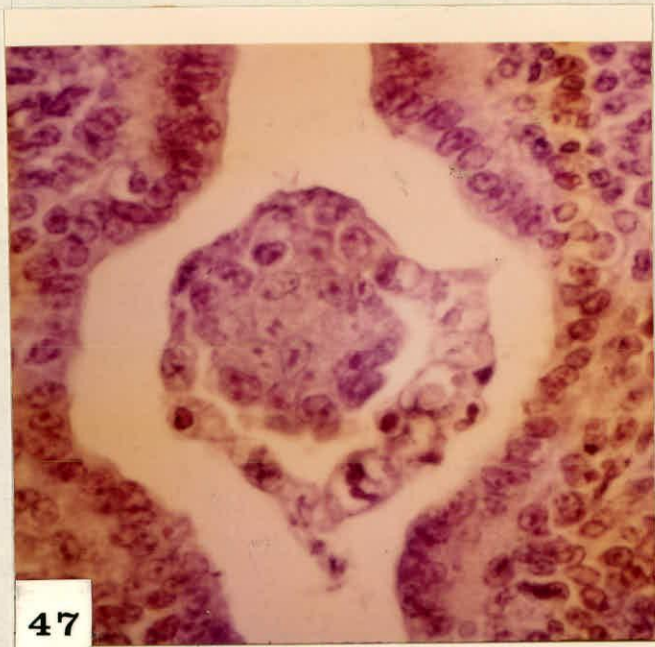
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T

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THE CONTROL OF UTERINE SENSITIVITY FOR EGG IMPLANTATION

AND DECIDUOMATA INDUCTION IN CYCLIC MICE AFTER TREATMENT

WITH MEDROXYPROGESTERONE ACETATE AND OESTRADIOL

ABSTRACT

Virgin cyclic mice were treated with the potent long-acting
progestins, medroxyprogesterone acetate (MAP) or melengestrol
acetate (MGA) at different stages during the oestrous cycle.   After
a variable number of days mice were either treated with oestradiol
followed approximately seven hours later by an injection of air into
their uterine lumina for deciduomata induction, or blastocysts were
transferred to their uteri and oestradiol was injected on the
following day.   Six days after egg transfer mice were laparotomised
and after a further eleven days they were autopsied.

The two variables that were important both for deciduomata
induction and egg-implantation were the MAP-oestradiol interval
length and the stage of the oestrous cycle during which the progestins
were injected.   Maximum uterine sensitivity both for deciduomata
induction and egg-implantation was obtained after the injection of
MAP during oestrus with a 4 day MAP-oestradiol interval.   Pooled
data from experiments when MAP was injected during oestrus with a
4 day MAP-oestradiol interval demonstrated that 34% of the trans-
ferred blastocysts developed to full term viable foetuses.   A
variable proportion (0-37%, mean 15.6%) of the swellings seen at
laparotomy from these experiments probably did not contain embryos.

The injection of MAP or MGA during oestrus (or during other
stages of the oestrous cycle) usually did not induce pseudopregnancy
that was accompanied by the persistance of active ayclic corpora
lutea.   Neither the incidence of 'active' corpora lutea nor the
ovarian weight influenced the probability of a positive response to
injected air or eggs.   'Pregnancy' was maintained by the activity

of the injected progestins.

It is concluded that the uterine sensitivity of early pregnancy can be mimicked in cyclic mice by treatment with exogenous progestins and oestrogen. However, this sensitivity was probably influenced by the release of endogenous oestrogen which was probably not completely inhibited by treatment with the progestins.

INTRODUCTION

Egg transfer experiments have delineated the period when the uteri of pregnant or pseudopregnant mice are receptive to transferred eggs (McLaren and Michie, 1956). Receptivity is lost at the time that coincides with the development of maximal uterine sensitivity to artificial intraluminal decidualizing stimuli on the 4th day of pregnancy (Finn, 1965; Hetherington, 1970). At the beginning of the 5th day the apposing uterine luminal walls have begun to fuse with concomitant changes in the morphology of the luminal epithelium (Potts, 1968; 1969; Nilsson, 1970). The period of uterine receptivity is naturally extended in pregnant mice suckling young (Enzmann, Saphir and Pincus, 1932) and can be experimentally prolonged by ovariectomy and progesterone treatment before noon on the 4th day of normal pregnancy (Yoshinaga and Adams, 1966; Humphrey, 1967b; Grant, 1968). During the extended receptive period blastocysts are maintained in a state of diapause, and implantation can be invoked both in lactating (Whitten, 1955; 1958; McLaren, 1968) and in ovariectomised (Yoshinaga and Adams, 1966) mice by an injection of sensitizing oestrogen. Lactationally delayed implantation in the rat is associated with high levels of plasma progesterone (Grota and Eik-Nes, 1967; Yoshinaga/and Greep, Moudgal 1971) and it is possible that the same situation applies in the mouse.

The current problem was to determine if progestin treatment of cyclic mice would render their uteri receptive to transferred eggs in such a way that subsequent sensitization and implantation could be controlled by the administration of exogenous oestrogen. Quantitative measures of uterine sensitivity are most precise when

intraluminal agents were used to induce decidualization (Finn, 1965; De Feo, 1967).   In the current experiments intraluminal injections of air were used to test for uterine sensitivity (Orsini, 1963; Hetherington, 1970).

One practical consequence of the hormonal induction of uterine receptivity would be its use as an alternate method of preparation of recipient mice for egg transfers.   Alternate that is to the induction of pseudopregnancy following a sterile mating with a vasectomised male.   Everett (1963) demonstrated that treatment of cyclic rats with progesterone beginning at oestrus induced pseudo-pregnancy.   Banik and Ketchel (1965) and Mantalenakis and Ketchel (1966) have evaluated hormonal methods of inducing pseudopregnant rats as recipients for egg transfer operations.   The present study evaluates one method of progestin treatment for the induction of uterine receptivity in cyclic mice.

MATERIALS AND METHODS

Virgin, cyclic 6-9 week old female mice of the Q strain were used.   Initially vaginal smears were made daily and the cycle stages were interpreted after Bronson, Dagg and Snell (1966).   In later experiments the cycles were followed by observation of externally visible vaginal changes, as the results of this method agreed with those from the smear data.   The four successive cycle stages recognized were proestrus, oestrus, metoestrus-1 (M-1) and metoestrus-2 (M-2) which included dioestrus.   Artificial lighting; $16\frac{1}{2}$ hours of light and $7\frac{1}{2}$ hours of darkness (centred on 01.30 hrs) was used for all experiments.   The room temperature was thermos-

tatically controlled and remained around 23°C.

Pentobarbitone sodium ('Nembutal';Abbott) was used as the anaesthetic for laparotomies and ovariectomies. Hormones were injected subcutaneously; 6α-methyl-17α-acetoxyprogesterone (MAP, 'Depoprovera'; Upjohn) in 0.05ml of vehicle; 17α-acetoxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione, (Melengestrol acetate, MGA; Upjohn) in 0.1ml of 2% methyl cellulose and oestradiol-17β (Koch-Light) in 0.1ml of arachis oil.

Deciduomata induction

MAP (2.5mg) was injected on a chosen stage of the oestrous cycle. Two to five days later 15ng of oestradiol was injected and approximately seven hours later mice were laparotomised and 0.02ml of air was injected into the lumen of the left uterine horn. The air was injected near the utero-tubal junction with a microsyringe (Hamilton) and was seen to distend the horn as it passed down the lumen. The presence of active (i.e. red) corpora lutea on the left ovary was noted and mice were autopsied three or four days later.

In one experiment mice were bilaterally ovariectomised during oestrus immediately before the injection of MAP. In another experiment mice were given an injection of 0.5μg of oestradiol and 2.5mg of MAP during chosen stages of the oestrous cycle.

At autopsy mice with decidual swellings in their left horns were considered to have responded, and the size of the response was estimated by subtracting the weight (in mg) of the right from that of the left horn. The ovaries were trimmed and examined under a dissecting microscope for corpora lutea and then weighed.

Egg transfer experiments

MAP (2.5mg) or MGA (1.0mg; in one experiment only) were

injected during a chosen stage of the oestrous cycle and after a
variable delay eggs (usually six per mouse) were transferred to one
or both uterine horns.   The eggs were flushed from the uteri of
donor mice on the 4hh day of pregnancy when they were usually at the
blastocyst stage and less often the late morula stage of development.
One day later mice were injected with 15ng of oestradiol-17β and
after a further five days laparotomised.   The presence of active
corpora lutea was noted and mice with uterine swellings were
allowed to recover and autopsied ten days later.   Those without
swellings at laparotomy were immediately autopsied and their uterine
horns flushed for non-implanted blastocysts.

At autopsy mice were treated as described for the deciduomata
induction experiments except that the number of live foetuses and
other uterine swellings were counted.   Parturition was delayed in
these mice and live young had to be obtained by caesarean section.
Some foetuses were revived and given to fostermothers.   The size of
the swellings that did not contain live foetuses were assessed
relative to those that contained normal live embryos to provide
an estimate of time of death of the embryos that they may have
contained.   Moles smaller than the size of normal uterine swellings
six days post coitum were not counted as dead embryos.   This was
necessary because in some treatments the number of swellings counted
at laparotomy exceeded the number of eggs injected, presumably
owing to induction of deciduomata.

Selected mice from both the deciduomata and egg transfer
experiments had their ovaries and small lengths of their uteri
sectioned, and some doubtful swellings from the latter experiments
and pieces of inguinal mammary tissue were sectioned.   Tissue for

histology was fixed in Bouins, embedded in paraffin, sectioned at 6-8μ and stained with haematoxylin (Harris's) and eosin.

Statistical methods

Data showing discontinuous variation were analyzed by Chi-square tests (2 x n or 2 x 2 contingency tables). Two by two tables with less than five observations in any cell were analysed by Fisher's exact method (Fisher, 1958) or by Yates method (see Snedecor, 1956). Comparisons made were orthogonal.

Data showing continuous variation were analyzed by 'Student's t test' and correlation and regression techniques (Snedecor, 1956). Percentages were transformed by arc sin $\sqrt{\%}$ method.

Results were considered significant if the likelihood of the difference having occurred by chance alone was equal to or less than 5%.

RESULTS

Deciduomata induction

1. **MAP injection at different stages during the oestrous cycle.** MAP was injected during each of the four stages of the oestrous cycle. Differences between experiments were not significant and the pooled data are given in Table 1. The proportions of mice with deciduomata was heterogeneous (P < 0.05), and the proportion was highest after the injection of MAP during oestrus. The quantitative response was consistently (although nonsignificantly) highest after the injection of MAP during oestrus, and lowest after injection of MAP during M-2 and dioestrus.

2. **The simultaneous injection of MAP and oestradiol at different stages during the oestrous cycle.** MAP and 0.5μg of oestradiol

TABLE 1.

AIR-INDUCED DECIDUOMATA IN CYCLIC MICE.

FOUR DAY MAP-OESTRADIOL INTERVAL[*]

MAP injection on:	Number of mice		Quantitative[‡] response (mg) $\pm$ S.E.
	Total	With deciduomata	
Oestrus	16	12	94.7 $\pm$ 24.4
Metoestrus 1	10	3	60.6 $\pm$ 58.1
Metoestrus 2[+]	13	2	10.5 $\pm$ 10.5
Proestrus	10	4	36.8 $\pm$ 23.3

[*] This table contains data pooled from several experiments.

[+] Includes mice in dioestrus.

[‡] Measured three days after the injection of air.

were both injected during each of the four stages of the oestrous cycle (Table 2). On the small number of mice used, the proportion with deciduomata showed no significant effect of the oestrous cycle, but the quantitative response showed significant variation (by observation), being lowest after injections during proestrus.

3. <u>Variable MAP-oestradiol interval.</u> All mice were injected with MAP during oestrus. Oestradiol and air were injected from two to five days later. Data pooled from all deciduomata-induction experiments is given in Table 3. Variation with the MAP-oestradiol interval was significant for the proportion of mice responding ($P < 0.05$), but not significant for the size of the response. A 4-day interval gave the highest proportion of mice responding and also the largest deciduomata.

4. <u>Variable MAP-oestradiol interval after ovariectomy (Table 4).</u> Mice were treated as above (see 3) except that they were ovariectomised and injected with MAP during oestrus and there was no 2-day interval. When the data were pooled from all the groups a higher proportion of ovariectomised than intact mice had deciduomata ($P < 0.05$). This difference was most notable for the 5-day group.

Although the quantitative response was consistently higher in the intact than in the ovariectomised mice, this difference was nonsignificant.

5. <u>Ovarian weight and the decidual response.</u> Data were pooled for mice given MAP during oestrus with 3- and 4-day MAP-oestradiol intervals.

There was no correlation between the decidual response and the ovarian weight ($r = +0.38$, $t_{19df} = 1.79$ N.S.)

The presence of active corpora lutea at the time of the air

TABLE 2.

AIR-INDUCED DECIDUOMATA IN MICE INJECTED

SIMULTANEOUSLY WITH MAP AND OESTRADIOL

Cycle day of injection	Number of mice		Mean quantitative response (mg) $\pm$ S.E.
	Total	With deciduomata	
Estrus	4	3	48.33 ± 12.47
Metestrus 1	4	4	80.50 ± 40.80
Metestrus 2	8	6	44.00 ± 15.30
Proestrus	4	2	4.00 ± 2.00

TABLE 3.

AIR-INDUCED DECIDUOMATA IN CYCLIC MICE.

MAP INJECTED DURING OESTRUS WITH VARIABLE MAP-OESTRADIOL INTERVAL[*]

MAP-Oestradiol interval	Number of mice		Mean quantitative response (mg) $\pm$ S.E.
	Total	With deciduomata	
2	4	1	69.0
3	16	10	74.1 $\pm$ 32.0
4	23	18	86.8 $\pm$ 18.4
5	13	5	58.4 $\pm$ 35.4

* This table contains data pooled from all deciduoma-induction experiments.

TABLE 4.

EXTENSION OF UTERINE SENSITIVITY FOR AIR-INDUCED DECIDUOMATA

AFTER OVARIECTOMY AND MAP TREATMENT DURING OESTRUS

Treatment				Number of mice		Mean response (mg $\pm$ S.E.)
				Total	With deciduomata	
MAP-Oestradiol interval 3 days, intact				5	5	47.00 $\pm$ 27.12
"	"	"	ovarX	5	4	15.75 $\pm$ 7.39
"	"	4 days, intact		5	3	63.33 $\pm$ 53.93
"	"	"	ovarX	9	9	43.00 $\pm$ 17.33
"	"	5 days, intact		6	2	132.00 $\pm$ 59.00
"	"	"	ovarX	6	6	32.67 $\pm$ 18.19

injection did not increase the probability of deciduoma induction ($X_2 = 0.02$, $_{1df}$, N.S.). There were only two mice with active corpora lutea at autopsy and neither of these had deciduomata.

Egg transfer experiments

1. The size of MAP & oestradiol doses. Early experiments indicated that there was no difference in the proportion of eggs represented by swellings five days after the oestradiol injection when mice were injected with 2.5, 5.0 or 7.5mg of MAP (Table 5). Therefore in subsequent experiments 2.5mg was used. Doses of 6, 15 and 37.5ng of oestradiol were used and assessed by the same criterion. Six was not different from 15ng and 37.5ng was significantly worse than 15ng ($P < 0.01$). A dose of 15ng of oestradiol was used in subsequent experiments.

2. MAP injection during the different stages of the oestrous cycle (four day MAP-oestradiol interval). The proportion of eggs represented by swellings after MAP had been injected during oestrus was higher than after its injection during M-1 ($P < 0.01$) (Table 6) and M-2 ($P < 0.05$). No live foetuses were recovered following the injection of MAP during either day of metoestrus and a higher proportion of transferred eggs developed to full term viable foetuses after MAP injected during oestrus than after its injection during proestrus ($P < 0.05$).

3. The simultaneous treatment with MAP and 0.5μg of oestradiol during metoestrus (four day MAP-oestradiol interval). The proportion of mice with swellings after egg transfer was higher when both MAP and oestradiol were injected (pooled data from two experiments) during M-2 than when only MAP was given (pooled data from three experiments,

TABLE 5.

IMPLANTATION OF EGGS TRANSFERRED TO MAP* TREATED RECIPIENT MICE.

FOUR DAY MAP-OESTRADIOL INTERVAL.

Treatment	Number of mice		No. of eggs transferred	% of eggs represented as swellings
	Total	With implants		
2.5mg MAP vs	4	4	22	36.3
5.0 " "	5	2	29	31.0
5.0 " " vs	3	2	18	16.7
7.5 " "	3	2	18	27.8
6.0ng oestradiol vs	5	4	30	53.4
15.0 " "	5	4	30	56.7
15.0 " " vs	4	4	20	40.0
37.5 " "	4	1	19	5.3

* Injected without regard to oestrous cycle stage.

TABLE 6.

IMPLANTATION AND SURVIVAL AFTER TRANSFER TO RECIPIENT MICE
TREATED WITH MAP OR MGA AT DIFFERENT STAGES OF THE OESTROUS CYCLE

A. MAP Stage of cycle	No.of mice	No. of eggs transferred	% of eggs represented as swellings	No.of viable foetuses as % of transferred eggs
Oestrus	13	76	64.5	32.9
Metoestrus 1	3	18	16.7	0
Metoestrus 2	5	30	3.2	0
Proestrus	5	30	56.7	16.7
B. MGA				
Oestrus	5	31	77.5	32.2
Metoestrus	5	33	15.1	0

Table 7, P < 0.01).   The proportion of eggs represented by swellings
was significantly higher (P < 0.001) when MAP and oestradiol were
injected than when MAP alone was injected, at M-2.   This proportion
was nonsignificantly higher after the addition of oestradiol to MAP
injected at M-1 (pooled data from two experiments).

4. Variable MAP-oestradiol interval (MAP injected during oestrus).
Data pooled from experiments comparing a 4-day MAP-oestradiol interval
with 3- and 5-day intervals is given in Table 8.   The proportion of
eggs represented by swellings was higher when this interval was four
days than when it was three days (P < 0.01) or five days (two experi-
ments, P < 0.05 and 0.001).   The lowest proportion of eggs represented
by swellings (by inspection) was observed after transfer with a
17-day MAP-oestradiol interval.

   There was no significant difference between the 3- and 4-day
intervals in the proportion of eggs developing to viable foetuses.
This proportion was higher (by inspection) for both the 3- and 4-day
intervals than for the 5-day interval.

5. The incidence of 'false' implantation sites.   Some uterine
swellings of mice killed immediately after laparotomy were found to
contain no embryos although the decidual tissue was healthy.   The
incidence of these 'false implantation sites' can only be derived
by indirect methods and was highest when the uteri were most
sensitive to transferred eggs, that is after MAP treatment during
oestrus with a 4-day MAP-oestradiol interval.

   The criteria for false 'implantation sites' were:

   (1) more swellings counted at laparotomy than eggs injected,

   (2) and/or the occurrence of small swellings at laparotomy that
       had either disappeared or that were smaller than normal

TABLE 7.

'IMPLANTATION' AFTER TRANSFER TO RECIPIENT MICE TREATED

WITH MAP AND OESTRADIOL DURING METOESTROUS

Treatment	Number of mice		No. of eggs transferred	% of trans-ferred eggs represented as swellings
	Total	With swellings		
Oestradiol + MAP at metoestrus 2	6	5	32	34.4
MAP at metoestrus 2	12	4	65	7.7
Oestradiol + MAP at metoestrus 1	6	4	24	45.9
MAP at metoestrus 1	3	1	18	16.6

TABLE 8.

'IMPLANTATION' AND SURVIVAL AFTER TRANSFER TO CYCLIC RECIPIENT MICE

TREATED WITH MAP DURING OESTRUS. VARIABLE MAP-OESTRADIOL INTERVAL.

MAP-Oestradiol interval (days)	No.of mice	No. of eggs transferred	% of eggs represented as swellings	No.of viable foetuses as % of transferred eggs
3	5	26	57.7	26.9
4	13	75	82.7	34.7
5	8	46	34.8	2.2
17	5	29	17.2	-

6-day postcoitum swellings, at the time of autopsy.

The pooled data from eight experiments for which MAP had been injected at oestrus with a 4-day MAP-oestradiol interval were examined according to these criteria. Approximately 16% of all swellings probably contained no embryos. This proportion was heterogeneous over the eight experiments (P $\angle$ 0.06). Thus, although 74% of all the eggs were represented by swellings the proportion of eggs actually implanting was approximately 58%. This proportion was statistically homogeneous over the eight experiments (P $\angle$ 0.60).

6. <u>Ovarian weight and the incidence of swellings and viable foetuses after the transfer of eggs to receptive mice</u>. Data were pooled from the eight experiments in which MAP was given at oestrus and the MAP-oestradiol interval was four days.

Ovarian weight was not significantly correlated with either the proportion of eggs represented by swellings (r = -0.07, t_{27df} = 0.40, N.S.) or the proportion of swellings that developed into viable full-term foetuses (r = +0.07, t_{27df} = 0.34, N.S.). Only four of these mice had active corpora lutea at autopsy. Their ovaries were heavier (16.00 $\pm$ 0.54) than those without active corpora lutea (10.40 $\pm$ 0.54: t_{27df} = 7.275, P $\angle$ 0.001).

The mean weight of the ovaries with active corpora lutea was not different from that of six normal mice (three each from the 19th and 20th days of pregnancy, i.e. 18.00 $\pm$ 1.44, t_{8df} = 1.302). All four mice with active corpora lutea had viable foetuses at autopsy and their uteri resembled those of normal full-term pregnant mice. Pregnant mice without active corpora lutea often had hypertonic uteri with the foetuses compressed in their chambers and situated close together.

7. Ovarian weight in 'receptive' and 'nonreceptive' mice.   The
ovarian weights from mice given MAP at oestrus with a 4-day MAP-
oestradiol interval were compared with those of mice treated the same
way except that they were given MAP at M-1, M-2 and dioestrus or
proestrus.   The differences were not significant either for the
experiments considered individually, or when the results were pooled
(pooled data t_{24df} = 0.976, N.S.).

Mice given MAP at oestrus with a 4-day MAP-oestradiol interval
did not have heavier ovaries than those with 3- or 5-day intervals
(pooled 4-day interval data versus pooled 3- and 5-day interval
data t_{20df} = 0.806, N.S.).

Histological changes

Mice treated with MAP of MGA did not ovulate.   Corpora lutea
were usually not present in the ovaries of mice killed 19-22 days
after treatment and were usually small and regressing 7-8 days after
MAP treatment.   The presence of large healthy corpora lutea were
confirmed in the ovaries of mice seen to have 'active' corpora with
a dissecting microscope.   The majority of all large follicles were
degenerating (Fig. 1) and all ovaries contained abundant interstitial
tissue.

The uterine stroma from mice killed 19-22 days after MAP
treatment, irrespective of whether they contained live foetuses or not,
was uniformly vacuolated with a low density of small stromal cells
with dark staining angular nuclei (Figs. 2 & 3).

Mice with live foetuses at autopsy showed some development of
mammary tissue (Figs. 4 & 5) while this was absent from mice with
embryos that died early in pregnancy (Fig. 6).

DISCUSSION

The injection of MAP (or MGA) during oestrus in the present experiments usually did not activate the cyclic corpora lutea of mice so that they became pseudopregnant. In rats however progestins, oestrogens, prolactin, androgens and a variety of chemicals can induce pseudopregnancy if injected during or beginning at oestrus (see review by Everett, 1966). The proportions of eggs that implant following transfer to pseudopregnant rats varies with the hormonal method of induction used (Banik and Ketchel, 1965; Mantalenakis and Ketchel, 1966). However the latter workers found that injection of MAP at oestrus and metoestrus was as effective a method for the induction of pseudopregnancy as mating with vasectomised males in terms of the proportion of transferred eggs developing to foetuses. In the present study the most suitable scheme both for the induction of deciduomata and for foetal development after egg transfer was the injection of MAP (or MGA) during oestrus followed by the injection of oestradiol four days later. The injection of MAP at other cycle stages was less successful, though injection during proestrus allowed the development of some viable full-term foetuses whereas treatment during metoestrus did not.

Priming oestrogen

The importance of injecting the progestins during oestrus (and to a lesser extent MAP during proestrus) on subsequent uterine sensitivity suggested that the time of their action relative to the release of ovulatory oestrogen was important. This was confirmed by the improvement in subsequent uterine sensitivity after the simultaneous treatment of metoestrus mice with MAP and 0.5μg of

oestradiol.   The endogenous release of oestrogen at ovulation
normally 'primes' the uterus for subsequent progesterone action
during normal pregnancy and pseudopregnancy.   Weichert (1928)
first demonstrated that deciduomata could be induced in ovariectomised
rats given 'corpus luteum extract' after oestrogen priming.   Priming
with oestrogen has since been demonstrated to be necessary for
maximal deciduoma formation in ovariectomised rats (reviewed by
De Feo, 1967) and mice (Finn, 1966) treated with progesterone.
However with regard to the implantation of transferred eggs in
ovariectomised nonpregnant mice and rats the situation is less clear.
Humphrey (1969) found that oestrogen priming lead to a slight but
nonsignificant increase in the number of eggs implanting in mice, and
Dickmann (1967) found that fewer eggs developed to mid-term foetuses
following transfer to rats treated with progesterone for five days
after ovariectomy during oestrus or proestrus than during the third
day of diestrus.

The low quantitative deciduoma response following the treatment
of proestrus mice with both MAP and oestradiol may have resulted
from the priming dose of oestrogen being too large.   As endogenous
levels of oestrogen are high during proestrus, the size of the
priming dose of oestrogen probably affects subsequent sensitivity.
Finn (1966) observed that smaller deciduomata were formed when the
priming oestrogen was reduced and Bloch (1968) reported that
implantation could be induced on the 5th day (the normal time in
nonlactating mice) by injecting oestrogen at the time of ovulation
in post parturient mice in addition to removing the litter.   Neither
the injection of oestrogen nor the removal of the litter, alone was
sufficient.

The MAP-oestradiol interval length

Transferred blastocysts have to be placed within the recipient uteri on the 3rd or 4th day of pregnancy or pseudopregnancy if they are to implant successfully (McLaren and Michie, 1956). The yield of live embryos was highest after transfer of $3\frac{1}{2}$ day post coitum (pc) eggs into uteri of $2\frac{1}{2}$ day pc recipients. Approximately half this yield was obtained with synchronous experiments using $2\frac{1}{2}$ day and $3\frac{1}{2}$ day pc eggs and recipients. Nonspecific deciduomata often result after the $3\frac{1}{2}$ day pc synchronous transfers and in addition eggs are thought to require time to recover from the 'shock' of the operation during which they fall behind the relative age of a synchronous uterus. Uteri of mice have also been demonstrated to be most sensitive to intraluminal inducers on the 4th day of pseudopregnancy (Finn, 1965; Hetherington, 1970). Thus uterine receptivity (for eggs or intraluminal stimuli) is lost at the time of maximum sensitivity. Studies with progesterone-treated mice that have been hypophysectomised (Bindon and Lamond, 1969) and ovariectomised (Finn, 1965; Humphrey, 1967b; Grant, 1968) suggest that a gonadotrophin release around 72 hours pc induces an ovarian oestrogen release about eight hours later that sensitizes the uterus for implantation.

In the present experiments the 4-day MAP-oestradiol interval between the progestin injection and the sensitizing dose of oestradiol gave the best results for deciduomata induction (Table 3) and 'implantation' (Table 8). The next best was the 3-day interval where the proportion of eggs that developed to full-term foetuses was not significantly lower than the 4-day interval. Intervals of two and five or more days were too short and too long, respectively. Relative to the timing of events during normal pregnancy, the

closest experimental approximation was with the 3-day interval, that is equating the oestrus cycle stage during which MAP was injected as being equivalent to the 1st day of pregnancy. For this interval, the eggs were transferred on the '3rd day of pregnancy' and the sensitizing oestrogen given on the '4th day'. Uterine receptivity associated with maximal sensitivity after the 4-day interval would suggest an extension of receptivity of about one day relative to normal pregnancy. The extension of receptivity has been observed with egg-implantation and deciduoma induction in ovariectomised nonpregnant mice. The duration of progesterone treatment given immediately after priming oestrogen that was most suitable for the implantation of transferred eggs was three or four days (Humphrey, 1969). Similarly Finn (1966) found that at least two and preferably four or five days of progesterone treatment were required before the injection of sensitizing oestrogen and intraluminal oil for deciduomata induction. Thus priming oestrogen may have set the limits for the duration of uterine receptivity. However in the present study there was a complicating factor of a possible endogenous oestrogen release occurring before the dose of sensitizing oestrogen with the 4-day MAP-oestradiol interval.

Ovariectomy extended the duration of uterine receptivity to air as a decidualizing stimulus. That is, a higher proportion of ovariectomised than intact mice responded when the MAP-oestradiol interval was five days (Table 4). The duration of uterine sensitivity for deciduomata induction was also extended in ovariectomised rats (De Feo, 1967). This difference between intact and ovariectomised mice together with other evidence suggests that the ovaries may have been secreting oestrogen despite treatment with MAP.

Treatment of mice that had been ovariectomised on the 5th - 8th day with MAP alone rarely permitted foetal survival to term (Grant, 1972d). Daily oestradiol treatment in addition to MAP was usually necessary. However in the present work full foetal development usually occurred in mice with no corpora lutea at autopsy. Although MAP acts to suppress gonadotrophin synthesis and release in the rat (Logethlo-poulous, Sharmer and Kraicer, 1961; Sulman and Danon, 1963; Schally, Carter, Saito, Arimura and Bowers, 1968; Labhsetwar, 1966;1969), it was about 50 times less active than oestrone in causing a 50% inhibition of ovarian weight gain in the intact female mouse of an ovariectomised-intact parabiotic pair over a 10-day test (Miyake, 1961). Oestrone was rated about 25 times as active in causing inhibition of ovulation in the rabbit (Pincus, 1965). Thus the large follicles in the ovaries of mice given MAP (Fig. 1) probably derived stimulus from released gonadotrophin and may have secreted oestrogen. Further the relatively high incidence of false implantation sites (i.e. 15.6%) in mice with a 4-day MAP-oestradiol interval could suggest that the uteri of these mice had already been sensitized. The 'false' sites were nonspecific deciduomata probably induced during the transfer of eggs. And uterine sensitivity at this time would presuppose a previous oestrogen release. Also both oestrogen and progesterone were required for maximal deciduoma response in the ovariectomised rat (Yochim and De Feo, 1962; 1963). Although the differences were not significant the quantitative deciduoma response was consistently higher in intact than in ovariec- tomised mice (Table 4). Finally neither 2.5 nor 5.0mg of MAP injected on the 1st day of pregnancy in intact mice delayed implan- tation swellings when mice were autopsied later on during pregnancy

(Grant, 1972d).   Evidently release of the oestrogen required for

egg implantation was not inhibited by these doses of MAP.   In

similarly treated rats MAP delayed implantation (Barnes and Meyer,

1964; Nutting and Sollman, 1967).

Thus it is probable that the treatment of intact cyclic mice

with progestins did not abolish endogenous oestrogen release.

Furthermore it is probable that more endogenous oestrogen was

released by the 5th than the 4th day after progestin treatment as the

incidence of 'false implantation' sites was higher with the 4-day

than with the 3-day MAP-oestradiol interval.

Maintenance of pregnancy

MAP and MGA are potent long acting progestins.   On the basis of

pregnancy maintenance in ovariectomised rats MAP is 20-50 times as

active as progesterone on a weight for weight basis (Stucki, 1958;

Revesz and Chappel, 1966).   On the same basis Duncan, Lyster,

Hendrix, Clark and Webster (1964) found that MGA was approximately

three times as potent as MAP.

The doses of 2.5mg of MAP and 1.0mg of MGA were sufficient to

maintain an entire pregnancy in mice.   This amount of MAP stopped

oestrous cycle activity in mice for about 47 days (Grant, 1972d).

Ovarian weight was not correlated with uterine sensitivity or with

the proportion of swellings that gave rise to foetuses.   Most

ovaries of pregnant mice did not contain visible corpora lutea, nor

were these found on histological sections.   Therefore 'pregnancies'

were maintained and the deciduomata were supported by the activity

of the exogenous progestins.   It was apparent however that after

MAP treatment the few mice with 'active' corpora lutea at autopsy,

had uteri that were more normal in appearance than 'pregnant' mice

without corpora lutea.   The absence of corpora lutea in MAP-treated
mice was associated with hypertonic uteri.   Foetuses were often
compressed and sometimes two foetuses were found in the same uterine
chamber with the smaller of the two often dead.   A similar increase
in uterine tonicity associated with pressure-induced foetal deformities
has been described in ovariectomised rats and mice whose pregnancies
were being maintained with progesterone (Carpent, 1962; Grant, 1968).
The concurrent daily administration of 2.5ng of oestradiol benzoate
and 2.0mg of progesterone reduced the degree of uterine hypertonicity
observed after progesterone treatment alone (Grant, 1968).   MAP
given to mice ovariectomised on the 7th day of pregnancy usually only
allows full foetal development of oestradiol if administered daily
(Grant, 1972d).   However uteri were also hypertonic at autopsy on
the 20th day of pregnancy after daily treatment with 37.5ng or
60.5ng of oestradiol.   Thus in the present experiment it is con-
sidered that uterine hypertonicity was due not to the relative absence
of oestrogen but to the absence of nonprogestin secretion(s) from
the luteinized ovaries.

Foetal survival

Foetal survival was always below that expected from the
implantation rates.   This discrepancy was more pronounced when MAP
was given during proestrus and after the 5-day MAP-oestradiol
interval than when MAP (or MGA) was given at oestrus with the 4-day
interval (see Tables 6 and 8).   Although part of this discrepancy
was due to the occurrence of 'false implantation' sites the
incidence of these was highest when the 'implantation' rate was
highest.   The size at autopsy of most of the dead implantation sites
in groups with the highest embryonic mortality suggested that they

62.

probably contained embryos. A comparison of the quantitative deciduomata and 'implantation' responses (Table 1 cf Table 6 and Table 3 cf Table 8) indicates that embryonic survival was highest when uteri were most sensitive to injected air. This suggests that the proportion of embryos surviving was a more sensitive indicator of uterine sensitivity than the rate of 'implantation'. Likewise the relatively high rate of embryonic mortality after MAP (or MGA) treatment during oestrus with the 4-day MAP (or MGA) interval is attributed to asynchrony between the time of egg transfer and the hormonal state of the uteri (as evidences by the occurrence of 'false implantation' sites). Mantalenakis and Ketchel (1966) obtained an implantation rate of 66% after egg transfer to rats on the 5th day of eight days of progesterone treatment starting during oestrus. Only 7% of the eggs transferred developed to viable young, however the authors did not state whether or not these rats remained pseudopregnant.

MAP toxicity per se was an improbable cause of high embryonic mortality as treatment of intact mice with 5.0mg on the 1st day of pregnancy or 2.5mg of MAP at intervals from the 1st to the 18th day did not adversely affect foetal survival on the 20th day of pregnancy (Grant, 1972d). Treatment of intact mice with MGA on the 7th to the 9th day of pregnancy also did not increase foetal mortality. However treatment of mice during early pregnancy with a single dose of 0.5mg of MAP aften lead to its interruption (i.e. abortion late in pregnancy or failure of mice to remain pregnant) (Grant, 1972d). This was not interpreted as evidence for MAP being embryo-toxic but for it acting as a luteolytic agent, with its own progestin activity being insufficient to maintain pregnancy.

Burdick (1942) has observed that corpora lutea fail after progesterone treatment early in pregnancy in the mouse.

The use of this hormonal method for the induction of uterine receptivity for egg transfers has the disadvantages of low foetal survival rates and the need for progestin injections at a precise stage of the oestrous cycle. The low survival rate probably resulted from an endogenous oestrogen release that interferred with uterine sensitization induced by exogenous oestradiol. The need for precise timing of the progestin injection is also probably related to this oestrogen release and to the duration of the interval between the release of ovulatory or 'priming' oestrogen and the transfer of eggs. MAP would seem less effective in suppressing gonadotrophin release in mice than in rats and this may be one reason why it did not induce pseudopregnancy in mice. This failure to effectively block gonadotrophin release could perhaps be overcome by the concurrent administration of a low dose of oestrogen insufficient to cause uterine sensitization.

EXPLANATION OF THE PLATE

Figs. 1-6 are photomicrographs of histological sections stained with haematoxylin and eosin.

Fig. 1.   A large Graafian follicle showing early degenerative changes (pyknotic granulosa cells) from the ovary of a mouse 19 days after treatment with 2.5mg of MAP during oestrus with a 4-day MAP-oestradiol interval.   One of five transferred eggs developed to a 'full-term' viable foetus.   This ovary contained no corpora lutea.   x219.

Fig. 2.   Vacuolated uterine stroma with small angular nuclei 19 days after treatment with 2.5mg of MAP during dioestrus with a 4-day MAP-oestradiol interval.   The one uterine swelling seen at laparotomy had disappeared by the time of autopsy.   x219.

Fig. 3.   Higher power view of the uterine stroma shown in Fig. 2.   x438.

Fig. 4.   Secretory mammary gland tissue 20 days after treatment with 2.5mg of MAP during oestrus with a 4-day MAP-oestradiol interval.   Four (of five) transferred eggs developed to 'full-term' viable foetuses.   (s = secretion).   x219.

Fig. 5.   Mammary gland tissue from the mouse whose ovary is shown in Fig. 1.   The ratio of glandular to nonglandular adipose tissue is lower than in Fig. 4.   x219.

Fig. 6.   Mammary gland tissue 19 days after treatment with 2.5mg of MAP during oestrus with a 5-day MAP-oestradiol interval.   Five uterine swellings were seen at laparotomy, however there were no live foetuses present at autopsy.   The ratio of glandular to nonglandular tissue was considerably lower than in Fig. 4 or in Fig. 5.   There was no evidence of secretion.   x219.
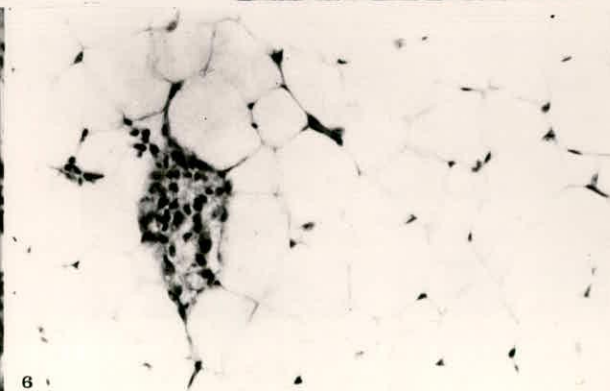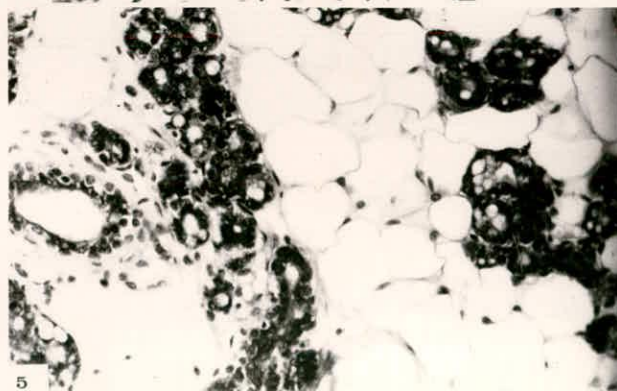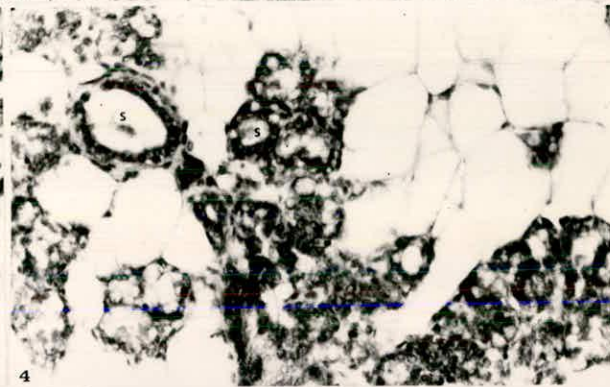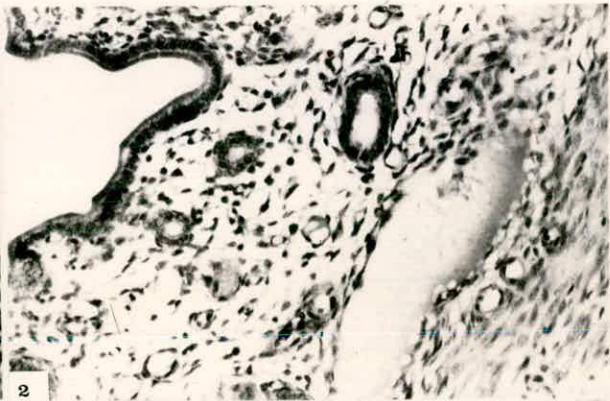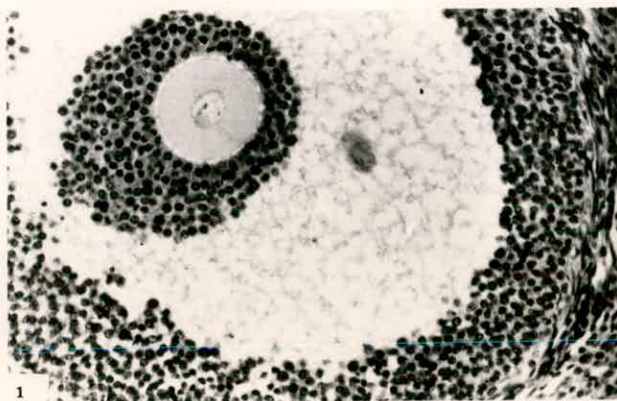
THE EFFECT OF TREATMENT OF PREGNANT MICE WITH MEDROXYPROGESTERONE

ACETATE AND MELENGESTROL ACETATE ON THE SUBSEQUENT

REPRODUCTION OF THEIR DAUGHTERS

ABSTRACT

Intact pregnant Q-strain mice were given either medroxyproges-
terone acetate (MAP) or melengestrol acetate (MGA) as a single sub-
cutaneous injection. Other mice were ovariectomised on the 5th-8th
day of pregnancy and immediately given a single dose of MAP or MGA.
Some of these mice were injected daily with oestradiol until the 20th
day of pregnancy. The daughters of these mice and the daughters of
mice prepared as recipients for transferred blastocysts by an
injection of MAP and oestradiol were examined for genital abnormal-
ities and their subsequent reproductive life was studied.

The injection of MAP (0.5 or 2.5mg on or before the 5th day of
pregnancy) interrupted pregnancy (i.e. mice were not pregnant or had
aborted before the 20th day) in a minority of mice. The majority of
mice given MAP (2.5mg) or MGA (0.5 or 1.0mg) had prolonged gestation
periods. Three of 13 mice given 0.5mg of MGA gave birth unassisted
on the 20th day. In other mice young were recovered by caesarean
section. MAP (0.5, 2.5 or 5.0mg) given before the 5th day of
pregnancy probably did not delay egg-implantation.

Ovariectomised mice usually required daily oestradiol in addition
to an injection of MAP or MGA before foetuses survived to the 20th
day of pregnancy.

Daughters of all classes of MAP-treated mice regardless of the
time of injection usually had abnormal external genitalia. The
incidence of these abnormalities in daughters of MGA-treated mice
was not significantly higher than seen in normal mice. The external
genitalia of MAP daughters ranged from normal to very abnormal and
the degree of abnormality was subjectively classified according to

the position of the urethral orifice, the size and shape of the
genital papillae, and the size of the vaginal orifice.

The reproductive history of the MAP daughters was highly
variable and usually not related to the method of treatment of their
mothers with MAP.   A possible exception was that the daily dose of
37.5ng of oestradiol following 2.5 or 5.0mg of MAP after ovariectomy
may have countered in part, some of the pathological effects of MAP.
Abnormal reproductive behavioural features displayed by the MAP
daughters included one or more of the following:

1.   Failure to mate (this was sometimes physically impossible due to
     some females having a very small common urethro-vaginal orifice).

2.   Retention of vaginal plugs for several days.

3.   Failure to become visibly pregnant after mating.

4.   Failure to litter after becoming visibly pregnant (i.e. foetal
     resorption and abortion).

5.   Highly variable litter size perhaps in some cases the result of:

6.   Bizarre maternal behaviour.   Postnatal young were sometimes
     stunted and up to the age of at least seven days were liable to
     be cannabalized.

7.   Mice that were markedly abnormal were also highly susceptible to
     reproductive tract infections.

The reproductive history of MGA daughters was probably not
different from that of normal mice.

The weight of the clitoral glands from MAP daughters was often
higher than in normal mice and was positively correlated with the
degree of abnormality of the external genitalia.   This correlation
was nonsignificant for normal mice and for MGA daughters.   Further
the ovarian weight was correlated with the clitoral gland weight in

both MAP and MGA daughters.   This correlation was nonsignificant in

normal mice.   Testosterone proprionate treatment caused a marked

increase in clitoral gland weight in normal mice.   Cystic ovaries

were often seen in MAP and less often in MGA daughters.

The correlation between clitoral gland weight and ovarian

weight may suggest that the ovaries were secreting androgens in both

MAP and MGA daughters.   The abnormalities of the external genitalia

seen in MAP daughters are consistent with androgen-induced changes

described in the literature.

The fertility of the sons of MAP or MGA treated mothers was not

adversely affected.

INTRODUCTION

(MAP)   (MGA)

Medroxyprogesterone acetate/and melengestrol acetate/are both potent, long-acting synthetic progestins. MAP was approximately twenty times as potent as progesterone (on a weight for weight basis) in the maintenance of pregnancy in ovariectomised rats (Stucki, 1958; Revesz and Chappel, 1966). By the same test melengestrol acetate was approximately three times as potent as medroxyprogesterone acetate (Duncan, Lyster, Hendrix, Clark and Webster, 1964).

The treatment of pregnant rats with medroxyprogesterone acetate has produced masculinizing changes in their daughters (Revesz, Chappel and Gaudry, 1960; Revesz and Chappel, 1966; Suchowsky, Turolla and Acari, 1967). These changes were not observed after the treatment of pregnant rats with melengestrol acetate (Duncan et al., 1964).

Although these compounds have the advantage over progesterone of requiring fewer injections for a given effect, their usefulness will depend on the incidence of harmful side-effects. In the present study, the effect of these compounds on pregnant mice and their offspring is examined.

MATERIALS AND METHODS

Mice of the Q strain were kept in artificial lighting; $16\frac{1}{2}$ hours light and $7\frac{1}{2}$ hours dark (centred on 01.30 hrs) and the temperature was thermostatically controlled at approximately 23°C. Vaginal smears were taken daily from virgin females and interpreted after Bronson, Dagg and Snell (1966). During metoestrus-1 (M-1) (the day following oestrus) some mice were injected subcutaneously with

2.5mg of progesterone (Koch-Light) in 0.1ml of arachis oil, 0.1ml of oil, 0.1mg, 0.5mg or 2.5mg of 6α-methyl-17α-acetoxyprogesterone (MAP, 'Depoprovera'; Upjohn) in 0.1ml of vehicle (diluted with water, except 2.5mg which was injected in 0.05ml of vehicle). One group was injected with 0.5mg of MAP without regard to the stage of the oestrus cycle. One day following the injections mice were placed with fertile males.

Other virgin females were put with fertile males and checked daily. The day on which a vaginal plug was found was called the 1st day of pregnancy. Synthetic progestin hormones were injected subcutaneously once only, on a selected day of pregnancy. On one of the first five days of pregnancy mice were injected with 0.5mg (in 0.1ml of vehicle plus water), 2.5mg or 5.0mg of MAP (in 0.05ml and 0.1ml of vehicle, respectively). On one day from the 5th-18th day of pregnancy other mice were injected with 2.5mg of MAP. Another group were injected on the 7th, 8th or 9th day of pregnancy with 0.5mg or 1.0mg of 17α-acetoxy-6-methyl-16-methylenepregna-4, 6-diene-3, 20 dione (melengestrol acetate, MGA; Upjohn) in 0.1ml of 2% methyl-cellulose.

Other mice were bilaterally ovariectomised on the 5th, 6th, 7th or 8th day of pregnancy. Immediately after the operation mice were either injected with 2.5mg or 5.0mg of MAP or following ovariectomy on the 7th day of pregnancy four mice were injected with 1.0mg of MGA. In addition to MAP or MGA some of the mice ovariec- tomised on the 7th day of pregnancy were injected daily for 12 days with 4.5ng, 37.5ng or 60.5ng of oestradiol-17β (Koch-Light) in 0.1ml of arachis oil.

Mice were checked regularly throughout the remainder of pregnancy

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and daily after the 19th day.   Most mice were anaethetised with

ether on the 20th day of pregnancy and the young were removed by

casesarean section.   Young were revived by warming and usually by

gassing with 95% O_2 5% CO_2 and then put with foster-mothers.   The

mothers were usually killed and if intact, their ovaries were

checked for corpora lutea and then fixed in formol-saline or Bouin's

fluid along with a piece of mammary gland tissue taken from the

inguinal region.   A minority of the mothers were allowed to recover.

Young mice were studied from mothers that had been injected

with MAP or MGA during pregnancy, following ovariectomy during

pregnancy, and from recipients prepared for egg transfers with MAP

and oestradiol (see Grant, 1972c).   They were weaned at 28 days

of age.   Periodic examinations were made of their external genitalia

and females were placed with normal fertile males when aged between

44-72 days and thereafter checked regularly.   Males, after they

had reached three months of age, were placed with normal females.

At autopsy some females were weighed and their external

genitalia were assessed on a subjective scale from normal (0) to

abnormal (1-5) according to the following criteria.

0.   The urethral orifice normally opens at or near the tip of

the genital papilla (GP) (or clitoris).   This was often slightly

forked with the orifice in the fossa of the cleft.   The modified

skin that covered the GP fused to form a medium raphae on its

dorsum.   Viewed from above or below the GP was cone-shaped in outline.

1.   The urethral orifice opened on the dorsum of the GP.   The

open urethra was represented by a narrow hyperaemic groove flanked

on either side by mucous membrane.   The modified skin did not fuse

medially to form a raphae.   The GP was shaped like a truncated

cone and sometimes enlarged (Pl. 1, Fig. 1).

2. The urethral orifice opened at or anterior to the vaginal
orifice (i.e. these mice were hypospadic). The open urethra formed
a prominent medial hyperaemic groove (Pl. 1, Fig. 2). The mucous
membrane was sometimes confined to the periphery of the dorsum of the
GP (with the modified skin around its periphery) which was bright
red in colour and bulged dorsally (i.e. the urethral-groove was
absent). The GP was as in 1.

3. As for 1 or 2 except that the GP was mis-shapen and contained
macroscopically visible papillae in the cleft of the forked tip.
The GP was square in outline.

4. As for 3 except that the GP was usually enlarged with
prominent papillae. In mice with a common-urethral orifice this
was often slightly smaller than usual (Pl. 1, Fig. 2).

5. These mice were always hypospadic with moderate to marked
stenosis of the common urethro-vaginal orifice. The GP was some-
times enlarged with papillae and usually square in outline.

The clitoral (preputial) glands and ovaries from these mice
were dissected and weighed. Selected reproductive organs from
normal and abnormal mice were fixed in Bouin's fluid for histology.

Tissue for histology was embedded in paraffin wax, sectioned
at 6μ and stained with haematoxylin (Harris's) and eosin.

Data showing continuous variation were analyzed with analysis
of variance or with correlation and regression techniques (Snedecor,
1956). Results were significant if the probability of their
having arisen by chance alone, was equal to or less than 5%.

RESULTS

Suppression of the oestrous cycle by the injection of progestins (Table 1)

Neither 2.5mg of progesterone nor 0.1mg of MAP significantly extended the time taken for mice injected at M-1 to mate (determined by the presence of a vaginal plug). MAP (0.5 and 2.5mg) injected at this time significantly extended the time relative to oil (by observation). MAP (0.5mg) given at M-1 resulted in a shorter interval to mating than the same dose given without regard to the oestrous cycle stage. Two (out of six) mice injected with 2.5mg of MAP on M-1 had not mated at the conclusion of the experiment and this dose was clearly the most effective at suppressing cyclic behaviour.

The subsequent reproductive behaviour of the five mice given 0.5mg of MAP without regard to the cycle stage was abnormal. The mean interval from the date of the vaginal plugs until the birth of the first litters was 38.6 days. The mean size of thr first and second litters (when seen up to three days after birth) was 5.4 and 8.2, respectively. Both litters (especially the first) were progressively reduced in size due to the mothers eating their young. The survivors had had their tails and limbs chewed.

The effect of MAP injection prior to and at the time of implantation in pregnant mice (Table 2)

Injections of MAP up to and including the 5th day of pregnancy always interferred with the duration of pregnancy. The smallest dose (0.5mg) either caused unobserved failure of pregnancy, abortion of late foetuses or prolonged the gestation period. The inter-

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TABLE 1.

SUPPRESSION OF THE OESTROUS CYCLE BY THE SUBCUTANEOUS INJECTION OF PROGESTINS, THE TIME (IN DAYS) [*]

FROM INJECTION OF THE PROGESTIN UNTIL MATING (A) AND SUMMARY OF ANALYSIS OF VARIANCE (B). [**]

A. Treatment	MAP (mg)				Progesterone	Arachis Oil
	0.1	0.5	0.5(a)	2.5	(2.5mg)	(0.1ml)
Number of mice	6	6	5	6	5	5
Mean No. days until mating	5.50	14.00	8.40	42.83(plus) [+]	5.00	3.00
(Standard error of mean)	±0.33	±1.25	±0.81	±7.82	±0.73	±0.04

B. Analysis of variance
   (excluding 2.5mg MAP)

Source	df	Mean squares
Between treatments	4	103.40
Residual	22	17.58

Comparisons: Oil vs 2.5mg of progesterone   N.S.

  " vs 0.1mg "   MAP   N.S.

0.5mg MAP (M-1) vs 0.5mg "   " (a) $P < 0.05$

[*]   MAP injected as Depoprovera (Upjohn) in 0.1ml of vehicle diluted with water; except 2.5mg which was injected in 0.05ml of vehicle.   Progesterone injected in 0.1ml of arachis oil.

[**]   Injection at metoestrus-1; except for 0.5mg(a) which was injected without regard to the cycle stage.

[+]   2 of the 6 mice had not mated at the conclusion of the experiment.

TABLE 2.

THE EFFECT OF MAP INJECTED PRIOR TO AND AT THE TIME OF IMPLANTATION IN PREGNANT MICE ON THE RATE OF EMBRYONIC DEVELOPMENT AND ON THE LENGTH OF GESTATION PERIOD.

Dose of MAP and the day of injection	No. of 'pregnant' mice			No. of foetuses 'born' spontaneously			No. of mice with prolonged gestation (Foetal viability on the 21st day)
	injected	(later pregnant)	aborting	alive	dead	their fate	
0.5mg 1st day of preg.	4	(3)	2	5(20th day)	15(19th day)	live foetuses eaten over 3 days	0
+ 2.5mg 1st day of preg.	2	(2)	0	Autopsy on the 16th day, mean foetal wt. 827.600, n = 25 " " " " " " placental " 387.840, n = 25			
+ Normal mice	-	(2)	0	" " " " " " " foetal wt. 855.826, n = 23 " " " " " " " placental " 377.454, n = 23			
5.0mg 1st day of preg.	2	(2)	0	0	0	-	2(21 alive & 1 dead)
5.0mg 1st day of preg.	4	(4)	0	} Autopsied from the 9th-13th day. Size of implantation sites as expected in normal pregnant mice.			
0.5mg 3rd day of preg.	4	(4)	0				
2.5mg 3rd day of preg.	4	(4)	0	0	25(20th-22nd day)	-	4 (Incomplete parturition in 3 mice, dead foetuses retained
0.5mg 4th day of preg.	1	(1)	0	0	0	-	1(9 dead on 21st day)
0.5mg 5th day of preg.	4	(0)					
2.5mg 5th day of preg.	4	(3)	1	?	?(15th day)	immediately eaten	2(? dead on 21st day)

* Day of finding the vaginal plug.

+ Foetal and placental weights not significantly different.

mediate dose (2.5mg) usually prolonged the gestation period though after injection on the 5th day one mouse aborted on the 15th day of pregnancy. The highest dose (5.0mg) always delayed parturition.

The injection of MAP on or before the 4th day of pregnancy probably did not delay implantation. Foetal and placental weights from normal mice and mice injected with 2.5mg of MAP on the 1st day were not signnificantly different (after analysis with the 't test') on the 16th day of pregnancy. Further the size of implantation sites in mice given 5.0mg of MAP on the 1st day were not smaller than normal sites of the same age when the mice were autopsied from the 9th-13th days of pregnancy.

The effect of the treatment of pregnant mice with a single injection of 2.5mg of MAP (Table 3)

Parturition was delayed in all MAP injected mice and young were removed by caesarean section on the 20th day of pregnancy. Except for two (out of five) mice injected with MAP on the 6th day of pregnancy all injected mice remained pregnant. The number of viable young in utero on the 20th day was not different from the number of young seen immediately after birth of primiparious normal stock Q females (means of 10.7 and 9.6 young/litter, respectively, each for 22 litters). The number of daughters successfully reared after fostering and the number of these with abnormal genitalia are shown in Table 3. Daughters with some degree of external genitalia abnormality were seen from mothers injected on all of the days from the 5th to the 18th day of pregnancy. The degree of abnormality was highly variable, even among sisters.

Corpora lutea were usually greyish coloured on the 20th day of

TABLE 3.

THE EFFECT OF THE TREATMENT OF PREGNANT MICE WITH MAP ON THE EXTERNAL GENITALIA AND SUBSEQUENT

REPRODUCTION OF THEIR DAUGHTERS

Pregnant mice given 2.5mg MAP on the:	No. pregnant mice	Mean no. viable young in utero on the 20th day	No. daughters reared with abnormal external genitalia (total)	No. that became pregnant	No. that littered	Observed litter size (range)	Days of cohabitation with a fertile male
5th day of preg.	1	8	4 (4)	4	4	7-12	59
6th " " "	2	12.5	3 (4)	-	-	-	0
7th " " "	3	?	8 (9)	7	6	4-9	41-117
8th " " "	1	13	6 (6)	6	6	3-11	70
9th " " "	2	?	11 (11)	6	3	2-11	98 (6 females only)
10th " " "	2	?	6 (7)	6	3	4-9	46-112
11th " " "	2	8.0	4 (4)	-	-	-	0
12th " " "	2	12.0	3 (5)	5	5	1-7	79
13th " " "	3	10.0	5 (5)	5	5	2-11	56-74
14th " " "	2	14, ?	4 (5)	5	5	2-12	83
15th " " "	2	11.5	4 (4)	-	-	-	0
16th " " "	2	?	5 (5)	5	4	4-11	77
17th " " "	2	?	4 (5)	5	5	5-10	73
18th " " "	1	11	3 (5)	5	5	7-9	58

* Reared after recovery by caesarean section on the 20th day of pregnancy.

pregnancy.   Sometimes none were seen.   Mammary development appeared

normal and the mothers that were allowed to recover suckled their

young.

The effect of the treatment of pregnant mice with a single injection (0.5mg or 1.0mg) of MGA (Table 4)

Parturition was delayed in 13 (of 19) mice injected with MGA on

the 7th-9th day of pregnancy.   The young from nine mice were removed

by caesarean section on the 20th day.   The remaining four had

littered spontaneously by the 20th day.

Five (of 19) mice remained pregnant after treatment with MGA.

The mean number of viable young in utero on the 20th day was

normal (9.84) though there was a reduction in litter size following

spontaneous delivery (5.63).

The proportion of the 39 daughters successfully reared that had

abnormal genitalia (i.e. 7.7%) was not different from that proportion

of 166 normal females that had similar abnormalities (i.e. 9.6%).

Further, the degree of abnormality seen in these two groups of mice

was comparable and was less extensive than that observed in MAP

daughters.

Foetal survival after the treatment of ovariectomised pregnant mice with MAP (or MGA), or MAP (or MGA) plus oestradiol (Table 5)

Only one mouse (of 18) given MAP alone after ovariectomy had

live foetuses on the 20th day of pregnancy.   No live foetuses were

seen in two mice given MGA alone.

Six of the 17 and one of two mice given daily oestradiol in

addition to MAP and MGA had live foetuses on the 20th day, respec-

tively.   The proportion of live foetuses from the total number of

TABLE 4.

THE EFFECT OF THE TREATMENT OF PREGNANT MICE WITH MGA ON THE EXTERNAL GENITALIA AND SUBSEQUENT REPRODUCTION OF THEIR DAUGHTERS

Treatment	No. mice injected	No. that remained pregnant	No. with prolonged gestation	No. that littered spontan-eously	Mean No. of viable foetuses on 20th day	No.of daughters reared with abnormal ext. genitalia (total)	No.of daughters that littered after one mating (total)	Observed litter size (range)
0.5mg MGA 7th-9th day of preg.	13	10	10	3	9.63[+]&5.50[*]	3 (27)	8 (8)	7-13
1.0mg MGA " " " "	6	4	3	1	10.33[+]&6.0[*]	0 (12)	7 (7)	6-13

+   Young removed by caesarean section.

*   Young born spontaneously.

TABLE 5.

FOETAL SURVIVAL AND THE REPRODUCTIVE HISTORY OF REMALE OFFSPRING FROM MICE OVARIECTOMISED AFTER IMPLANTATION AND GIVEN MAP (OR MGA) ⊕R MAP (OR MGA) PLUS DAILY OESTRADIOL UNTIL THE 20TH DAY OF PREGNANCY

MAP treatment	No. of mice Total	with live foetuses	Mean litter size (live only)	No. of females reared with abnormal genitalia (total)	Mean degree of abnormality	No. that mated	No. that became pregnant	No. that littered	Observed litter size	Total No. young seen	Total No. weaned	Total No. days cohab. with ♂
OvarX 6th day preg. 2.5mg MAP	5	0										
" 7th " " " "	8	1	8	3(3)	4.3	3	3	0				
" 7th " " 5.0mg "	3	0										
" 8th " " 2.5mg "	2	0										
" 7th " " 2.5mg " + 4.5ng oestradiol daily	4	1	10	3(3)	5.0	1	1	1	4-13	27	27	90
" 7th day preg. 5.0mg MAP + 4.5ng oestradiol daily	2	0										
" 7th day preg. 2.5mg MAP + 37.5ng oestradiol daily	3	2	8.0	2(2)	4.0	2	2	2	1-12	44	38	256
" 7th day preg. 5.0mg MAP + 37.5ng oestradiol daily	4	1	12	3(4)	2.3	4	4	4	1-13	112	105	919
" 7th day peeg. 2.5mg MAP + 60.5ng oestradiol daily	4	2	11.5	3(3)	3.0	1	1	1	7-10	35	34	144
MGA treatment												
OvarX 7th day preg. 1.0mg MGA	2	0										
" 7th " " " " + 37.5ng oestradiol daily	2	1	4	0								

implantation sites was higher for 2.5 than for 5.0mg of MAP with 4.5ng of oestradiol (χ^2 = 2.47, 1df, NS) and with 37.5ng of oestradiol daily (χ^2 = 6.424, 1df, P < 0.02).

The correlation between clitoral gland weight and other parameters (Table 6)

The mean degree of abnormality of the external genitalia was significantly higher (by observation) for the MAP daughters than for normal mice and for MGA daughters. The degree of abnormality of MAP daughters was variable and included mice with normal genitalia.

The clitoral gland weight of MAP treated daughters was higher than in normal mice and was significantly correlated (r = +0.718, $t_{19\ df}$ = 4.506, P < 0.001) with the degree of abnormality of the external genitalia. This correlation was nonsignificant for MGA daughters and normal mice. In normal mice and MGA daughters the clitoral gland weight was significantly correlated with the body weight (r = +0.649, $t_{8\ df}$ = 2.419, P < 0.05 and r = +0.601, $t_{23\ df}$ = 3.611, P < 0.01, respectively). This correlation was nonsignificant for MAP daughters. The clitoral weight was also significantly correlated with the age at autopsy for MGA daughters (r = +0.423, $t_{23\ df}$ = 2.243, P < 0.05). This correlation was nonsignificant for normal mice (for which the age range was considerably smaller than for the MGA daughters) and for the MAP daughters.

The treatment of normal ovariectomised mice with progesterone and testosterone proprionate significantly (by observation) increased the weights of the clitoral glands relative to those of normal untreated mice. Testosterone was probably responsible for this increase as the treatment of ovariectomised mice with 2.5mg of MAP

TABLE 6.

THE EFFECT ON THE WEIGHT OF THE CLITORAL GLANDS OF DAUGHTERS (OF VARIABLE REPRODUCTIVE STATUS) AFTER TREATMENT OF THEIR MOTHERS DURING PREGNANCY WITH MAP OR MGA; WITH CORRELATIONS BETWEEN THE CLITORAL GLAND WEIGHT AND AGE, BODY WEIGHT AND THE DEGREE OF EXTERNAL GENITALIA ABNORMALITY; AND THE EFFECT OF TESTOSTERONE ON CLITORAL GLAND WEIGHT.

	No.of Mice	Mean age (days)*	Mean body wt.(g)	Mean clitoral gland weight (mg)	Mean degree of 'abnormality'	Correlation between clitoral gland weight and		
						Age	Body wt.	Degree of 'abnormality'
Normal mice [+]	10	83.30 ± 9.13	25.90 ± 2.07	3.80 ± 0.42	0	+0.044, NS	+0.649 P < 0.05	–
Daughters of mice treated with MAP	21	180.05 ± 19.41	31.57 ± 1.82	5.79 ± 0.61	2.76 ± 0.39	+0.382, NS	+0.150 NS	+0.718 P < 0.001
Daughters of mice treated with MGA	25	204.00 ± 16.16	31.88 ± 1.06	5.08 ± 0.39	0.36 ± 0.19	+0.423 P < 0.05	+0.601 P < 0.01	+0.247 NS
Ovariectomised normal mice treated daily with 0.75mg of testosterone proprionate [**]	4	60.00 ± 0.00	31.25 ± 0.75	14.125± 0.69	0.25	–	–	–

* ± standard error.

+ Mice from untreated mothers that were not 'abnormal'.

** Ovariectomised on the 3rd day of pregnancy and treated with 2.5mg of progesterone daily in addition to testosterone.

did not increase the weight of their clitoral glands. Furthermore,
the glands of pregnant and lactating mice were not heavier than
expected.

The correlation between the ovarian weight and other parameters (Table 7)

Ovarian weight of MAP daughters was significantly correlated
($r = +0.682$, $t_{14\ df} = 3.495$, $P < 0.01$) with the degree of abnormality
of the external genitalia. This correlation was nonsignificant for
the MGA daughters and for normal mice.

The ovarian weight of both MAP and MGA daughters was signific-
antly correlated with the clitoral gland weight ($r = +0.569$, $t_{14\ df} =
2.591$, $P < 0.05$ and $r = 0.44$, $t_{21\ df} = 2.276$, $P < 0.05$, respectively).
This correlation was nonsignificant in the normal mice. The
ovarian weight was also correlated with the age for the normal mice
and for MAP daughters ($r = +0.698$, $t_{7\ df} = 2.585$, $P < 0.05$ and
$r = +0.723$, $t_{14\ df} = 3.922$, $P < 0.01$, respectively). This correlation
was nonsignificant for the MGA daughters.

The reproductive behaviour of MAP daughters (see Tables 3, 4 & 5)

Irrespective of the method or time of treatment, all mice
treated with MAP during pregnancy had daughters with a variable
degree of external genitalia abnormality. Likewise the reproductive
performance of these mice varied.

Although the numbers were small, treatment of ovariectomised
mice with daily oestradiol in addition to MAP lessened the degree of
abnormality and improved the subsequent reproductive performance
relative to females in the litter for the one mouse given MAP alone
(Table 5). The intermediate dose of oestradiol (i.e. 37.5ng) was

TABLE 7.

THE EFFECT ON THE OVARIAN WEIGHT OF DAUGHTERS (EXCLUDING PREGNANT OR SUCKLING
MICE) AFTER TREATMENT OF THEIR MOTHERS DURING PREGNANCY WITH MAP OR MGA;
WITH THE CORRELATIONS BETWEEN THE OVARIAN WEIGHT AND AGE, BODY WEIGHT,
CLITORAL GLAND WEIGHT AND THE DEGREE OF EXTERNAL GENITALIA ABNORMALITY.

	Normal Mice	Daughters of MAP treated mothers	Daughters of MGA treated mothers
No. of mice	9	16	23
Mean age (days)	84.78 ± 10.07	192.75 ± 24.01	213.91 ± 15.93
Mean body wt. (g)	24.00 ± 0.93	31.06 ± 2.12	32.48 ± 1.07
Mean clitoral wt. (mg)	3.61 ± 0.42	5.50 ± 0.73	5.22 ± 0.42
Mean ovarian wt. (mg)	12.11 ± 1.72	13.91 ± 1.08	16.15 ± 1.29
Mean degree of abnormality	0	2.94 ± 0.47	0.39 ± 0.21
Correlation between ovarian wt. and:			
Age	0.698 P < 0.05	+0.723 P < 0.01	+0.261 NS
Body weight	+0.231 NS	+0.428 NS	+0.257 NS
Clitoral weight	+0.304 NS	+0.569 P < 0.05	+0.444 P < 0.05
Degree of abnormality	–	+0.682 P < 0.01	+0.057 NS

* ± standard error.

better in this regard than 4.5ng or 60.5ng.

However, the reproductive performance of most MAP daughters was abnormal. The degree of abnormality was variable and not invariably related to the degree of external genitalia abnormality. MAP daughters produced fewer young over a given period than normal mice for one or more of the following reasons.

1). Failure to mate. This was physically impossible for some mice due to their very small common urethro-vaginal orifices. These mice showed no externally visible cyclic vaginal changes. Their external genitalia abnormality rating was 5.

2). Retention of vaginal plugs. Normal mice usually lose their vaginal plugs on the 1st day of pregnancy and rarely on the 2nd. Some MAP daughters retained plugs for at least four days. Occasionally abnormal mice (types 4 and 5) were damaged during mating; e.g. blood soaked vaginal plugs were recovered and the autopsy data suggest that some vaginae may have been ruptured. The vaginal walls of these mice appeared pliable and thin. Retained vaginal plugs were moulded to the shape of the anterior vagina (they were shaped rather like wooden clothes pegs).

3). Failure to become pregnant (visibly or by palpation) after finding a vaginal plug. These mice often displayed vaginal plug retention.

4). Failure to litter after becoming pregnant (visibly and by palpation). This was a common failing and affected mice with a variable range of genital abnormalities. Laparotomies confirmed that pregnancies failed due to the resorption and abortion of embryos after the 12th day of pregnancy.

5). Highly variable litter size (see Tables 3 & 5). Infantophagia

77.

was in part responsible and it is probable that the number of

young born varied widely.   As at laparotomy late in pregnancy,

dead implantation sites of variable size were seen.   Although some

young were stunted, healthy ones were occasionally eaten up

to the age of at least seven days.

6).   Mice with type 5 genital abnormalities were more than usually

susceptible to reproductive tract infections.

The reproductive behaviour of MGA daughters

This was probably not different from that of normal mice

(see Table 4).

The fertility of sons of MAP-treated mothers

Individual sons from mothers treated with 2.5mg of MAP on one

day from the 6th-18th day (excepting the 7th day) of pregnancy and

from a mother given 5.0mg of MAP sired at least one litter.

Changes in the internal reproductive organs of MAP daughters

At autopsy gross pathological changes were usually associated

with type 5 external genitalia abnormalities.

(i) The vagina and uterus

The vaginal walls were pliable, appeared thinner than normal and

were easily ruptured by a probe.   Unless directed along the roof of

the vagina in severely abnormal mice, the probe would usually enter

the pelvic urethra.   Occasionally in these mice intact septae were

present that divided the vaginal into anterior and posterior fluid

filled compartments.   (It is not known if these septae were

developmental faults or if they resulted from healed vaginal ruptures

after copulation).   These compartments and 'vaginal cysts' often

78.

contained viscous cloudy fluid.   Cysts and abscesses were sometimes attached to the outer vaginal walls.   These occasionally produced externally visible swellings in the perineum.   In rare cases these abscesses ruptured externally through the skin and lead to the formation of wet gangrene.   One mouse had a short thin-walled tube connecting the anterior of the cervices to the bifurcation of the uterine horns.   Another had the mid-third of one uterine horn represented by a thin fibrous cord (segmantal hypoplasia).   Endo-metrial cysts containing clear fluid of varying sizes were relatively common.

(ii) The ovaries

Mice with ovarian cysts containing clear or sanguineous fluid were often found.   Occasionally cysts larger than the expected size of the ovary were observed with pale atrophic 'lace-like' ovarian tissue on the surface.   Adhesions between the ovarian bursae and ovarian tissue were observed.

(iii) The clitoral glands

These are paired bean shaped subcutaneous glands situated on either side of the GP.   Two collecting ducts open, one on each of the tips of the fork arms at the end of the GP.   External pressure in normal mice caused the extrusion of a thin white coloured secretion.   In MAP daughters with abnormal genitalia a thick oily clearish secretion could be extruded.   They were usually enlarged in these mice and it was difficult to dissect them free of surrounding connective without expressing some of their contents.

The histological changes in the reproductive organs of MAP daughters

Reproductive organs from two MAP daughters with type 5 abnormalities of their external genitalia were compared with those of

two normal mice.   The mothers of both daughters were ovariectomised

and given MAP and daily oestradiol (4.5ng and 60.5ng daily).

Neither daughter had showed visible evidence of being pregnant after

cohabitation with fertile males.   The age of the daughters at

autopsy was 172 and 295 days.

The ovaries: These were extensively luteinized with fewer

follicles present than in normal ovaries.   The centres of some of

the corpora lutea from ⬭ one mouse contained a large amount of

yellowish pigment (Lutein ?).

The uteri: A minority of nuclei from the luminal and to a lesser

extent from the glandular epithelium were abnormally variable with

regard to their size, shape and alignment.   Small foci of abnormal

cells were situated next to normal cells (Pl. 2, Figs. 9, 10, 11, 12

& 13).   Localized areas of the luminal epithelium from the uteri of

one mouse were vacuolated (Pl. 2, Figs. 10, 11 & 13).

The pathological changes observed in the stroma included

glandular hyperplasia, fibrotic changes, regional variation in

cellular density and an abnormal variation in stromal cell nuclear

shape.   A minority of glands in both uteri were hyperplastic (Pl. 2,

Fig. 3).   In one uterus the density of cells in the inner region of

the stroma, i.e. that surrounding the lumen, was higher than in the

outer regions (Pl. 2, Figs. 8 & 11).   The stromal nuclei varied in

shape from normal ovoid through small angular to long spindle-shaped

nuclei.   The abnormal shaped nuclei were usually stained more

heavily (Pl. 2, Fig. 6).

Abnormal changes were observed in local sectors of the inner

circular muscle layers.   In one uterus part of the circular muscle

layer was absent and the stromal tissue occupied the gap and made

contact with the outer longitudinal muscle layer (Pl. 2, Fig. 4).

In the other uterus thin dark-staining nuclei (pyknotic ?) were

observed (Pl. 2, Fig. 6).

The vaginae: Both vagina were smaller in cross-section than in

normal mice. In one vagina the germinal layer of the epithelium was

vacuolated (Pl. 3, Fig. 16). In the other the squamous epithelium

was incompletely keratinized (Pl. 3, Fig. 17). Bilateral cysts were

attached to the posterior vagina in one mouse. One was filled with

a clear fluid and the other with pus (Pl. 3, Fig. 20). Glandular

elements and concretions were present in these cysts.

The urethra: The nuclei of the transitional epithelium of the

urethra were smaller and more angular than in normal mice.

The clitoral glands: These resembled normal glands except

they contained very much more secretion in large 'cysts' (Pl. 3,

Figs. 18 & 19).

The adrenal glands: One pair of adrenal glands from one of the

above mentioned nulliparous masculinized mice (autopsied at 172 days

of age) was sectioned and compared with the glands from a normal

nulliporous oestrus mouse (autopsied at 72 days of age).

The X-zone from the normal mouse was prominent and healthy

(Pl. 4, Figs. 21 & 23). In the masculinized mouse it usually was a

thin zone with irregular shaped nuclei (Pl. 4, Figs. 22 & 24), and areas

of X-zone tissue were also present amongst cells of the zona

fasiculata and of the zona glomerulosa where they were sometimes

attached to the adrenal capsule (Pl. 4, Fig. 26). Further at the

border of the medulla and the X-zone many vacuoles of varying sizes

were observed (Pl.4, Fig. 22). These were not present in the

normal mouse (Pl. 4, Fig. 21) nor are they present in photomicrographs

of adrenals from other normal mice (Starkey and Schmidt, 1938; Jones,

1949). Also blood sinouses in the medulla of the adrenals were

larger in the masculinized than in the normal mouse and occasionally

extended well into the zona fasiculata (Pl. 4, Fig. 26).

DISCUSSION

In the rat MAP acts to depress gonadotropin synthesis and

release (Logethetopoulos, Sharma, and Kraicer, 1961; Sulman and

Danon, 1963; Schally, Carter, Arimura and Bowers, 1968; Labhsetwar,

1966; 1969). It also acts to depress gonadotrophin release in the

mouse (Miyake, 1961) and at a dose of 2.5mg prevented ovulation in

mice for at least 20 days (Grant, 1972c). Thus in the present

experiments MAP probably suppressed the oestrous cycles by acting at

the level of the hypothalamo-pituitary axis. The mode of action of

MGA in rats appears similar to that of MAP (Duncan, Lyster, Hendrix,

Clark and Webster, 1964).

The mean duration of 38.6 days between the finding of a vaginal

plug after the injection of 0.5mg of MAP irrespectiveoof the cycle

stage and the birth of the first litter suggests that these mice

mated twice. Further, they ate some of their young and the

survivors were mutilated. Less young were eaten and mutilated

after their second litter. Dzuik (1960) observed that six of 32

mice that mated after being fed MAP mixed with their food to

synchronize their oestrous cycles failed to become pregnant. There

is also a reduction in fertility associated with the first mating

in cows after the administration of MAP (Collins, Smith, Hauser and

Casida, 1961) or MGA (Zimbelman and Smith, 1966) to synchronize

oestruus cycles.   There are probably a number of reasons for this

reduction in fertility (see Hill, Lamond, Hendricks, Dickey and

Niswender, 1971).

The expected time of parturition was usually altered or the

pregnancy failed 'silently' after the administration of MAP from the

1st to the 5th day of pregnancy (Table 2).   One mouse that received

0.5mg of MAP on the 1st day of pregnancy gave birth at the expected

time.   Silent failure of pregnancy was more prevalent with 0.5mg

of MAP than with higher doses.   Treatment of mice with one dose of

2.5mg of MAP on one day from the 5th to the 18th days of pregnancy

delayed or inhibited parturition (with the exception of one mouse

that aborted after treatment on the 5th day and two mice that failed

to remain pregnant after doses on the 6th day) and was usually

associated with pale 'inactive' corpora at autopsy on the 20th day.

The effect of progestin treatment during early pregnancy is

reported to be variable.   Small daily doses of progesterone were

luteolytic in the mouse (Burdick, 1942) and the daily treatment of

intact rats with MAP (Barnes and Meyer, 1964) or of rats hypophy-

sectomised on the 1st day of pregnancy with progesterone (Ahmad,

1971) caused involution of the corpora lutea.   Also Smith and

Robinson (1969) observed that treatment of cyclic ewes beginning

during oestrus with 19α-fluro-11β-hydroxy-17α acetoxypregn-4-ene-3,

20 diol ('Cronolone') prevented the corpora lutea of ewes that

ovulated from becoming fully functional.   This was despite the fact

that the weight and diameter of these corpora lutea were not

significantly below that of fully functional corpora lutea.   Treat-

ment beginning on the 4th, 8th or 12th day of the oestrous cycle did

not reduce the progesterone output of cyclic corpora lutea.   The

corpora lutea of mice treated at various times during pregnancy with 2.5mg of MAP were probably nonfunctional at autopsy. Their size was variable and they were usually pale coloured. This amount of MAP however is sufficient to maintain the pregnancy in the absence of a live corpora lutea (Grant, 1972c) while 0.5mg was normally insufficient and if it caused luteolysis the pregnancy would then fail. On the other hand large doses (up to 4mg daily) of progesterone administered to mice during the first nine days of pregnancy had no effect on the current litter or on the gestation length (Martin, 1963; see also Deanesly, 1966).

The delayed and/or inhibited parturition associated with MAP and MGA treatment was probably due to excess exogenous hormone being available. Barnes and Meyer (1964) observed that MAP treatment delayed or inhibited parturition in pregnant rats as did treatment of mice during the second half of pregnancy with progesterone (Hall and Newton, 1947).

The relative failure of MAP or MGA alone to support pregnancy in ovariectomised mice compared with the addition of daily oestrogen suggests that these compounds are acting like progesterone. Foetal survival in ovariectomised mice (Humphrey, 1967a; Grant, 1968) rats (Stucki, 1958) and hamsters (Harper, Prostkoff and Reeve, 1966) was improved when oestrogens were given in addition to daily progesterone.

Treatment of pregnant mice with MAP produced a variable masculinizing effect on the external genitalia of their daughters. Furthermore, the subsequent reproductive performance of these daughters was usually suboptimal and the most severely masculinized were usually sterile.

The administration of androgens or a variety of synthetic

progestins during pregnancy in several species has been associated
with masculinization of their female offspring (see reviews by
Dorfman and Shipley, 1956, and Duncan, Wyngarden and Cornette, 1967).
MAP has caused masculinizing changes in daughters of treated pregnant
guinea pigs (Foote et al., 1968) and rats (Revesz et al.,
1960; Revesz and Chappel, 1966).   Suchowsky et al. (1967) reported
that in the rat these MAP- induced, but not testosterone-induced,
abnormalities could be prevented by the concurrent administration of
oestradiol.   They claimed that the antioestrogenic effect of MAP
was responsible for the interference in the development of the female
sex organs.   Judged by the inhibition of the oestrous cycle in the
intact rat and the inhibition of a continuous oestrus smear in
ovariectomised rats treated with oestradiol undecylate, Suchowsky and
Baldratti (1964) and Suchowsky et al. (1967) estimated the anti-
oestrogenic activity of MAP to be 75 and 95 times as potent as
progesterone, respectively, on a weight for weight basis.   However,
Dorfman and Kincl (1963) found that MAP was less than twice as
active as progesterone as an antioestrogen when they used the weight
change in the uterus of oestrone stimulated mice as the endpoint.
In the present study the administration of daily oestradiol in
addition to MAP following ovariectomy probably helped to prevent
some masculinizing changes and improved the subsequent reproductive
performance (see Table 5) of these daughters.

The possibility that the masculinization of females results
from an androgenic effect of MAP that directly influences the
development of the female tract cannot be excluded.   The reported
observations however on the effect of MAP on the weight increase in
castrated immature rats of the ventral prostrate, seminal vesicles

and the levator ani muscle are not in agreement. Suchowsky et al.
(1967) observed that MAP and progesterone were about equally andro-
genic while Revesz and Chappel (1966) reported that MAP stimulated
a greater weight increase in these organs than progesterone did.
Logethetopoulos et al. (1961) and Sulman and Danon (1963) could
detect no stimulatory effect of MAP on the ventral prostrate and
seminal vesicles after prolonged administration beginning with
immature rats.

The MAP daughters resembled the least severely affected female
rats described by Greene, Burrill and Ivy (1939) after androgen
administration to their mothers during pregnancy. Wolffian duct
derivatives were often well developed in masculinized female rats
and of these the ventral prostrate had the lowest androgen threshold.
The glandular tissue attached to the vagina (Pl. 3, Fig. 20)
resembles prostrate tissue and the cystic form of these glands
suggests that excretory ducts were not patent. These workers also
noted that with high doses of androgen that the posterior vagina
was absent though it was present in daughters of females given lower
doses. In the present study vaginal stenosis was regarded as a
serious abnormality and presumably represents an intermediate stage
between its normal size and its absence. Greene et al. considered
that a hypospadic phallus and a common urethro-vaginal orifice
represented a state of arrested development, as the phallus of the
normal new born female rat was hypospadic.

The pathological effects described in the uterus, vagina and
ovary in the present study have all been produced by the prolonged
administration of androgens together with oestradiol to intact and
ovariectomised adult rats (Korenchevsky and Hall, 1940). Some of

these effects can be induced by the injection of ovariectomised rats
with oestradiol esters alone.   For example epithelial vacuolation
(Pl. 2, Fig. 11), subepithelial oedema (Pl. 2, Fig. 7), cysts in the
epithelial layer (Pl. 2, Figs. 10 & 13) and a more fibrous stroma
(Pl. 2, Fig. 6).   Other changes were seen when oestradiol esters
were injected into intact rats.   These included, an irregular
development of the circular muscle layer (Pl. 2, Fig. 4) and cystic
enlargement of some glands (Pl. 2, Fig. 3).   Korenchevsky and Hall
observed that most of these pathological effects were enhanced after
treatment of rats with androsterone or testosterone esters plus
oestradiol esters.   In addition new changes were observed, e.g.
subepithelial stromal cellular infiltrations (Pl.2, Fig. 8) and
'dropsical vacuolation' of the otherwise well developed vaginal
epithelium (Pl. 3, Fig. 16).   The addition of progesterone treatment
to that of oestradiol and testosterone esters lessened the degree
of abnormality seen.   Stromal cysts were smaller and metaplastic
changes of the uterine luminal epithelium (not seen in the present
study) were few or absent.   The ovaries of rats given simultaneous
injections of testosterone and oestradiol esters contained numerous
cysts of varying size.   Treatment with oestradiol esters did not
induce cysts.   Some of the MAP and a minority of the MGA daughters
had large ovarian cysts at autopsy.   A second stage of the andro-
genic action on the ovaries was that of luteinization and the third
stage was characterised by ovarian atrophy.   The ovaries sectioned
in the present study appear more heavily luteinized than normal.
The phalli of the more abnormal female rats resembled small penes
of males.   The enlarged genital papillae (clitorides) seen in the
present work were probably androgen stimulated as the clitorides of

ovariectomised rats were enlarged and developed prominent papillae after androgen treatment (Hall; 1938).

· It is possible that the irregularly sized and aligned nuclei of the glandular and luminal epithelium (Pl. 2, Figs. 9 & 12) represented the prelude to vacuolation.

In androgen-treated rats, clitoral gland hypertrophy was an invariable consequence (Salmon, 1938; Noble, 1939, see Dorfman and Shipley, 1956; Stucki and Forbes, 1960). In ovariectomised rats these glands involute. Clitoral glands of normal ovariectomised mice in the present study responded markedly to the action of testosterone prioprionate to become very much heavier than in normal untreated mice (Table 6). Therefore, the increased size of these glands in most MAP and some MGA daughters would indicate that they were maintaining an abnormally high level of androgen secretion. Furthermore, the changed nature of the secretion in the MAP daughters (i.e. a thick oily secretion) from the thin white exudate obtainable from normal mice was observed by Salmon (1938) after the administration of testosterone proprionate to rats.

The significant correlation between the clitoral gland weight and the degree of abnormality may indicate that the progressively higher androgen levels that stimulated clitoral gland hypertrophy also caused progressive disturbance of the external genitalia. Furthermore, the significant correlation between the ovarian and the clitoral gland weights may mean that the glands were responding to an ovarian secretion that increased with the size of the ovary. Perhaps due to cyst formation. Although ovarian weight was significantly correlated with the age of MAP daughters it was also significantly correlated with degree of abnormality. On the other

hand both the ovaries and clitoral glands could be responding to

adrenal androgen. Although this possibility cannot be excluded the

one pair of adrenal glands that were sectioned from a severely

abnormal MAP daughter were not grossly enlarged and showed no

cortical hyperplasia (Pl. 4, Fig. 22), as may be likely if androgens

were being produced in significant quantities (see Dorfman and

Shipley, 1956). The juxtamedullary adrenal cortical zone (Pl. 4,

Fig. 21), the X-zone, disappears in females during the first

pregnancy (see Jones, 1952) and in nulliparous mice it slowly

regresses as mice age (Deanesly, 1928). Its disappearance is

probably due to androgen secretion perhaps of adrenal (Deanesly,

1958) or of ovarian origin (Jones, 1949; 1952; Kirby, 1966) as

androgen treatment usually causes its rapid regression (see Dorfman

and Shipley, 1956). The partial regression of the X-zone in the

masculinized mouse (Pl. 4, Fig. 24) could be interpreted as normal

for a six month old nulliparous mouse. However the prompt androgen

induced regression of the X-zone depends upon the age at which

treatment begins. Starkey and Schmidt (1938) observed that injections

of testosterone proprionate caused the rapid removal of the X-zone in

24-28 day old mice. The response in 8-16 day old mice was however

variable. Treatment that began at 8-9 days of age prevented the

formation of the X-zone, while treatment begun at 15-16 days did

not induce complete degeneration. These authors noted 'an area of

degenerating tissue was observed at the medullary border of the

X-zone that extended towards the periphery or cortical edge in

varying degrees'. Thus the presence of X-zone tissue amongst cells

of the zonae fasiculata and glomerulosa suggests that androgen

secretion began in the present masculinized mouse at a time when it

would not either prevent the formation or induce the complete disappearance of the X-zone. The X-zone is very sensitive to androgen treatment and Jones (1952) estimated that 20μg of testosterone was sufficient to mimick the degeneration observed during pregnancy. In the experiments of Starkey and Schmidt (1938) 1.8mg of testosterone proprionate injected over three days to 15-16 day old mice did not cause complete X-zone regression. Thus the disorganized X-zone seen in the present study may have persisted despite high levels of androgen secretion.

Maternal and foetal adrenal cortical hyperplasia produced by the administration of an inhibitor of 3β-hydroxysteroid dehydrogenase to pregnant rats was associated with clitoral hypertrophy of female and hyporspadia of male offspring (Goldman, Bongiovanni and Yakovac, 1966). The simultaneous administration of the inhibitor and corticosterone prevented adrenal cortical hyperplasia and clitoral hypertrophy (Goldman and Yakovac, 1966). The clitoral hypertrophy was interpreted as being due to androgen secretion by the hyperplastic adrenal cortices.

In summary, MAP, and to a lesser extend MGA, may have altered the steroidogenic pathways in daughters of treated pregnant females such that their androgen output was permanently elevated. It is unlikely that an androgenic effect of MAP per se would have persisted to exert a continuing influence on the clitoral weight of the daughters.

EXPLANATION OF THE PLATES

PLATE 1

Fig. 1.   This photograph shows the urethra opening into a hyperaemic groove (G) on the dorsum of the enlarged genital papilla of an abnormal (Type 1) mouse.   Although this mouse was fertile the litter sizes were small (6 and 3) and a third pregnancy failed because of probable abortion.   The mother of this mouse was injected with 2.5mg of MAP on the 12th day of pregnancy.   O = vaginal orifice.   x 3.1.

Fig. 2.   This is an example of an abnormal mouse (Type 4) with hypospadia.   The urethra opens into the vagina anterior to the common urethro-vaginal orifice.   There is a prominent hyperaemic groove along the dorsum of the genital papilla (GP).   The size of the GP is normal although it is squarish in outline and has a deeply forked tip containing papillae in the cleft (obscured in this photograph).   The urethro-vaginal orifice was slightly smaller than usual. This mouse was an infertile daughter of the one ovariec-tomised mouse to have live foetuses at 'term' after treatment with 2.5mg of MAP alone.   x 3.1.
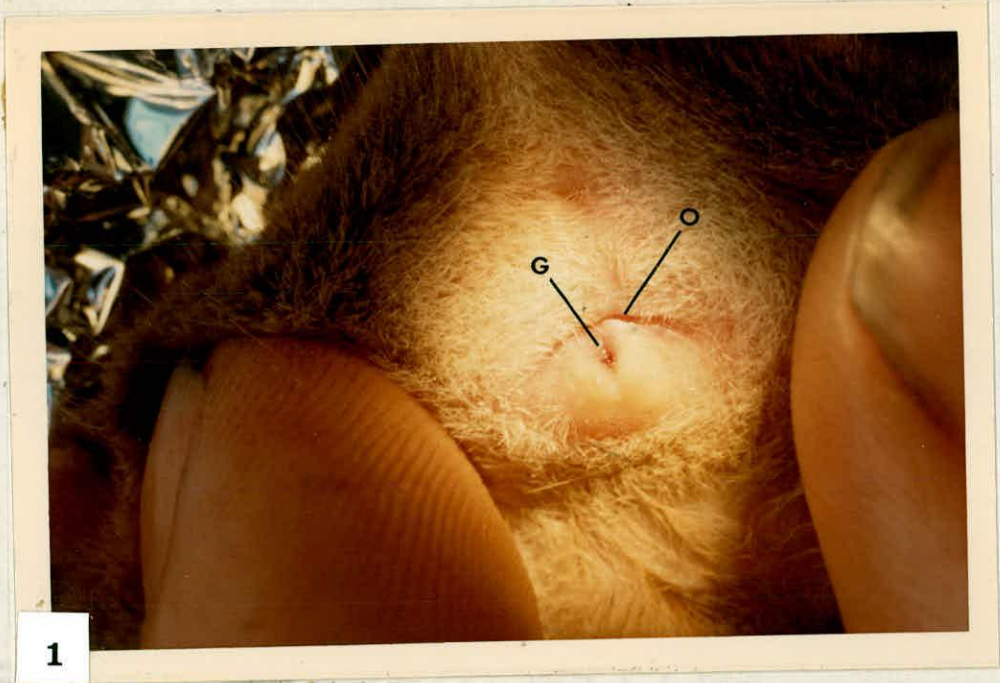
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PLATE 2

Figs. 3-13 are photomicrographs from histological cross-sections of uterine tissue.   Fig. 5 is from a normal mouse and all the other Figures are from two masculinized mice (Figs. 3, 4, 9 & 12 are from the same mouse).   All sections were stained with haematoxylin and eosin.

Fig. 3.   The lumen of the hyperplastic gland (G) is of a comparable size to the uterine lumen (L).   x 96 .

Fig. 4.   There is a prominent gap in the inner circular layer of the myometrium which contains glands and other stromal tissue.   Below this gap there is also a smaller break in the circular muslce layer. (L = uterine lumen).   x 96 .

Fig. 5.   Stromal tissue containing a gland (G) is situated below the inner circular muscle layer of the myometrium from a normal mouse. x 480.

Fig. 6.   This photograph shows the equivalent tissues of a masculinized mouse to those depicted in Fig. 5.   The nuclei from both the stromal and the muscle cells are irregularly shaped (generally smaller) and more densely stained than in the normal uterus.   Some of these nuclei appear pyknotic.   Furthermore, the stromal connective tissue is more dense (fibrotic) in this Figure than in Fig. 5.   x 480.

Figs. 7 & 8.   There is a prominent gap between the luminal epithelium and the underlying stroma which probably contained oedematous fluid. The stromal tissue situated between the lumen (L) and the circular muscle layer (M) in Fig. 7 is highly condensed.   Also the density of the central stromal cells (i.e. those near the lumen (L) is higher than in the peripheral regions (Fig. 8).   x 480.

Fig. 9.   Note the variation in size and shape of the nuclei of the luminal epithelium.   There are normal ovoid shaped nuclei and small angular shaped nuclei with crinkled membranes.   The nuclei in the stromal cells and the glandular epithelium (G) appear normal. x 480.

Figs. 10 & 13.   Localized lengths of the luminal epithelium are vacuolated and there are local areas of oedema in the underlying stroma with the formation of cysts.   The epithelial nuclei are sometimes orientated irregularly (Fig. 10) and often abnormally shaped.   The small dark staining nuclei are pyknotic.   x 480 .

Fig. 11.   Note the variable height of the luminal epithelial cells and the local areas of vacuolation.   x 240 .

Fig. 12.   Two uterine glands surrounded by stromal cells.   Compare the shape and size of the nuclei of the epithelial cells from the gland on the left hand side which appears normal, with those of the gland on the right.   x 480 .
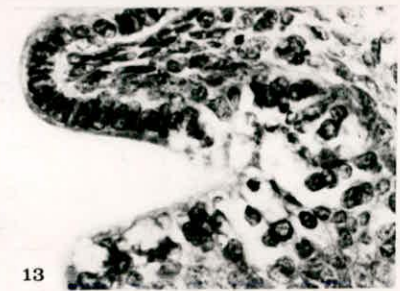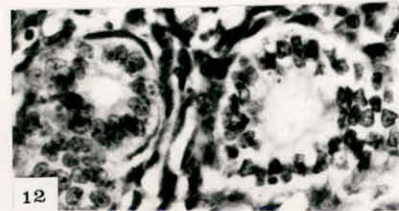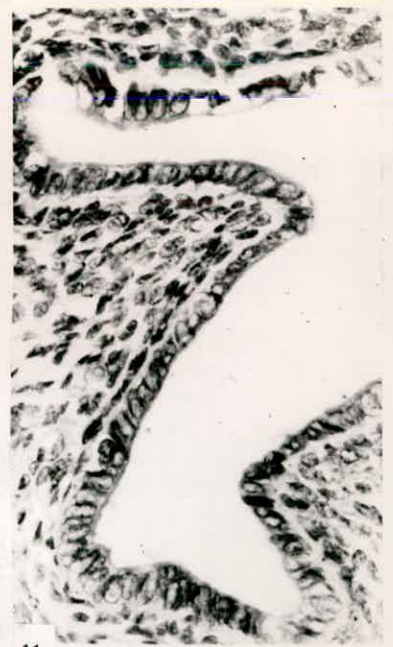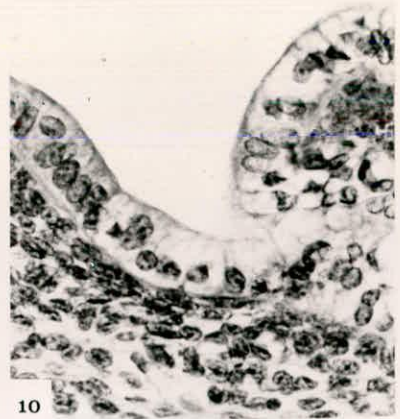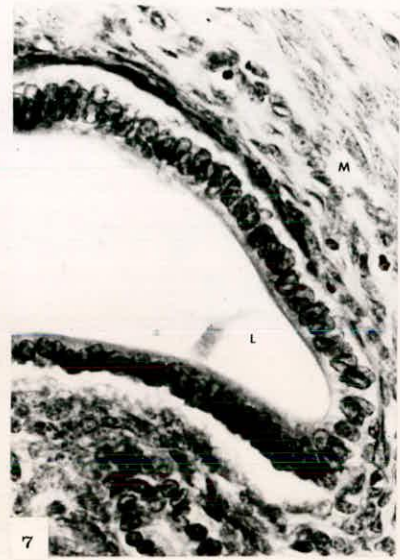
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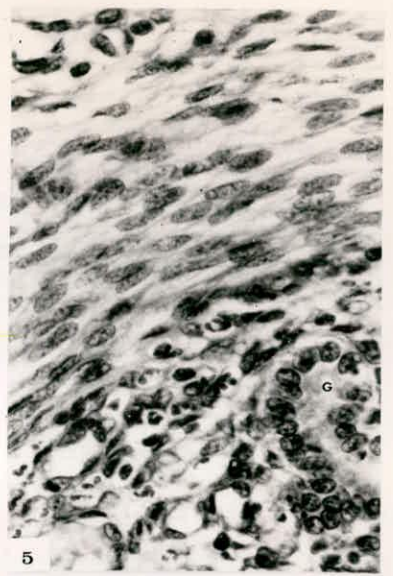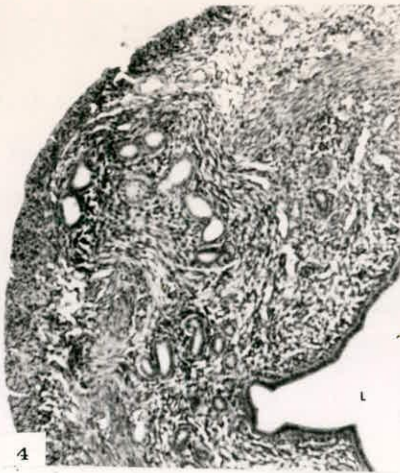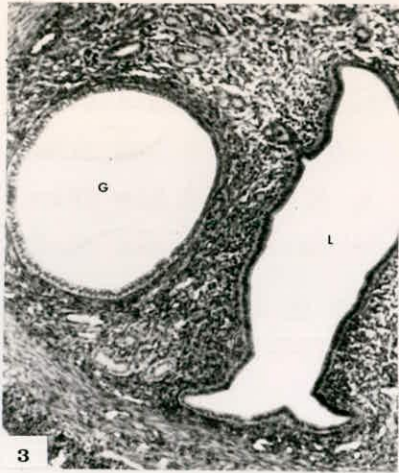

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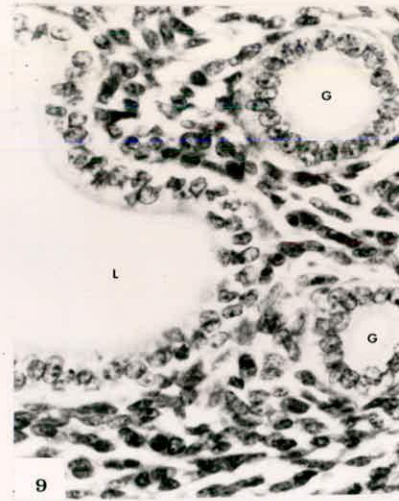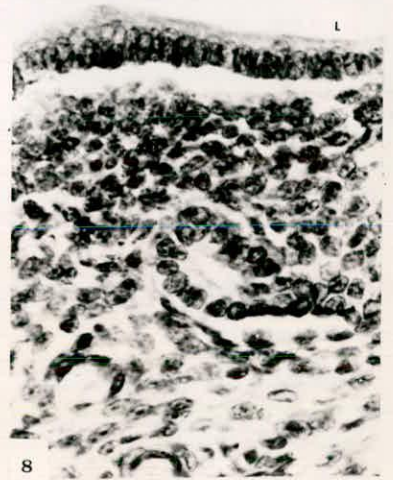
PLATE 3

Figs 14-20 are photomicrographs of histological sections stained with H & E.   Figs. 14-17 are of cross-sections of the vaginal epithelium and the underlying stroma.   x 710 .

Fig. 14.   Mucified epithelium from a normal mouse on the first day of metoestrus (M-1).

Fig. 15.   Keratinised epithelium from a normal oestrus mouse.

Fig. 16.   Mucified epithelium from a masculinized mouse.   Note the dropsical vacuolation of the germinal epithelial layer.

Fig. 17.   Partially keratinized epithelium from another masculinized mouse.   The cross-sectional area of this vagina was only a little larger than that of the urethra.

Fig. 18.   Clitoral gland from a masculinized mouse.   Note the large cysts which contained an oily secretion.   x142 .

Fig. 19.   Clitoral gland from a normal mouse.   x142 .

Fig. 20.   Male type accessory gland attached to the vagina of a masculinized mouse.   The peripheral glandular elements resemble prostrate tissue.   The large cysts contained pus.   x 142 .

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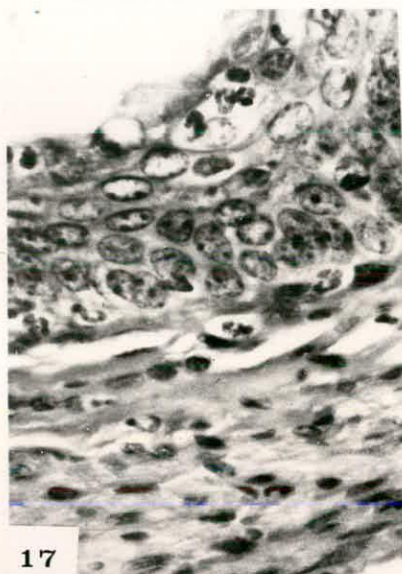
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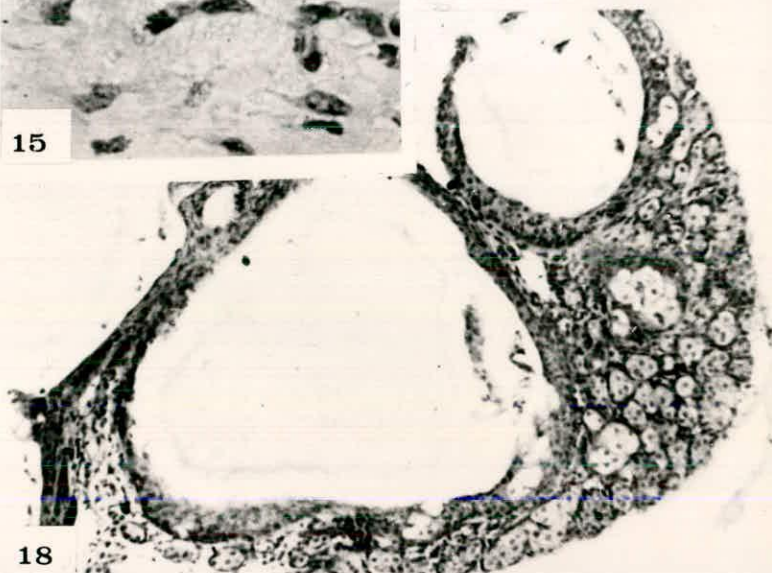
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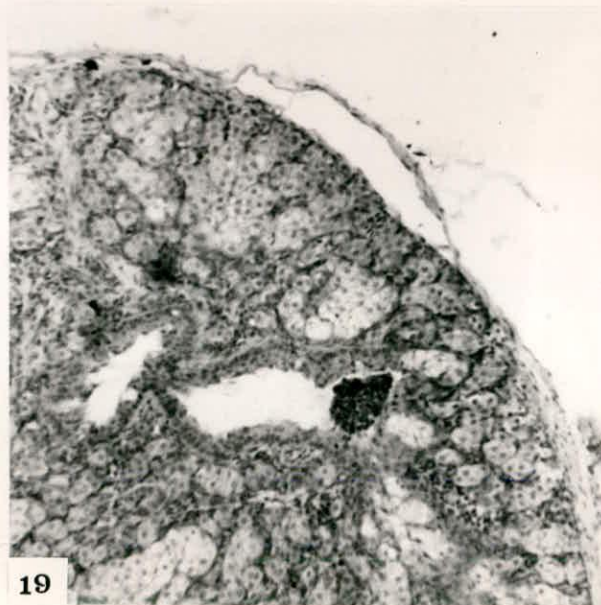
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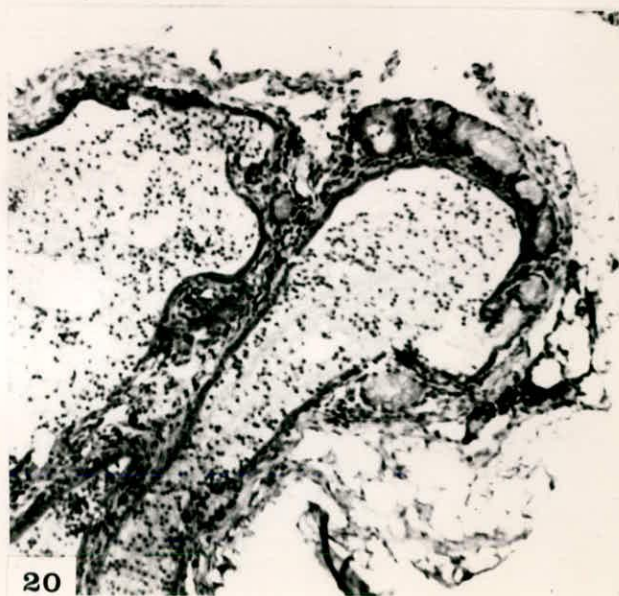
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PLATE 4

Figs. 21-26 are photomicrographs of histological sections of adrenal glands stained with H & E.   Figs. 21 & 22 x115 , Figs. 23-26 x288 .

Figs. 21 & 23.   Gland from a normal mouse.   G = zona glomerulosa, F = zona fasiculata, X = X-zone, M = medulla.

Figs. 22, 24, 25 & 26.   Gland from the masculinized mouse.   The X-zone is very much smaller, although streaks of X-zone tissue are present in the zona fasculata (Fig. 25) and zona glomerulosa (Fig. 26).   Further, there are numerous large blood sinouses situated between the medulla and the X-zone (Figs. 22, 24 & 26).
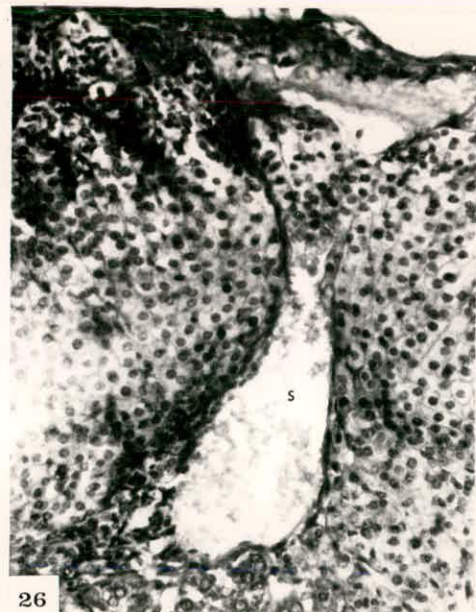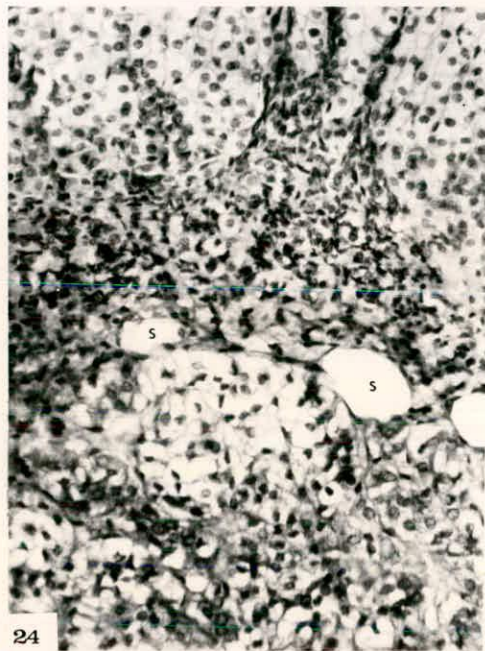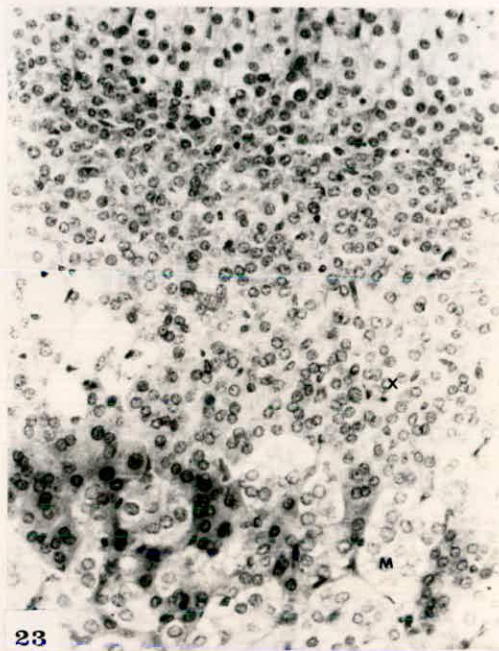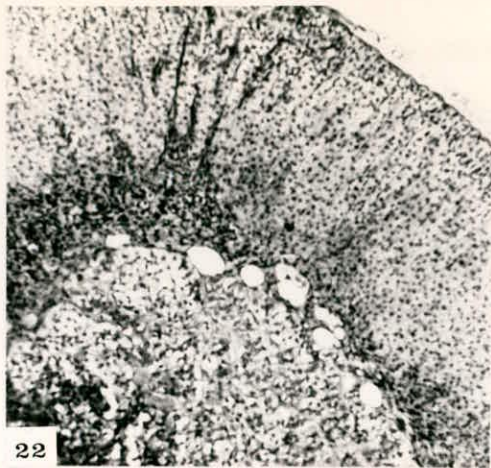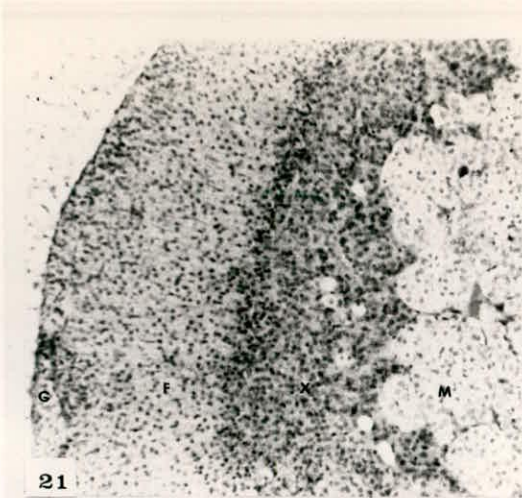
EGG IMPLANTATION IN OVARIECTOMISED MICE AFTER TREATMENT

WITH PROGESTERONE AND MEDROXYPROGESTERONE ACETATE.

THE EFFECT OF PROGESTERONE CONTAMINATED WITH ANDROGENIC

MATERIAL AND OF THE TIME OF OVARIECTOMY.

ABSTRACT

It was confirmed that the exogenous ovarian hormones required for egg-implantation depend on the time of ovariectomy. Mice ovariectomised on the 3rd day of pregnancy required both progesterone and oestrogen. Those ovariectomised during the afternoon of the 4th day, only required progesterone.

Egg-implantation following ovariectomy on the 3rd day of pregnancy in mice treated with two commercial brands of progesterone was probably due to the presence of contaminants. One brand contained approximately 20% contamination with a testosterone-like androgen. Ovariectomy on the 3rd day followed by treatment with progesterone and testosterone proprionate permitted egg-implantation.

Although high levels of progesterone treatment, following ovariectomy on the 3rd day of pregnancy with a preparation which usually did not induce implantation, did permit implantation. This was unpredictable and the number of implants was reduced.

Medroxyprogesterone acetate (MAP) behaved as a pure progesterone with regard to implantation following ovariectomy. After ovariectomy on the 3rd day of pregnancy and a single injection of MAP blastocysts remained in a state of diapause and could be activated by an injection of oestradiol up to the 50th day. Embryonic survival in ovariectomised mice treated with MAP decreased with advancing pregnancy after nondelayed oestradiol induced implantation.

Synergism between oestradiol and progesterone for egg-implantation in ovariectomised mice could not be demonstrated. This confirms that there probably are specific minimal requirements for both progesterone and oestrogen for implantation, and that those for oestrogen cannot be compensated for by an increase of progesterone.

INTRODUCTION

Egg implantation in rats and mice is generally considered to be primarily controlled by the ovarian steroid hormones, progesterone and oestrogen (see reviews by Shelesnyak, 1960; Mayer, 1963; Psychoyos, 1966; Nalbandov, 1971). On the basis of indirect evidence, oestrogen is thought to act on the progesterone-dominated uterus to initiate implantation. Evidence from ablation studies in the mouse has demonstrated that the oestrogen secretion is probably dependent on pituitary gland involvement early on the 4th day of pregnancy (Bindon and Lamond, 1969; Bindon, 1969) and subsequently on the activity of the ovary until about noon on the 4th day (Humphrey, 1967b; Grant, 1968). On the basis of incorporation of tritiated uridine into the uterus (Miller, Owen and Emmens, 1968) and from the changes in the distribution of mitotic figures in uterine tissues (Finn and Martin, 1969b) as indices of oestrogenic activity, these workers conclude that there is a gradual release of oestrogen starting well before the 4th day.

Whatever the nature of the oestrogen release there is general agreement that when implantation is delayed, either naturally as during lactation (Whitten, 1955; McLaren, 1968) or after ovariectomy and progesterone treatment (Yoshinaga and Adams, 1966; Humphrey, 1967b; Dickson, 1969) the oestrogen requirements for egg implantation are very small. Given as a single subcutaneous dose Humphrey found that $2.5 \times 10^{-3} \mu g$ of oestradiol-17β was sufficient and in ovariectomised superovulated mice treated with progesterone Smith and Biggers (1968) estimated that the minimum effective dose of oestradiol benzoate for implantation was $2.91 \times 10^{-2} \mu g$.

Ablation studies during which the ovaries have been removed
have however, given rise to inconsistent results.   Implantation in
ovariectomised pregnant mice after treatment with progesterone has
been found to be prevented by ovariectomy on or before the 3rd day
of pregnancy (Yoshinaga and Adams, 1966; Humphrey, 1967b; Grant, 1968)
while implantation has occurred after ovariectomy on the 2nd and 3rd
days of pregnancy (Smithberg and Runner, 1956; McLaren, 1971; Grant,
unpublished).   The problem then to be investigated in the present
study was to establish under what circumstances progesterone treatment
after ovariectomy on the 3rd day would allow implantation, and
assuming that oestrogen was required for implantation to find out the
source of this hormone.

The first possibility examined was that residual oestrogen, that
is oestrogen remaining after ovariectomy, was acting to allow
implantation.   It was noted that relatively high doses of proges-
terone were required for implantation following ovariectomy on or
before the 3rd day of pregnancy (McLaren, 1971; Grant, unpublished)
and it was postulated that the relative excess of exogenous proges-
terone may be synergizing with residual oestrogen to allow implan-
tation.   The corollary of this hypothesis would be that the
minimum amount of oestrogen required for implantation was variable
and decreased with increasing progesterone.   Instances of synergism
between oestrogen and progesterone have been cited by Courrier (1950)
in the mouse, rat and rabbit and by Hisaw and Hisaw (1961) in lower
primates.   Furthermore, Yoshinaga (1961) demonstrated that local
injections of progesterone as well as oestrogen into the adipose
tissue immediately adjacent to the uterus induced local implantation.
More recently Yoshinaga and Greep (1971) using this technique

reported that local oestrogen injections on the 3rd day of pregnancy induced a precocious refractory zone in the adjacent horn but only when systemic progesterone levels were elevated.   Bloch (1968) has observed that oestrogen injections at the time of post-partum ovulation as well as the temporary removal of the litter allow implantation at the expected time of normal pregnancy.   This author believed that the oestrogen released at the time of ovulation was sufficient for subsequent implantation in lactating mice.

Although there is no information available concerning the persistance of oestrogen following ovariectomy in the mouse, oestrogenic effects do not disappear immediately after the removal of the ovaries.   Ovariectomy during proestrus does not shorten the duration of vaginal cornification in mice (Brambell and Parkes, 1927; Grant, unpublished).   Urinary excretion of oestrogen in the rabbit did not immediately cease following ovariectomy and adrenalectomy (Takahashi, 1961).   This author described oestrogen retention in several organs especially in fat tissue of rabbits after these operations.   Also the administration of priming oestrogens in ovariectomised mice (Finn, 1966) and rats (De Feo, 1967) had a delayed effect on subsequent deciduomata formation.   The other possibility examined to explain implantation following early ovariectomy was to check progesterone solutions used for contaminants, especially for oestrogens.

MATERIALS AND METHODS

Virgin Q strain females aged from six to ten weeks were placed with fertile Q males and checked daily.   The day during which the

vaginal plug was found was called the 1st day of pregnancy.
Lighting was automatically controlled ($16\frac{1}{2}$ hr light and $7\frac{1}{2}$ hr dark
centred on 0130 hr) and the temperature was maintained at approxim-
ately 22°C, except for the experiment summarised in Table 3, when
the light was 'on' for 14 hr and 'off' for 10 hr.   This experiment
was carried out in the Medical Research Council's Clinical Research
Unit, Harrow.

Bilateral ovariectomy was performed with pentobarbitone sodium
('Nembutal'; Abbott) as the anaesthetic,at various times before egg
implantation.

Hormones were injected subcutaneously in 0.1ml of vehicle
unless stated otherwise.   Micronized oestradiol-17β, progesterone
and testosterone (all Koch-Light) were dissolved in arachis oil and
progesterone was also suspended in 2% methylcellulose for injection
and in one experiment pure progesterone was placed directly under
the skin.   Commercial brands of progesterone ('Lutocyclin'; Ciba
and 'Progestin'; Organon) were injected in undiluted vehicle (oil-
based) or after dilution with arachis oil.   MAP (6α-methyl-17α-
acetoxyprogesterone, medroxyprogesterone acetate; Upjohn) was
injected as undiluted 'Depoprovera' or after dilution with distilled
water.   Injection volumes of 0.05ml or 0.1ml were used.

In one series of experiments the three brands of progesterone,
each in an oily solution, were extracted with sodium hydroxide to
remove any oestrogen contaminants before use.   The progesterone
solution together with an equal volume of ethyl acetate were poured
into a separating funnel.   Normal sodium hydroxide from 10-100
times the volume of the progesterone solution was passed through the
funnel.   The ethyl acetate fraction containing the oily progesterone

solution was then washed with distilled water, separated and warmed until all the ethyl acetate had evaporated off.

Samples of these three brands of progesterone were also subjected to a chromatographic analysis after column separation to determine the type and amount of various steroidal compounds present. This study was carried out by Dr. T. Holmdahl, Dept. of Obstetrics and Gynaecology, University of Uppsala, Sweden.

Fifteen minutes prior to autopsy some mice were injected intravenously with 0.2ml of 0.5% Pontamine Blue (6BX) (Gurr) in saline to detect early implantation swellings (Finn and McLaren, 1967). At autopsy the number of implantation sites and their size (relative to plastic embedded benzol benzoate cleared uteri from normal pregnant mice at known times post coitum) were recorded. Sites that were misshapen and blue-black in colour were considered dead. Uteri without sites were flushed with 0.9% phosphate buffered saline to recover any free blastocysts.

Statistical analysis of experiments in which ovariectomised mice were treated with graded doses of progesterone and oestradiol was by analysis of variance on the angular transformed unweighted proportion of mice with implantation sites for a single level of both hormones. The assumptions of normality of data in each treatment and of equal variance from treatment to treatment were barely justifiable.

Comparisons between groups of mice that received graded levels of MAP and oestradiol following ovariectomy (see Table 10) were orthogonal. Differences between number of implantation sites were analysed by use of Student's 't test' and proportional survival differences were analysed by 2 x 2 Chi-square contingency tables

(Sned̂ecor, 1956).

Possible synergism between progesterone and oestradiol for implantation

In all three experiments mice were ovariectomised on the 3rd day of pregnancy and given daily graded doses of progesterone. Graded doses of oestradiol were injected on the 4th day of pregnancy. In the first two experiments Ciba progesterone (25mg per ml diluted with arachis oil) was used as the progesterone and was injected from the 3rd to the 10th or 11th day (inclusive) of pregnancy. In the third experiment Koch-Light progesterone suspended in methylcellulose was injected from the 3rd to 7th day (inclusive) of pregnancy.

The highest doses of both progesterone and oestradiol used in the first experiment (Table 1) each had a significant (by inspection) effect on implantation. When the dosage of progesterone was 0.5mg or higher, 15/18 mice had implantation sites after treatment with $7.5 \times 10^{-3} \mu g$ of oestradiol and 5/16 with $1.3 \times 10^{-3} \mu g$ oestradiol. When the progesterone dosage was 1.25mg per day oestradiol was not required for implantation. The lowest dose of progesterone, viz. 0.25mg, was probably around the lower limit compatible with implantation irrespective of the oestradiol level. These effects on implantation were additive and there was no evidence for interaction (by inspection) between the lower levels of oestradiol and progesterone that may have signified synergism.

The three dose levels of progesterone used in the second experiment were above the minimum required for implantation when the

TABLE 1.

IMPLANTATION AS SCORED ON THE 11TH DAY OF PREGNANCY, AFTER OVARIECTOMY ON THE 3RD DAY,

FOLLOWING TREATMENT WITH GRADED DOSES OF 'PROGESTERONE' AND OESTRADIOL*.

Dose of oestradiol (μg) given on the 4th day of pregnancy	Daily dose of 'progesterone' (mg) given from the 3rd -10th days (inclusive)					
	0 (oil)	0.25	0.50	0.75	1.00	1.25
0 (oil)	0, 3, 0	0, 4, 0	0, 2, 2	0, 4, 0	0, 4, 1	3(12.0), 2, 0 100%
1.0×10^{-5}	0, 4, 0	0, 3, 1	0, 3, 1	1(11), 3, 0 100%	0, 4, 0	4(8.5), 0, 0 100%
5.0×10^{-5}	0, 4, 1	0, 4, 1 1PB+	1(10), 4, 0 0%	0, 4, 0	0, 4, 0	2(13.0), 1, 0 1PB+
2.5×10^{-4}	0, 3, 1	0, 4, 1	0, 4, 1	0, 4, 1 1PB+	0, 4, 2	3(11.0), 1. 0 100%
1.3×10^{-3}	0, 1, 3	0, 4, 1	1(13), 3, 1 53%	0, 4, 0 1PB+	0, 4, 0 2PB+	4(13.0), 0, 0 98%
7.5×10^{-3}	0, 3, 1	1(6), 3, 0 0% 1PB+	3(13.0), 1. 0 61%	4(7.8), 0, 0 78%	3(6.7), 3, 1 100%	5(10.4), 0, 0 98%

*   The entries in a cell, from left to right, represent no. of mice with implants,(mean no.), no. of mice without implants but with free blastocysts, no. of mice with neither implants nor blastocysts (these were treated as failed pregnant mice).   The %age in the 2nd row is the % of implants that were alive at autopsy.   1PB+ means that 1 mouse with free blastosysts was Pontamine Blue positive.

** One mouse had implants in one horn and free blastocysts in the other.

oestradiol dose was 7.5×10^{-3} µg (Table 2 cf Table 1). The aim was
to establish if this would remain equally true for these three levels
of progesterone when the dose of oestradiol was gradually reduced.
Mice that received '6.0×10^{-3} µg' of oestradiol were omitted from the
analysis summarized in Table 4. Assuming that 60% of the mice were
expected to have implantation sites the probability, P, that none
out of 15 would have had sites is unlikely (i.e. P (o/15) = $(0.6)^{15} \doteqdot$
0.00001). The interaction between progesterone and oestradiol and
the differences between the progesterone levels were nonsignificant
(Table 4, A). This suggests that there was no synergism between the
two hormones for implantation and that the significant (P $<$ 0.001)
proportional increase in response with increasing oestradiol levels
was an additive effect.

The third experiment includes two sections that were performed
separately (Table 3). In the first section low progesterone doses
(i.e. 0.125 and 0.25mg) were combined with a wide range of oestradiol
levels (i.e. 5.0×10^{-6} - 5.0×10^{-1} µg). As before the interaction
and progesterone 'F ratios' were nonsignificant, and differences
between the oestradiol levels were significant (P $<$ 0.05, Table 4,B).
In the second section of this experiment the only significant
difference (by inspection) was between the oestradiol levels.

Implantation in ovariectomised mice treated with 'progesterone' alone

All mice were ovariectomised on the 3rd day of pregnancy and
given daily progesterone treatment from the 3rd to the 7th days,
inclusive. In the first experiment the effects of different brands
of progesterone and the effect of 'oestrogen' extraction with sodium
hydroxide were tested.

TABLE 2.

IMPLANTATION, AS SCORED ON THE 11TH–12TH DAY OF PREGNANCY, AFTER OVARIECTOMY ON THE 3RD DAY,

FOLLOWING TREATMENT WITH GRADED DOSES OF 'PROGESTERONE' AND OESTRADIOL[*].

Dose of oestradiol (μg) given on the 4th day of pregnancy	Daily dose of 'progesterone' (mg) given from the 3rd–10th or 11th days (inclusive)				% of mice with implants
	0.50	0.75	1.00	Totals	
1.5×10^{-3}	0, 3, 0	2(6.5), 4, 0 94%	1(9), 3, 1 0%	3(7.3), 10, 1 55%	21
3.0×10^{-3}	2(8.0), 2, 0 100%	1(6), 3, 1 100%	2(12.0), 2, 0 100%	5(9.20), 7, 1 100%	26
4.5×10^{-3}	1(10), 2, 1 100%	3(10.3), 1. 1 97%	4(6.0), 1, 0 58%	8(8.13), 4, 2 68%	57
6.0×10^{-3}	0, 3, 2	0, 4, 1	0, 4, 1	0, 11, 4	0
7.5×10^{-3}	4(6.5), 1[**], 0 96%	3(9.7), 2[**], 0 100%	4(12.0), 0, 0 81%	11(9.19), 3, 0 90%	79
Totals	7(7.13),11, 3	9(8.78),14, 3	11(9.54),10, 2		

* Interpreted as Table 1.

TABLE 3.

IMPLANTATION AS SCORED ON THE 8TH DAY OF PREGNANCY, AFTER OVARIECTOMY ON THE 3RD DAY, FOLLOWING TREATMENT WITH GRADED DOSES OF PROGESTERONE AND OESTRADIOL[*].   DATA FROM TWO EXPERIMENTS ARE DIVIDED BY THE DOUBLE LINE.

Dose of oestradiol (μg) given on the 4th day of pregnancy	Daily dose of progesterone (mg) given from the 3rd-7th days (inclusive)			
	0.125	0.250	0.500	2.500
0	0, 3, 0	0, 4, 0	0, 4, 0	0, 4, 1
5.0×10^{-6}	0, 5, 0	0, 5, 0	0, 3, 0	0, 2, 1
5.0×10^{-4}	2(4.5), 3, 0 55%	3(7.7), 3, 1 [**] 100%	2(9.5), 0, 0 100%	3(10.0) , 0, 0 100%
5.0×10^{-2}	0, 3, 1	1(3), 4, 1 [**] 0%	2(7.5), 0, 0 100%	3(11.0) , 0, 0 100%
5.0×10^{-1}	0, 2, 1	2(8.5), 0, 2 0%	—	—

*   Interpreted as for Table 1.

TABLE 4.

IMPLANTATION IN OVARIECTOMISED MICE AFTER GRADED DOSES OF
PROGESTERONE AND OESTRADIOL.   SUMMARY OF ANALYSES OF VARIANCE
WHERE 'A' AND 'B' REFER TO DATA SHOWN ON TABLES
2 AND 3, RESPECTIVELY.

	A				B			
Source of variation	df	MS	F ratio	P	df	MS	F ratio	P
Progesterone	2	265.4	1.34	NS	1	599.1	3.06	NS
Oestradiol	3	1931.0	10.09	<0.001	4	620.9	3.18	<0.05
Interaction	6	237.7	1.24	NS	4	196.0	1.00	NS
Within	∞	191.3*			∞	195.4*		

*   The variance of the angular values was calculated from $\dfrac{820.7}{n}$
(Fisher and Yates, 1948).   For these analyses n was equal to the
harmonic mean.

It is apparent (Table 5) that the brand of progesterone used had a significant (by inspection) effect on implantation. Implantation occurred in only one of 21 mice given Koch&Light progesterone, while it occurred in 23 of 32 and in 7 or 25 mice treated with Ciba and Organon brands, respectively. 'Oestrogen' extraction did not significantly (by inspection) reduce the incidence of implantation, regardless of the volume of sodium hydroxide used. The results were therefore pooled for each brand of progesterone used.

The results of the chromatographic analysis of Ciba progesterone showed that in one sample of '25mg' per ml that approximately 20% of the steroidal content present was identified as a C19 compound, possibly testosterone. Oestrogens were not detected. Neither androgenic nor oestrogenic contaminants were found in a sample of Koch-Light progesterone of 25mg per ml.

To test for a quantitative effect of progesterone on implantation, Koch-Light progesterone was chosen as implantation with this compound alone was less frequent than with the other two brands. As shown in Table 6 implantation could be obtained with very high doses of progesterone. The effect however was variable. Three of 12 mice implanted eggs with 4.0 or 8.0mg daily in arachis oil, whereas none of three mice had implants after daily treatment with 10.0-15.0mg suspended in methylcellulose. Although this difference may have been due to the different vehicles used, the excipient per se was not important as seven of 16 mice implanted eggs when given 50 or 100mg of pure micronized progesterone subcutaneously on the 3rd day of pregnancy. Progesterone at these levels induced narcosis and some mice given 100mg were still lethargic three days after the operation.

TABLE 5.

THE EFFECT OF THE TYPE OF PROGESTERONE AND THE ABSENCE OF AN EFFECT OF ITS 'OESTROGEN EXTRACTION'

ON EGG-IMPLANTATION FOLLOWING ITS ADMINISTRATION TO MICE OVARIECTOMISED ON THE 3RD DAY OF PREGNANCY.

Progesterone	Dose of pro-gesterone (mg/day;3rd-7th day)	No. of Mice	No.[+] with implant-ation sites	Mean No. of implan-tation sites	No. with free bla-stocysts	Mean No. of blas-tocysts	No.with both implantation sites and blastocysts
Koch-Light in 0.1ml of arachis oil	2.0	6	1	16	4	5.50	0
Koch-Light in 0.1ml of arachis oil ('Oestrogen-freed')	2.0	5	0	-	4	4.75	0
Koch-Light in 0.1ml of arachis oil	2.5	10	0	-	9	6.89	0
Ciba in 0.1ml of vehicle	2.5	19	15	9.87	0	-	0
Ciba in 0.1ml of vehicle ('Oestrogen-freed')	2.5	13	8	9.13	7	4.28[*]	2
Organon in 0.1ml of vehicle	2.5	14	3	6.0	10	4.60[**]	1
Organon in 0.1ml of vehicle ('Oestrogen-freed')	2.5	11	4	8.0	7	4.86	1

+   Mice autopsied on the 8th day of pregnancy.

*   Blastocysts from three mice were partially transformed.

**   Blastocysts from one mouse were partially transformed.

TABLE 6.

EGG-IMPLANTATION AFTER TREATMENT WITH PROGESTERONE FOLLOWING OVARIECTOMY

ON THE 3RD DAY OF PREGNANCY.

Progesterone	Dose of progesterone (mg/day; 3rd–7th day)	No.[+] of mice	No. with implantation sites	Mean No. of implantation sites	No. with freeeblastocytes	Mean No. of blastocytes
Koch-Light in 0.1ml of 2% methylcellulose	0.5	4	0	–	4	5.25[*]
Koch-Light in 0.1ml of 2% methylcellulose	2.5	5	0	–	4	2.50[*]
Koch-Light in 0.1ml of arachis oil	4.0	9	1	2	8	6.75
Koch-Light in 0.2ml of arachis oil	8.0	3	2	13.50	1	7
Koch-Light in 0.1ml of 2% methylcellulose	10.0–15.0	3	0	–	3	6.67
Koch-Light as micronized powder	50mg[**]	8	3	5.30	5	4.80
Koch-Light as micronized powder	100mg[**]	8	4	5.25	4	7.75

+   Mice autopsied on the 8th day of pregnancy.

*   Blastocysts lost from one mouse.

** As a single subcutaneous dose on the 3rd day of pregnancy.

Implantation after simultaneous treatment with progesterone and testosterone proprionate (TP)

All mice were ovariectomised on the 3rd day of pregnancy and treated simultaneously with 2.5mg of Koch-Light progesterone and 0.03, 0.15 or 0.75mg of TP daily from the 3rd to 7th days inclusive (Table 7). None of the mice given progesterone alone or in combination with the two lower doses of TP had implantation sites. Implantation however, occurred in all four mice given progesterone and 0.75mg of TP. These implantation sites were macroscopically healthy and their mean number was not below that expected after oestradiol treatment on the 4th day of pregnancy.

The effect of the time of ovariectomy on implantation in mice treated with progesterone alone

Mice were ovariectomised either on the afternoon of the 3rd day, the morning of the 4th day or the afternoon of the 4th day of pregnancy (Table 8). All mice were treated with 0.5mg daily of Koch-Light progesterone from immediately after ovariectomy up until the 8th day of pregnancy. At autopsy on the 8th day the four mice ovariectomised on the 3rd day of pregnancy had free blastocysts only (mean per mouse 6.5). Of the mice ovariectomised on the 4th day of pregnancy the four operated on during the afternoon all had implantation sites (mean per mouse 11.0)whereas three of four mice operated on during the morning had both implantation sites (mean 5.0) and free blastocysts, and all four mice had free blastocysts (mean 3.3). Thus mice ovariectomised during the morning of the 4th day were intermediate with respect to the number of blastocysts recovered relative to mice ovariectomised on the 3rd day and with respect to

TABLE 7.

EGG-IMPLANTATION AFTER THE SIMULTANEOUS ADMINISTRATION OF PROGESTERONE AND TESTOSTERONE

PROPRIONATE TO MICE OVARIECTOMISED ON THE 3RD DAY OF PREGNANCY.

Treatment [*]	No. of mice	No. with implantation sites	Mean no. of implantation sites	No. with free blastocysts	Mean no. of blastocysts
2.5mg P + 0.01ml oil daily	4	0	-	4	2.5
2.5mg P + 0.03mg TP daily	4	0	-	4	8.8
2.5mg P + 0.15mg TP daily	4	0	-	4	5.0
2.5mg P + 0.75mg [**] TP daily	4	4	9.75	-	-

Abbreviations: P = progesterone (in 0.1ml oil); TP = testosterone proprionate (in 0.1 or 0.05ml oil) [**];

   Oil = arachis oil.

[*] Hormones injected daily from 3rd - 7th day (inclusive) of pregnancy; mice autopsied on the 8th day.

TABLE 8.

THE EFFECT OF THE TIME OF OVARIECTOMY DURING EARLY PREGNANCY ON EGG-IMPLANTATION.

MICE WERE INJECTED (SUBCUTANEOUSLY) WITH 0.5MG OF PROGESTERONE DAILY

FROM THE TIME OF THE OPERATION TO THE 7TH DAY (INCLUSIVE) OF PREGNANCY.

Treatment	No.of mice	No. with implantation sites (IS)	Mean no. of IS	Approx. age of IS (day pc)	No. with free blastocysts	Mean no. of blastocysts	No. with both IS and blastocysts
OvarX 3rd day of preg. 1445 hrs	4	0	–	–	4	6.5	0
OvarX 4th day of preg. 0900 hrs	4	3	5.0	$7\frac{2}{3}$	4	3.3	4
OvarX 4th day of preg. 1445 hrs	4	4	11.0	$7\frac{2}{3}$	0	–	0

the number of implantation sites after ovariectomy during the
afternoon of the 4th day of pregnancy.

Likewise mice ovariectomised on the afternoon of the 3rd day of
pregnancy and immediately given a single injection of 1.0-20.0mg of
MAP (Table 9) had not implanted by the 7th or 8th day. However 19
mice ovariectomised on the afternoon of the 4th day of pregnancy and
immediately given 1.0 or 2.5mg of MAP all had implantation sites at
autopsy from the 7th to 13th days. (The mean number per mouse for
six mice killed on the 7th day of pregnancy was 11.33).

Delayed implantation in ovariectomised mice treated with MAP

All mice were ovariectomised during the 3rd day of pregnancy and
immediately given a single dose of 1.0-20.0mg of MAP (Table 9). Of
the 26 mice laparotomised during the 7th or 8th day of pregnancy none
had implantation sites and at varying times from the 7th to 50th day
of pregnancy mice were given a single injection of 5.0 x 10^{-3}µg of
oestradiol. Three to nine days after the oestradiol injection
mice were injected with Pontamine Blue and autopsied. When oestra-
diol was given on the 7th to 14th day of pregnancy after treatment
with 1.0-2.5mg of MAP, implantation could be readily induced.
Although implantation occurred when oestradiol treatmednt was given
on the 18th to 50th day of pregnancy after treatment with 5.0-20.0mg
of MAP, fewer mice responded and there was evidence that in some
mice implantation occurred before treatment with oestradiol. This
was derived from comparison of the size of the implantation sites at
autopsy relative to the standards. Also the number of implantation
sites following oestradiol treatment on the 50th day of pregnancy was
reduced relative to its treatment at earlier stages and one of these

TABLE 9.

DELAYED IMPLANTATION IN MICE OVARIECTOMISED ON THE 3RD DAY OF PREGNANCY, AND IMMEDIATELY INJECTED WITH A SINGLE DOSE OF MAP, AFTER THE ADMINISTRATION OF OESTRADIOL.

Dose of MAP (mg)	No. of mice	Laparotomy		Oestradiol (5ng) injected once on day(s)	Autopsy on day(s)	No. mice with implants (mean no.)	% of implants alive	No. of mice with implantation sites that were		No. mice without implants but with free blastocysts (mean no. blastocysts)	Remarks
		days(s)	Implants*					Oestrogen induced**	Before Oestrogen		
1.0	4	–	–	7th–11th	11th–14th	4(8.75)	68.6	4	0	0	
1.5	5	7th	0	7th–8th	11th–14th	3(11.66)	68.6	3	0	0	2 mice died at laparotomy. Both had free blastocysts.
2.0	4	7th	0	11th–14th	19th	3(10.00)	43.4	3	0	0	1 mouse died at laparotomy. It had free blastocysts.
2.5	4	7th	0	11th–13th	17th–19th	3(10.66)	34.4	3	0	1(10)	The inner cell mass was missing from one blastocyst and smaller than usual in some others.
5.0	4	7th	0	18th–21st	25th	3(11.66)	48.5	2	1	1 (8)	
10.0	4	8th	0	33rd	37th	2(13.00)	50.0	1	1	0	2 mice died between the 3rd–7th days. All live implants in one mouse.
20.0	5	8th	0	50th	54th–59th	3(4.66)	57.0	3	0	1 (4)	All live implants in one mouse. One mouse with neither implants nor eggs, had 2 endometrial cysts (equal in size to 2/3 days p.c. swellings.

* All mice were Pontamine Blue negative (except mice given 1.0mg that were not laparotomized and therefore not tested).

five mice (treated with 20.0mg of MAP) that had neither implantation sites nor eggs had two prominent endometrial cysts at autopsy.

The number of implantation sites that were probably alive at autopsy varied from 34-69%. Higher survival rates were associated with the shorter intervals between ovariectomy and oestradiol treatment.

Implantation and embryonic survival following MAP treatment after ovariectomy

Mice were ovariectomised and treated with MAP (i.e. 5, 10 or 15mg) on the 3rd day of pregnancy (Table 10). They were given a single dose of oestradiol (6.0×10^{-3}, 15.0×10^{-3} or $37.5 \times 10^{-3}\mu g$) on the 4th day, laparotomised on the 12th day and autopsied from the 12th to the 19th day of pregnancy.

The mean number of implantation sites in mice treated with 15mg of MAP was significantly ($P < 0.05$) lower than the mean of the combined groups that received 5 and 10mg of MAP. Differences in the mean numbers of implantation sites between the groups given 5 and 10mg of MAP and between the different oestradiol levels were nonsignificant.

The proportion of sites that probably contained live embryos at laparotomy was higher ($\chi^2 = 19.197$; $P < 0.001$) in mice given 15mg of MAP than in mice given the two lower doses. This proportion was not different ($\chi^2 = 2.351$; P NS) between groups given 5 and 10mg of MAP. The level of oestradiol given on the 4th day of pregnancy significantly affected embryonic survival on the 12th day. Proportional survival was lower ($\chi^2 = 24.290$; $P < 0.001$) with 6.0×10^{-3} than with $15.0 \times 10^{-3}\mu g$ of oestradiol. This difference between the combined proportion of the two lower doses and $37.5 \times 10^{-3}\mu g$ was

TABLE 10.

IMPLANTATION AND EMBRYONIC SURVIVAL AFTER TREATMENT WITH MAP AND OESTRADIOL

FOLLOWING OVARIECTOMY ON THE 3RD DAY OF PREGNANCY.

Dose of oestradiol on the 4th day of pregnancy (µg)	Dose of MAP on the 3rd day of pregnancy (mg)			
	5	10	15	Mean ± standard error
6.0×10^{-3}	2(11.00)	2(15.5)	3(10.66)	12.14 ± 1.22 44.1%
15.0×10^{-3}	4(13.00)	3(13.66)	4(11.50)	12.64 ± 0.88 78.2%
37.5×10^{-3}	3(12.00)	3(12.33)	4(8.25)	10.60 ± 1.14 65.3%
Means ± S.E.	12.22 ± 0.92 51.6%	13.63 ± 0.94 62.1%	10.09 ± 1.02 83.6%	

* Each cell contains the number of mice with implants with the mean number of these bracketed.
The % ages given in the overall means refer to the percent of implants that probably contained
live embryos at laparotomy on the 12th day of pregnancy.

nonsignificant ($\chi^2 = 0.003$; P NS).

At autopsy all embryos were dead except for three surviving singletons in mice treated with $15.0 \times 10^{-3} \mu g$ of oestradiol that were killed on the 18th day of pregnancy. Two of the latter mice received 15mg of MAP and the third was treated with 5mg. Mice aborted dead embryos from the 12th day of pregnancy onwards and both at laparotomy and autopsy implantation swellings were spaced close together. Many horns were rigid and the larger foetuses (alive and dead) occupied more than one chamber.

DISCUSSION

Synergism in the sense that a relative deficiency of either oestradiol or progesterone for egg-implantation could be compensated for by an excess of the other could not be demonstrated. Instead the evidence supports the concept of fixed minimal requirements for both oestradiol and progesterone. However the possible existence of synergism cannot be excluded as it may depend on a particular ratio of hormones that was not obtained in the present study (see Courrier, 1950; De Feo, 1967). Further, the numbers of mice used were small considering the variation encountered and the presence of an androgen contaminant in the progesterone injected in the first two experiments was a complicating factor.

The occurrence of approximately 20% 'contamination' with an androgenic substance that resembled testosterone in the Ciba brand of progesterone was unexpected. An oestrogen contaminant was considered to be the most likely to cause unexplained implantation though the negative results following 'oestrogen extraction' and the

failure to detect oestrogen after chromatographic analysis seem to obviate this possibility. There was a strong suggestion that the implantation observed in 72% of mice treated daily with 2.5mg of Ciba progesterone after ovariectomy on the 3rd day of pregnancy was due to the testosterone-like contaminant. Less than 4% of mice similarly treated but given instead 2.0 or 2.5mg of Koch-Light progesterone implanted eggs. Neither androgenic nor oestrogenic contamination was found in this compound. Furthermore, the concurrent administration of testosterone proprionate and Koch-Light progesterone induced implantation in mice ovariectomised on the 3rd day. As the level of testosterone used to induce implantation (0.75mg, Table 7) was 50% higher than should have been required if 20% of 2.5mg of Ciba 'progesterone' was a testosterone-like contaminant, further experiments are planned to establish the lowest dose required. Humphrey (1967b) has reported that 2.5mg of testosterone proprionate was sufficient as a single dose, to induce implantation in ovariectomised progesterone treated mice. It is not known if testosterone per se induced implantation or if it was converted to oestrogens as suggested by Varavudhi(1969) in the rat. However this worker considered that the ovaries were required for conversion.

It is likely that the Organon brand of progesterone was also contaminated, perhaps with a nonoestrogenic substance(s) as the incidence of implantation following ovariectomy on the 3rd day was higher after its use than after treatment with an equivalent amount of Koch-Light progesterone. A chromatographic analysis of this compound is at present being carried out. The possibility of other brands of progesterone being contaminated is probably very real as Bergstöm (1971) has observed that certain brands of progesterone

cause implantations in situations where others did not (see also McLaren, 1971).

Implantation following ovariectomy on the third day of pregnancy did however occur occasionally with high doses of Koch-Light progesterone. Vinson and Jones (1963) have demonstrated that in vitro adrenal cortical tissue can convert progesterone to oestrogens. Whether or not such conversion took place in the present studies is not known. Implantation occurs in oestrogen-deficient hamsters (Prasad, Orsini and Meyer, 1960; Orsini and Psychoyos, 1965), presumably due to the action of progesterone alone.

Treatment of mice ovariectomised on the 3rd and 4th day of pregnancy with the near physiological dose of 0.5mg daily of Koch-Light progesterone confirmed the existence of a time differential effect of ovariectomy on implantation (Yoshinaga and Adams, 1966; Humphrey, 1967b; Grant, 1968). Ovariectomy on the 3rd day prevented implantation whereas ovariectomy on the 4th allowed a full response. The intermediate response following ovariectomy on the morning of the 4th day when uteri contained both free blastocysts and implants is thought to have been due to an abrupt removal of the source of oestrogen. Variation in the developmental rate of blastocyst within the same uteri (Dickson, 1966b; Finn and McLaren, 1967; Grant, 1968) may mean that the most advanced were able to begin to implant before oestrogen became limiting. MAP behaved as 'pure' progesterone in regard to the time differential effect on implantation.

For pregnancy maintenance in rats MAP has been demonstrated as a long-acting, potent progestin (Stucki, 1958; Revesz and Chappel, 1966). The present study shows that blastocysts can remain viable after ovariectomy and treatment with MAP on the 3rd day up to the 50th

day of pregnancy.   Although this was considerably longer than

blastocysts can survive in the uteri of ovariectomised nontreated

mice (Weitlauf, 1968) both the implantation number and the survival

rate was low.   Even with shorter delay periods survival was well

below 100% and it is probable that oestrogen would be required to

improve this (see below).   The occurrence of endometrial cysts seen

on the 50th day of pregnancy in one mouse again suggests that large

doses of MAP to produce extended delay periods may be harmful.

Daughters of pregnant mice treated with MAP often develop endometrial

cysts (Grant, 1972d).   Delayed implantation induced by oestrogen

following ovariectomy on the 3rd day has been previously demonstrated

in mice (Yoshinaga and Adams, 1966; Dickson, 1969).   Yoshinaga and

Adams treated mice daily with progesterone and Dickson injected 1.0mg

of MAP on the 3rd or 8th day or 1.0mg on both of these days of

pregnancy.   Two injections of MAP was associated with larger

implantation sites on the 12th day than with a single injection.

The inference was that survival was better with 2.0mg of MAP than

with 1.0mg.   Similarly in the present work higher doses of MAP were

associated with a higher survival rate on the 12th day of a non-

delayed pregnancy (Table 10).   There was however a drastic increase

in the embryonic mortality rate after this irrespective of the dose

of MAP.   The failure of MAP to maintain pregnancy was probably not

due to insufficient progestin being available.   Half the smallest

dose of MAP (i.e. 2.5mg) used in the present study would maintain

foetal survival until the 20th day of pregnancy after ovariectomy

and MAP treatment on the 7th day of pregnancy, provided daily

oestradiol was administered (Grant, 1972d).   Further, 2.5mg of MAP

was sufficient to maintain an entire pregnancy after egg transfer

to virgin mice treated with MAP and oestradiol.   The ovaries were

the probable source of oestrogen in these mice (Grant, 1972c).

Foetal survival in progesterone treated rats (Stucki, 1958) and mice

(Humphrey, 1967a; Grant, 1968) was improved by concurrent oestrogen

treatment.   The uterine rigidity associated with the close spacing

of implantation chambers observed after MAP treatment resembles the

uterine 'hypertonicity' described in ovariectomised progesterone

treated rats (Carpent, 1962) and mice (Grant, 1968).

The reduction in the number of implantation sites following

treatment with 15mg of MAP compared with lower doses may have been

in part due to MAP toxicity.   Although MAP has been demonstrated

to be toxic in the rat this effect seems to operate to reduce

survival of post-implantation embryos (Barnes and Meyer, 1964;

Nutting and Sollman, 1967).

The lowest dose of oestradiol (i.e. 6.0×10^{-3} µg, Table 10)

injected on the 4th day of pregnancy following ovariectomy and MAP

treatment on the 3rd day was associated with reduced embryonic

survival later in pregnancy compared with 15×10^{-3} µg of oestradiol.

There was however no difference in the number of implantation sites

induced by these doses of oestradiol.   This may indicate that

6.0×10^{-3} µg was too low a dose and that the implantation rate per

se was less sensitive as an indicator of hormonal imbalance at the

time of implantation than subsequent survival.   Embryonic survival

was believed to be a more sensitive measure of optimal uterine

sensitivity than implantation following egg transfer to virgin mice

treated with MAP and oestradiol (Grant, 1972c).

APPENDICES

APPENDIX 1.

Autotransplantation of small lengths of uterine horn in pregnant mice

INTRODUCTION

A basic requirement for an _in vitro_ implantation system is a method of culturing blastocysts in contact with uterine epithelium. Such a system may have more relevance to the _in vivo_ situation if the normal relationships of the constituent uterine tissues are maintained (Lasnitzki, 1965). Thus a simple starting position for these studies would be to assess the possibility of culturing small lengths of uterine horn taken from pregnant mice at a stage when the blastocysts were free and spaced within the uterine lumen or when they were beginning to implant. However the diameter of such uteri is large and difficulties could be expected in the supply, necessarily by diffusion, of nutrients and oxygen to the centre of the explant (Trowell, 1959).

It was therefore decided to autotransplant lengths of uteri from pregnant mice into various situations on the assumption that adequate survival of the uterine lengths would be most likely in body fluids of host mice.

MATERIALS AND METHODS

Q strain mice at $3\frac{1}{2}$ days and $4\frac{1}{2}$ days post coitum (pc) were anaesthetised with sodium pentobarbitone ('Nembutal', Abbott) and laparotomised. Those at $4\frac{1}{2}$ days pc were injected intravenously with 0.20ml of 0.5% of Pontamine Blue (5BX) (Gurr) to locate regions of uteri where eggs were beginning to implant (Finn and McLaren,

1967). The broad ligament was cut near its attachment onto the uterus so as to free a length of uterine horn. This free length of horn was then cut into lengths measuring 2-5mm which included the blue areas of $4\frac{1}{2}$ day pc uteri. The small lengths were then put back into the peritoneal cavity away from the cut ends of the parent uterine horn. A number of small lengths were pushed against a shallow cut in the spleen. Other small and the larger lengths were positioned subcutaneously. The skin wounds were then sutured and mice were autopsied three to eight days later. At autopsy the lengths of uteri were removed and fixed in formol-saline, sectioned at 8μ and stained with haematoxylin and eosin. The survival of the lengths of uteri was subjectively assessed by estimating the percentage of viable cells on histological sections.

RESULTS

Twenty seven lengths of uteri from 15 mice were sectioned and the results are shown in the Table. Six mice that contained nine lengths were not pregnant at autopsy. Mean survival estimates for lengths from pregnant and nonpregnant mice at autopsy were 37% and 30%,respectively.

Lengths transplanted subcutaneously and against the cut surface of spleen survived better than those put back into the peritoneal cavity.

Eleven lengths transplanted at $3\frac{1}{2}$ days (pc) contained decidual tissue at autopsy, whereas none of the five Pontamine Blue positive lengths from the $4\frac{1}{2}$ day pc uteri had decidual cells. Lengths with decidual tissue had a higher mean survival rate (56%) than those

TABLE

AUTOTRANSPLANTATION OF SMALL LENGTHS OF UTERI

BEFORE AND AT THE BEGINNING OF IMPLANTATION IN PREGNANT MICE

		No. of lengths transplanted	No. of mice	Mean % survival	No. with deciduomata	No. with embryonic tissue
Blastocyst age at autotransplantation (days)	$3\frac{1}{2}$	22	12	38	11	2
	$4\frac{1}{2}$	5	3	23	0	0
Interval to autopsy (days)	3	3	2	9	0	0
	4	3	2	19	3	0
	5	19	10	42	8	2
	8	2	1	43	0	0
Position of transplant						
subcutaneous		6	4a	50	3	0
spleen		4	4a	40	2	1
omentum		17	12a	29	5	1
Host at autopsy						
pregnant		18	9	37	4	2
nonpregnant		9	6	30	3	0

a – Some mice contained more than one transplanted length of uterus.

without decidual tissue (21%).

Live embryonic tissue was seen in two lengths that were trans-
planted at $3\frac{1}{2}$ day pc and autopsied five days later.   One length
was attached to the spleen and had about 30% of its cells alive and
the other was attached to omental fat and had about 75% of its cells
alive.   Although both lengths contained healthy decidual tissue
their trophoblast giant cells were not in contact with this but
were surrounded by dead or dying stromal tissue and many red blood
cells (Fig. 1).   The length attached to the spleen contained a
small abnormal embryo that was about 1/3 of the size of a normal 9th
day pregnant embryo.   Unhealthy embryonic ectoderm and endodermal
cells were surrounded by a thick Reichert's membrane (Figs. 1 & 2).
Most of the giant cells were healthy.   The length attached to the
omentum contained healthy and unhealthy giant trophoblast cells only.

DISCUSSION

The outermost muscle cells survived in all autotransplanted
lengths and in those with no decidual cells a variable amount of
central stromal tissue and often part of the inner muscle layer was
dead.   Healthy luminal epithelium was only present when the under-
lying stroma was decidualized.   The dead tissue was usually replaced
with numerous red blood cells.   Variable numbers of leucocytes and
pyknotic nuclei also occupied the remaining connective tissue
framework.   Occasionally the lumina of the lengths were distended
with sanguineous fluid.   Unless the stromal tissue was decidualized
it degenerated and was infiltrated by red blood cells and macrophages.

The position of autotransplantation affected the subsequent

survival of the lengths. Those positioned subcutaneously or attached to the spleen survived better and were usually better vascularized than those attached to the omentum. Also, perhaps as a consequence of better vascularization, a higher proportion of the lengths placed subcutaneously and attached to the spleen (five of ten) had decidual tissue than lengths attached to the omentum (five of 17).

Survival of lengths was no better in mice that remained pregnant than in those that did not do so after the transplantation operation. Although the two lengths that contained embryonic tissue were found in mice that remained pregnant the numbers are too small to determine whether or not this was by chance alone. Eggs will develop in extrauterine sites irrespective of the hormonal status of the host (Fawcett, Wislocki and Waldo, 1947; Kirby, 1970). Eggs enter the uterus at about three days pc (Lewis and Wright, 1935) so there was a reasonable chance that lengths removed 12 hours later would contain eggs. The extensive central necrosis and the host reaction that this precipitated probably both mitigated against implantation and further development. Further, it is not possible to know if the eggs that did develop invaded through the luminal epithelium or whether they attached to cut ends of the lengths of uteri.

When seen, decidual cells were usually healthy and viable and lengths with decidual cells were seen in all transplantation positions and in mice that were both pregnant and nonpregnant at autopsy. In two lengths cell survival was very good and comparable with that of tissue from the undamaged control horns. However it is uncertain as to whether all decidual tissue survived. One length had only two viable decidual cells surrounded by dead stromal

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tissue and another had dead multinucleate cells which may have been decidual or trophoblast cells. Despite this uncertainty, it is clear that stromal cells that decidualized survived very much better than those that did not. Mechanical damage associated with the cutting of the horns into lengths was the probable decidualizing stimulus. None of the five lengths transplanted at $4\frac{1}{2}$ days post coitum had decidual tissue whereas 11 of the 22 transplanted at $3\frac{1}{2}$ day pc did so. Stimuli on the 4th day pf pregnancy are the most effective for inducing deciduomata (Finn, 1965). Evidently the Pontamine Blue positive lengths of uteri transplanted at $4\frac{1}{2}$ day pc did not continue to decidualize. The subsequent degenerative changes probably killed the embryos these areas would be expected to contain (Finn and McLaren, 1967).

Although both lengths that contained embryonic tissue also contained decidual tissue this was not adjacent to the trophoblast cells which were situated in areas where most of the cells were dead. (Fig. 1). It is uncertain whether the trophoblast tissue had a predilection for dead tissue or whether the host tissue died as a result of trophoblast activity.

In conclusion autotransplanted lengths did not survive well unless decidualization of the stroma occurred and decidual cells were seen in less than half of the transplanted lengths. The central stromal necrosis observed may not have been due simply to the diameter retarding diffusion of nutrients and oxygen to the centre of the lengths. As decidualized lengths had larger diameters but much better survival than nondecidualized lengths, thus the relevance of the survival results of autotransplanted lengths to what may be expected in vitro is obscure. However it is probable

that the diameter of lengths from pregnant mice would be too large

to culture _in vitro_ and it would seem unrealistic to expect decid-

ualization on the scale seen _in vivo_.

Fig. 1.   Photomicrographs of histological sections after staining

with haematoxylin and eosin.   The small abnormal 9th day

embryo (E) surrounded by healthy trophoblast giant cells

(T) which are invading necrotic spleen tissue (S).   x 400.

Fig. 2.   A higher power view of the embryo surrounded by a

prominent Reichart's membrane (R).   T = trophoblast giant

cells and rbcs = red blood cells.   x 800.
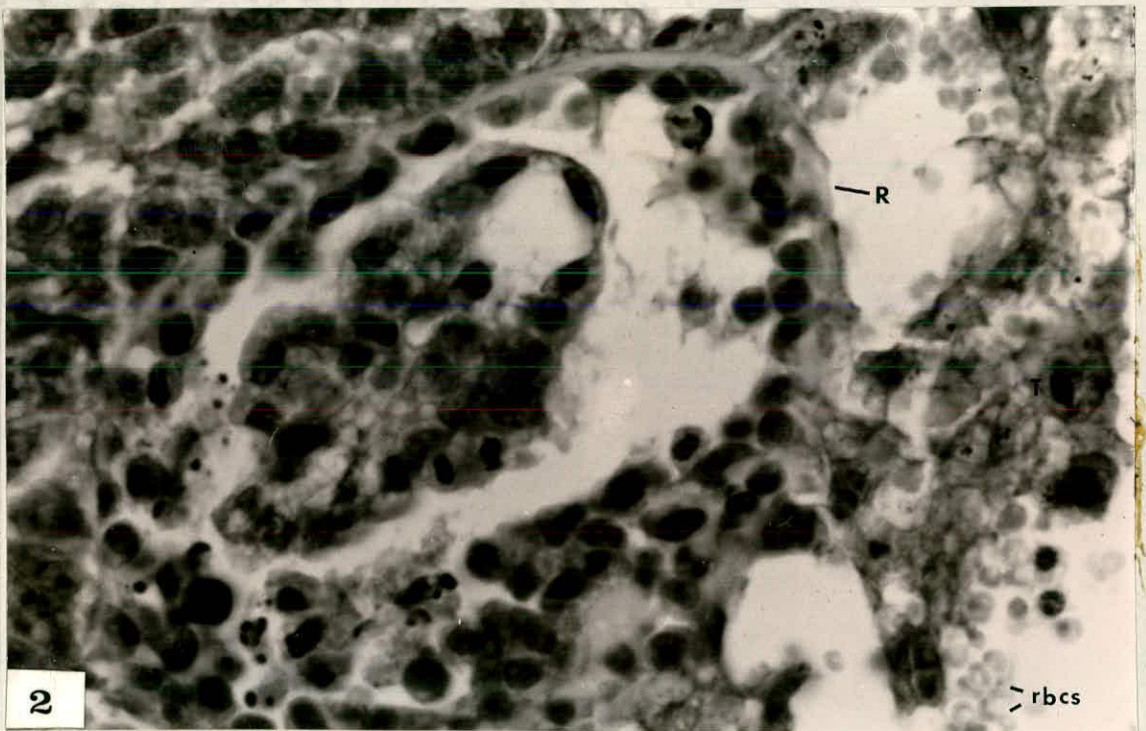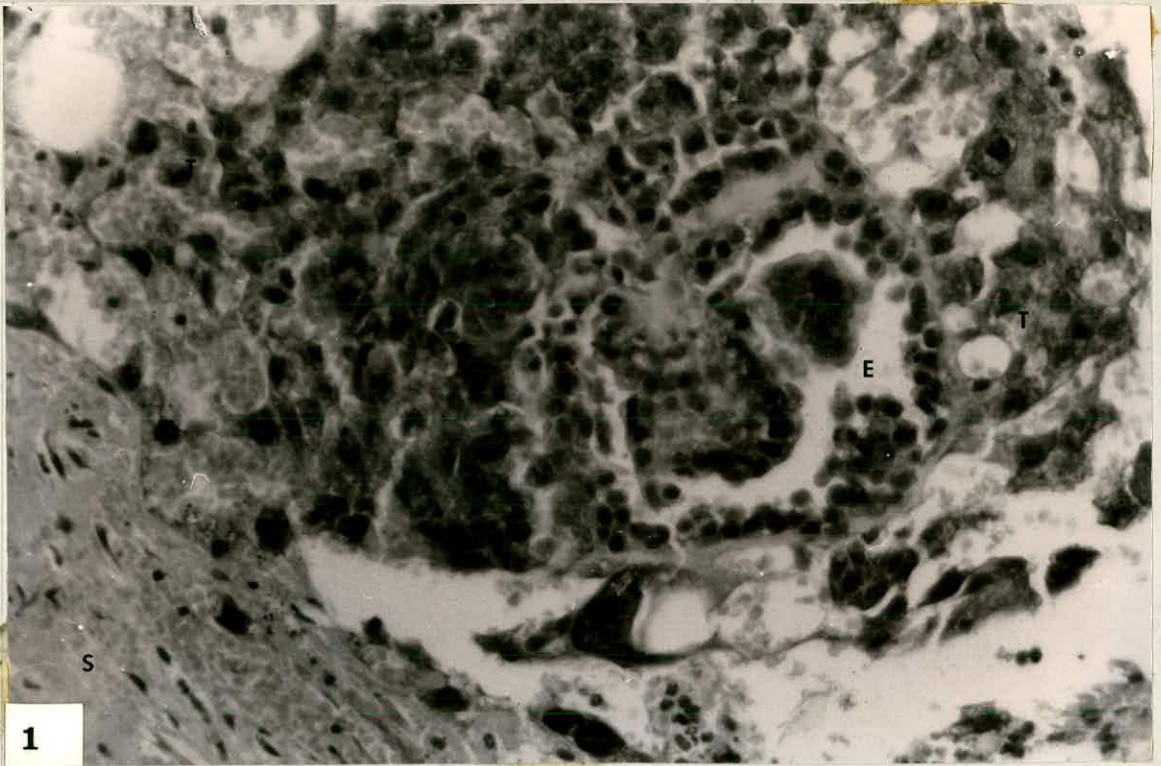
APPENDIX 2.

Development of a culture system to study egg implantation in vitro

INTRODUCTION

An outline is presented here of the methods and materials used, most of which were unsuccessful, in attempts to culture blastocysts in contact with uterine epithelium. General proficiency in culture techniques increased with time, to the disadvantage of the procedures used earlier.

The first requirement was to achieve adequate survival of uterine tissue after at least two days in culture. In retrospect the survival of about 70% of the cells initially present in the explant was considered adequate. The culture techniques used are described elsewhere (Grant, 1972a and b) and were based on those developed by Trowell (1954; 1959) and Baker and Neal (1969).

Parallel to the above studies attempts were made to establish what type of blastocyst would be suitable, and what methods of blastocyst culture were compatible with the most successful methods for uterine culture.

Uterine culture

1. Opened out lengths of uterine horns. Uterine horns from pregnant, cycling and ovariectomised mice were cut into small lengths measuring 1-3mm. These were then slit open and cultured with their epithelial surfaces uppermost on the supporting grids. The aim was to culture blastocysts on the epithelial surface.

This method of culture had the following disadvantages:

a). Tissue damage during the preparation of explants.

The uterus is a tough elastic organ which is difficult to cut into small lengths and the tissue present near the borders of explants usually survived least well after culture.

b). Myometrial activity.

Myometrial contractions continually humped the epithelial surface and attempted to reclose the lengthwise cut of the lengths of uteri.   Consequently the explants did not initially lie flat on the grid.   Further, the explants moved about during culture, sometimes falling off the edge of the grid and drowning.

These contractions made it difficult to place and keep eggs on the epithelial surface.

c). Fluid surface tension on the explants.

When the media height was optimal for survival of the explants there was a thin film of media extending over the epithelial surface.   Unless eggs could be put in surface depressions they were rapidly removed by the surface tension of the media over the explant.   Glenister (1971) found that hamster eggs were lost in this way from the surface of endo-metrial strips.   Eggs could be put on such explants by adjusting the level of the media so that it was considerably higher than the surface of the explant and allowing them to sink down into surface depressions.   The excess media was then drawn off.   Occasionally an egg remained on the explant for the duration of culture, but these were washed off by the dilute Nile Blue Sulphate added to stain eggs for identifi-cation prior to fixation.

d). Survival according to the endocrine state of the donor.

Opened out lengths from mice on the 4th or 5th day of pregnancy were too thick to culture adequately. They usually developed extensive central necrosis. Nor did lengths from mice taken on any day of the oestrous cycle regularly survive well. Those removed during diestrus survived best.

Lengths from adult mice that had been ovariectomised one week or more prior to their culture often showed adequate survival. The best survival was obtained after the culture of lengths that were cut from a uterine horn slit open in vivo two to three days prior to culture in mice that had been previously ovariectomised.

Lengths flattened and became thinner during culture, a process which tended to eliminate initial surface depressions. The thickness of the epithelium varied considerably and was often columnar in central and cuboidal in the outer regions.

e). The effect of explant survival on blastocysts.

A few eggs remained on the cut surfaces of segments of explanted kidney tissue and attached to explanted strips of ventral abdominal wall muscle during culture. As the survival of these tissues was not superior to many explants it was concluded that inadequate survival per se did not necessarily kill the blastocysts and that they were preferentially lost from uterine explants.

2. The culture of intact uterine horns. Uterine horns from long-term ovariectomised adult or from immature mice could be maintained in culture so that survival was adequate for three days: uterine horns from ovariectomised and immature mice have previously been

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cultured by Lostroh (1963; 1964; 1966). However a disturbingly high proportion had unilateral off-central necrosis affecting the stromal tissue nearest the grid and often extending to part of the luminal epithelium. This necrosis probably resulted from oxygen lack (see Trowell, 1959) and was usually prevented by stretching the uterine horns taut and pinning them down onto the grid in this position. The advantages of this method were:

a). The stretching of the horns reduced the diameter and lessened the incidence of off-central necrosis.

b). The horns could be positioned at the correct height relative to the grid (see Trowell, 1959).

c). Pinning prevented movement about the grid (see Trowell, 1959).

d). Eggs injected into the horns were not subject to drying out or surface tension problems and their position within the lumina of uterine horns was more akin to the situation in vivo.

e). Also the pins prevent the uterine contractions from expelling the injected eggs.

f). The culture of uterine horns from immature mice did not require prior treatment of the donors and was routinely adopted.

3. The culture of lengths from which the myometrium had been removed. Theoretically the removal of the myometrium before the culture of lengths should lessen the chance of central necrosis by allowing thinner explants, secondly it would prevent troublesome movement of the explants.

However, survival was very poor after part of or all of the

myometrium had been removed.   The techniques used for removal of the myometrium were clumsy and probably inflicted considerable damage on the stromal and epithelial tissues.   Glenister has successfully cultured strips of rabbit and hamster (Glenister, 1961; 1971) endometrium, and Everett (1962) was able to culture strips of guinea pig endometrium.   Thus the adoption of less traumatic methods probably would have allowed the successful culture of strips of mouse endometrium.

Culture medium

The following culture media were tested for organ culture of uterine explants and the outgrowth of blastocysts (Gwatkin, 1966) maintained in drops of media under oil (Biggers, Whittingham and Donahue, 1967):   Eagle's, Basal and Minimum Essential (Standard and Glasgow Modification), Medium 199 and Trowell's T_8 medium. All media were supplied by Flow Laboratories Ltd., Ayrshire.

Uterine explants survived best in Trowell's T_8 medium.   Eggs would outgrow most readily in Eagle's Basal medium with added foetal calf serum.   They also outgrew in Eagle's Minimum Essential (Standard) and in Trowell's T_8 medium provided foetal calf serum was present.

1. The effect of foetal calf serum.   Levels of foetal calf serum from 0-20% were added to Trowell's T_8 medium for the culture of uterine explants.   In specific experiments to test the effect of the level of foetal calf serum on explant survival no consistent differences were obtained.   Adequate survival was possible in the absence of serum.   However, an analysis of results over many experiments showed that serum probably helped survival and in any case was required for blastocyst outgrowth.   As egg 'implantation'

was observed first in cultures with 20% foetal calf serum, this
level was chosen in subsequent experiments.

2. The effect of hormones.   Hormones were added to Trowell's T_8
medium at the following levels, 0.1, 1.0, 2.0, 3.0, 4.0, 5.0 and
10µg/ml of progesterone, and 0.03, 0.1, 1.0, 2.0, 3.0, 4.0 and
5µg/ml of oestradiol.   Also both progesterone and oestradiol were
added together at levels of 0.1, 1.0, 2.0, 3.0, 4.0 and 5µg/ml
each hormone per ml and 10µg of progesterone with 1 and 2µg of
oestradiol/ml of culture medium.   At none of the levels used did
any hormonal treatment have an obvious beneficial effect on the
survival of cultured uteri.

The addition of oestradiol to the media for outgrowing blasto-
cysts (5 or 10µg/ml Trowell's T_8 medium) decreased the time taken
for eggs to attach and begin to outgrow, but the differences were
not significant.   The highest level of oestradiol, i.e. 10µg/ml of
media, slowed the rate of spread of the outgrowths relative to that
observed with 5µg/ml of oestradiol.   This measurement was not
different for the control versus oestradiol treatments comparison.
The effect of progesterone on outgrowing eggs was dependent on the
treatment of the blastocyst donor mice and on the dose.   Proges-
terone treatment (5 or 10µg/ml) speeded the attachment and the high
dose increased the rate of outgrowth of blastocysts flushed on the
5th day from pregnant mice ovariectomised on the 4th day.   Proges-
terone (5µg/ml) did not speed attachment of blastocysts flushed from
mice ovariectomised and given 2.5mg of MAP ('Depoprovera', medroxy-
progesterone acetate; Upjohn) on the 3rd day of pregnancy.   Proges-
terone alone (5µg/ml) and together with oestradiol (5µg/ml) slowed
the rate of outgrowth.

The hormonal treatment in use at the time when eggs first 'implanted' was 5μg/ml of culture media of both progesterone and oestradiol.   These levels were not changed in subsequent experiments although in some treatments only one hormone was used.

The oxygen concentration for culture

Cultured uterine explants survived well in an atmosphere of 95% O_2 and 5% CO_2.   However eggs did not survive well in this atmosphere.   Although blastocysts flushed on the 5th day from mice ovariectomised and given MAP on the 3rd day of pregnancy will outgrow, they did so slowly and contained dead cells.   Blastocysts flushed on the 5th day after ovariectomy on the 4th day of pregnancy usually did not outgrow at all in 95% oxygen.   Both types of blastocysts grew well in 95% air and 5% CO_2.

Around 30% oxygen allowed blastocysts from ovariectomised, MAP-treated mice to outgrow quite well and usually was adequate for uterine survival.

Blastocysts for culture

When cultured in drops of media under oil, blastocysts flushed on the 4th day of pregnancy required about four days in culture before they all outgrew.   Blastocysts flushed on the 5th day from mice ovariectomised on the 4th day of pregnancy usually took three days to reach this stage of development.   Blastocysts flushed on the 5th day from mice ovariectomised and given MAP on the 3rd day of pregnancy, usually outgrew after only two days in culture. Thus the latter class of blastocysts were preferable from the point of view of time taken to begin outgrowth.

Of the blastocysts from ovariectomised mice, those from MAP-

treated donors survived higher oxygen levels. The speed at which they attached and started to outgrow was not affected by progesterone or oestradiol. This suggested that they were healthier at the beginning of culture than blastocysts from non-treated ovariectomised mice. Consequently this type of blastocysts was chosen for subsequent culture experiments.

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