OVA-IMPLANTATION, EMBRYONIC SURVIVAL AND EMBRYONIC SPACING IN OVARIECTOMISED MICE AFTER PROGESTERONE AND OESTROGEN TREATMENT

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GENERAL INTRODUCTION

This investigation is concerned with the ovarian hormonal requirements for ova-implantation, post-implantation survival, and the spacing, of the developing young within the uterus of the ovariectomised pregnant mouse. Most attention is directed towards establishing the effectiveness of the ovarian hormone progesterone in maintaining pregnancy in mice ovariectomised before ova-implantation. Emphasis is placed on the time relationships between the start and interruption of normal pregnancy by ovariectomy. The establishment of continued pregnancy in ovariectomised mice with progesterone treatment then allowed a study of the survival rates and of the spacing of embryos within an altered maternal hormonal environment.

Accordingly the thesis presentation is divided into the three sections:

I. the ovarian hormonal requirements for ova-implantation;

II. the ovarian hormonal requirements for post-implantation survival;

III. the ovarian hormonal requirements for embryonic spacing within the uterus.

Section I includes a subsection (section I(a)) which deals with the time of ova-implantation in intact mice and a consideration of the health and mating behaviour of the experimental mice.

The purpose of this introduction is to provide a general framework on which each of the outlined aspects of reproduction can be more closely examined.

1. The hormonal Requirements for Ova-implantation

(a) Neuroendocrine considerations

The secretion of the main ovarian hormones, the oestrogens, oestradiol and oestrone, and the progestagen, progesterone (Pincus, 1965a) is under neuroendocrine control.

The ovary functions as an integral part of a complex neuroendocrine hierarchy, the hypothalamo-hypophyseal-gonadal axis (Everett, 1961). Evidence for the neural control of the ovary has been reviewed by Harris (1960), Everett (1961, 1964) and Guillemin (1964). Neural directions to the ovary are indirect and are initiated from the hypothalamus. Small polypeptides, or releasing factors, capable of stimulating the release of the hypophyseal gonadotropins, luteinizing hormone (LH) (Schally and Bowers, 1964) and of follicle stimulating hormone (FSH) (Igarashi, Nallar and McCann, 1964) have been isolated from beef and rat hypothalamic extracts, respectively. The releasing factors are probably synthesized in the hypothalamic neurones and released by neurosecretion into the hypophyseal vascular portal system to reach the adenohypophysis (B. Scharrer, 1967).

Of the two gonadotropins, FSH is considered to be primarily responsible for the phase of follicular growth (reviewed by Rowlands and Parkes, 1966) and an accelerated release of LH is probably the stimulus responsible for ovulation (reviewed by Everett, 1965; Ramirez and Sawyer, 1965).

Prolactin, the principal luteotropic hormone in rats and mice (Rothchild, 1966) is released from the adenohypophysis probably in response to a hypothalamic stimulus evoked during mating (Everett and Quinn, 1966). Prolactin is needed before the corpora lutea become competent to secrete sufficient progesterone to allow implantation to occur (reviewed by Eckstein and Zuckerman, 1956).

Although it is probable that both FSH and LH are required for the

secretion of oestrogen from the ovaries of the immature mouse (Eshkol and Lumenfeld, 1967) and immature rat (Lostroh and Johnson, 1966), LH alone was sufficient for the secretion of this hormone from the luteinized ovaries of the mature rat (Macdonald, Armstrong and Greep, 1966).

(b) Pré-implantation phenomena

After successful fertilization, the egg is subjected to a period of tubal passage during which cleavage occurs, and a period of intra-uterine existence during which development and growth of the blastocyst supervenes. While the rate of cleavage is independent of ovarian hormonal influence (Brinster, 1963) the rate of tubal passage is liable to ovarian hormonal changes. The effects of oestrogen and progesterone on tubal transport have been reviewed by Austin (1963), Adams (1965) and Pincus (1965b). Exogenous oestrogens can either speed, slow or stop ('tube-lock') egg transport (see Chang and Harper, 1966) while progesterone probably acts to slow transport during normal pregnancy. Egg transport is apparently normal in ovariectomised rats (Alden, 1942a) and in ovariectomised mice (Smithberg and Runner, 1956). The correct timing of the arrival of the eggs into the uterus is crucial if further development is to be successful. Premature arrival may lead to expulsion through the vagina, while delayed entry in the rat precluded the uterus from responding in a favourable way to the presence of the blastocyst (Psychoyos, 1966).

After entry into the uterus the eggs are spaced throughout the length of the uterine horns and in the mouse each comes to occupy an antimesometrial crypt (Snell, 1956). Ova spacing in the rabbit is about even and is thought to be an ordered event, in that the blastocysts and the uterus each play a part (Böving, 1956). There is some doubt as to how evenly the blastocysts are spaced in the mouse and uterine movements have been considered to play the major role in spacing the blastocysts (McLaren and Michie, 1959;

Wilson, 1963).

The intra-uterine requirements for ova-implantation are strict, both with regard to the development status of the ovum and the stage of hormonal preparation of the uterus. This is in marked contrast to the relative ease with which mouse ova will 'implant' in extra-uterine sites regardless of the endocrine condition of the host (Fawcett, Wislocki and Waldo, 1947; Kirby, 1965a).

In the nonlactating pregnant mouse and rat, the ova and the uterus develop in phase with one another. In these species progesterone acting after, though synergistically with oestrogen is the normal sequence of hormonal action during the period of free ovum existence (Courrier, 1950). The progesterone-dominated phase is characterised by endometrial proliferation, enhanced glandular secretion of 'uterine milk' and by reduced myometrial activity (see reviews by Mayer, 1960; and Reynolds, 1965). Rabbit blastocysts before implantation are rich in coenzymes and vitamins and are capable of a marked degree of metabolic selectivity (Lutwak-Mann, 1963). Ovariectomy immediately prior to implantation was associated with obvious disturbances in the metabolic behaviour and morphological changes in rabbit blastocysts and produced failure of implantation (Lutwak-Mann, Mays and Adams, 1962).

Implantation in several species (e.g. the mouse, rat and rabbit) is associated with the proliferation and differentiation of the uterine stromal connective tissue to form decidual tissue which ultimately provides an implantation chamber for each ovum (see reviews by Mossman, 1937; Amoroso, 1952; and Shelesnyak and Kraicer, 1963). Deciduomata, as decidual tissue localizations invoked by methods or agents other than implanting blastocysts are known, may be produced in the uteri of pregnant or pseudopregnant mice and rats by a variety of artificial stimuli during

a limited period of the pre-implantation, or progestational stage of pregnancy. It is during this limited period of uterine sensitivity that changes associated with implantation are initiated. Both in the rat and the mouse maximum uterine sensitivity to decidualizing stimuli follow the action of both progesterone and oestrogen (De Feo, 1963; Shelesnyak and Kraicer, 1963; and Finn, 1965, 1966a, respectively).

The function of decidual tissue is obscure and has been discussed by McLaren (1965). It may play a nutritive role as far as the blastocyst is concerned and a defensive role in protecting the uterus from trophoblastic invasion.

Oestrogenic and progestational hormones may not normally act independently of one another. Evidence for synergism and antagonism between the two hormonal groups has been discussed by Courrier (1950) and Hisaw and Hisaw (1961). Experiments have shown that the results of the interactions depend on both the absolute amounts and the ratio of oestrogen to progesterone present, the duration of the action, the target tissue and the species under consideration.

Pincus (1965c) has discussed the effectiveness of many steroidal hormones and nonsteroidal compounds in interrupting the progestational phase of early pregnancy. One of the most potent groups of antifertility agents are the natural oestrogens. Others active in this way are the so-called antioestrogens, pro-oestrogens, antiprogestagens and to a lesser extent progestagens. Their effectiveness would appear to result from their ability to alter the delicate balance and sequence of action of the endogenous ovarian hormones, either due to their own effects and/or to interference with the actions of endogenous oestrogens and progestagens. Among these substances can be found examples that inhibit proliferation of the endometrium, promote alterations in the motility of the

reproductive tract, inhibit deciduogenesis and alter the nature of tubal and uterine secretions.

(c) Methods of investigating the hormonal requirements for ova-implantation in rodents and the 'oestrogen surge' hypothesis

The methods used are broadly divisable into two main groups: studies concerned with normal pregnancy and its physiological variants; and studies involving the interruption of pregnancy and the dissociation of the blastocyst/uterus relationship.

Ovarian hormonal levels have been estimated during normal pregnancy by the largely subjective method of noting changes in the morphological characteristics of the reproductive tract and comparing these with the known changes in the response of these tissues to oestrogens and progesterone (reviewed by Deanesly, 1966). With the advent of more sensitive biochemical methods attempts have been made to estimate directly the plasma levels of progestagens in the pregnant rat (Fajer and Barraclough, 1967). Plasma oestrogen levels are low during pregnancy and Grota and Eik-Nes (1967) were unable to detect these with any accuracy during late pregnancy and early lactation in the rat.

Delayed implantation, during which the blastocysts remain free in the uterine lumen for an extended period, occurs naturally in some species and in lactating pregnant mice and rats. The length of the delay is roughly proportional to the number of suckling young and for lactating mice implantation is delayed about one day for each pup suckled (Enzmann, Saphir and Pincus, 1932; Turpeinen, 1943). Pregnant lactating mice have been used in attempts to define the hormonal needs for blastocyst survival and implantation (Bloch, 1958, 1959, 1965; Whitten, 1955, 1958).

Experimental studies on how ovarian hormones are involved in ova-implantation have concentrated on the uterus alone, to a lesser extent on

the egg alone and on the reciprocal relationships between the uterus and the blastocyst. Reversible interruption of implantation has been achieved by removal of or by altering the functioning of the endocrine glands that constitute the hypothalamo-hypophyseal-gonadal axis.

The artificial induction of delayed implantation in the rat has been responsible for much of the information that has accrued concerning the hormonal requirements for ova-implantation in this species. This work has been reviewed by Mayer (1963) and Psychoyos (1966). Implantation can be delayed by manipulation of the hypothalamo-hypophyseal-gonadal axis before the afternoon of day 3* of pregnancy so that the uterus remains in a 'neutral' progestational state. Maintenance of this progestagen-dominated phase can be effected by ovariectomy and the administration of exogenous progesterone (Cochrane and Meyer, 1957; Mayer 1959; Nutting and Meyer, 1963).

Hypophysectomy and autotransplantation of the hypophysis to a site remote from the hypothalamus (Everett, 1956) or the administration of tranquilizers (Psychoyos, 1963; Mayer, 1965) before day 3 of pregnancy delays implantation and both these procedures are compatible with the continued release of prolactin which in turn maintains ovarian progesterone secretion. The delayed blastocysts, free in the uterine lumen do not immediately die, in fact Cochrane and Meyer (1957) observed that blastocysts were maintained in a viable state for 45 days and Mayer and co-workers

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* The day on which evidence of mating is found (e.g. a vaginal plug, or sperm found in vagina) is defined as day 0. Results of other workers are adjusted to coincide with this usage.

found that rat blastocysts remained alive in the uterus for up to seven days in the absence of either ovarian or adrenal hormones (Mayer, 1959). Implantation can be brought about during the period of delay by the concurrent administration of progesterone and a small single dose of oestrogen.

From these and other studies there has arisen the concept of a neuroendocrine hierarchal control of ova-implantation (see reviews by Mayer 1965; Shelesnyak and Kraicer, 1963). The hypohysis, presumably under the influence of hypothalamic releasing factors, in particular LH - releasing factor releases LH which in turn acts on the ovary long enough to cause an ephemeral discharge of oestrogen late on day 3 of pregnancy. Oestrogen acting during the progestational phase of pregnancy and in particular an 'oestrogen surge' (proposed by Shelesnyak, 1959, 1960; see also Shelesnyak and Kraicer, 1963) is regarded as essential for implantation in the rat and causes the uterus to change from what Psychoyos (1966) calls the 'neutral' to the 'receptive' state and is also associated with changes in form of the blastocyst (Yasukawa and Meyer, 1966). Work by Macdonald, Armstrong and Greep (1967) and Hayashida and Young (1965), supported the involvement of the hypothalamus and in particular the release of LH in rats. The former authors found that rats that were hypophysectomised on day 1 of pregnancy and had their pituitaries autotransplanted were subsequently able to implant ova when given exogenous LH. While Hayashida and Young inhibited implantation by the daily injection of an antiserum to LH for 5 days, starting immediately after breeding. Studies by Zeilmaker (1963) and Psychoyos (1963) during which hypophysectomy and tranquilizers, respectively, were used to determine the time limits of pituitary involvement in implantation, showed that inactivation of the pituitary had to be completed before day 3 of pregnancy if implantation was to be delayed under conditions that allowed continued

progesterone secretion. The earliest time after which ovariectomy did not delay implantation, again in the advent of progesterone being available, was about 12 hours later (Mayer, 1963). This suggests that there is a definite time ordered sequence of action of the neuroendocrine factors controlling ova-implantation.

Comparatively, the mouse has been subjected to fewer investigations than the rat. However, recent work by Bindon and Lamond (1968) would suggest that as in the rat, pituitary involvement occurs during a definite period of early pregnancy. Hypophysectomy before this time together with progesterone administration prevented ova-implantation, while the same treatment later on in pregnancy was compatible with implantation. The effect of ovariectomy on implantation in the mouse is also less certain than in the rat and will be dealt with in detail elsewhere.

Ova transfer experiments with ovariectomised recipients have been used to establish the hormonal needs for subsequent implantation in the mouse (Humphrey 1967; Smith 1966) in the hamster (Orsini and Psychoyos, 1965) and in the rat (Dickmann, 1967).

Pseudopregnant animals have been used extensively to investigate the role of the uterus in ova-implantation, especially with regard to the onset, duration and hormonal control of uterine sensitivity to decidualizing stimuli in the rats (Shelesnyak and Kraicer, 1963; Shelesnyak, 1965; De Feo, 1963; Yochim and De Feo, 1963; Finn and Keen, 1962) and in the mouse (Finn and Hinchliffe, 1964, 1965; Finn, 1965, 1966a). The assumption generally made was that the maintenance of the corpora lutea of pseudopregnancy allowed the establishment of a hormonal environment simulating that of normal pregnancy. There are however, many ways in which to induce a state of pseudopregnancy and there is evidence to suggest that the actual endocrine conditions prevailing in pseudopregnant rats differ according to

the methods used to induce this state (Banik and Ketchel, 1965; Fajer and Barraclough, 1967). Further, the hormonal requirements for deciduomata formation may differ according to the decidualizing stimulus employed (Finn, 1965).

The development of ova in ectopic sites has allowed emphasis to be placed on the egg in the absence of the uterus (Fawcett, Wislocki and Waldo, 1947; Kirby, 1965a, b, 1966). Dickson (1966a) has studied changes in the form of the implanting blastocyst and compared these with changes occurring after ovariectomy in the mouse (Dickson, 1966b, c). Also Yasukawa and Meyer (1966) have noted oestrogen-dependent changes in the shape of rat blastocysts prior to implantation.

2. Embryonic Survival

Variations in the reproductive performance of the female mouse can result from changes in the number of eggs ovulated, the fertilization rate, the implantation rate and in the embryonic and foetal survival rates. This present study is concerned with the implantation rate and post-implantation survival until day $12\frac{1}{2}$ of pregnancy when the endocrine balance has been upset by ovariectomy before implantation and attempts made to correct this state by the administration of exogenous hormones. Emphasis is placed on the relative rates of survival between intact and ovariectomised pregnant mice rather than on the magnitude of the prenatal losses. An approximate guide as to the efficiency of reproduction can be derived from a comparison between the numbers of corpora lutea and the number of implanted embryos. Further, of the many casual factors able to lower the efficiency of reproduction only those referable to a defective maternal endocrine balance will be considered.

(a) Pre-implantation Survival

Social influences, mediated by olfactory sensitive pheromones (literally

'carriers of excitation') influence the oestrous cycle and the luteotropic process in the mouse (see review by Bruce and Parkes, 1965). The pheromones are probably produced under the influence of testosterone and secreted in the urine of male mice (Dominic, 1965; Bruce, 1965). The exposure of recently mated female mice to 'strange males' especially those from another strain, leads to failure of both pregnancy and pseudopregnancy (the 'Bruce Effect'). It is likely that the pheromones inhibit the endogenous release of prolactin because the administration of this luteotropin allows the female to maintain her pregnancy (Bruce and Parkes, 1960).

Progesterone administration was required before implantation occurred in intact prepubertal mice stimulated to ovulate and mate after gonadotropin treatment (Smithberg and Runner, 1956). It has been observed that daily handling of pregnant mice reduced the percentage of mice that remained pregnant (Runner, 1959) and that inbred mice that showed vaginal plugs often did not show implantation sites (Runner, 1960). In each case, failure of the animals' luteotropic processes was thought to be responsible for the pregnancy failures. Progesterone administration prevented the embryocidal effect of handling the mice and actually allowed a higher percentage of these animals to maintain their pregnancies than of the nonhandled controls. Pregnancies were recorded at 18 days post coitum or by the birth of living young so some losses may have been post-as well as pre-implantational.

The stresses of high temperature and hypoxia inhibit implantation in the intact but not in the adrenalactomised rat, suggesting that implantation may have been prevented by adrenal hormones (Fernandez-Cano, 1959). The combined stresses of suckling two young and burning on the hindleg of nursing rats on the day 3 of pregnancy caused a delay in implantation, whereas acting individually, neither stress delayed implantation (Canivenc and Mayer, 1955a, 1955c). McClure (1963) found that starvation of mice

and rats at or about the time of implantation prevented pregnancy.

(b) Post-implantation survival

Amoroso (1955) and Deanesly (1966) have reviewed literature concerned with the role of the endocrine glands during pregnancy. There is a changing relationship between the components of the hypothalamo-hypophyseal-gonadal axis as gestation progresses, due largely to the ability of the placenta to secrete hormones. Available evidence suggests that perhaps the uterus should be considered as an integral part of this axis. When acting via a local (Bland and Donovan, 1965; Melampy, 1966) or by way of a systemic influence (Nalbandov, 1966; Greep, 1966), the uterus is able to influence the life span of the corpora lutea and thus the availibility of progesterone. In both the guinea pig (Bland and Donovan, 1965) and the mouse (Kirby, 1965b), it has been argued that as a result of the interaction between the trophoblast and the decidual tissue, luteal function is stimulated. In both these species, ectopic pregnancies do not influence the oestrous cycle and Kirby (1965b) has shown that mice are able to carry extra- and intrauterine pregnancies concurrently. Luteotrophic substances have been demonstrated from both mouse and rat placentae (Amoroso, 1955; Deanesly, 1966) and are probably responsible for the maintenance of pregnancy in the mouse after hypophysectomy in the latter half of gestation (Gardner and Allen, 1942). Fajer and Barraclough (1967) measured an increase in the amount of ovarian progestagens secreted on day 13 of pregnancy in the rat and suggested that it may be due to the activity of placental luteotropin. There is indirect evidence that the rat and mouse placentae are able to secrete oestrogens and small amounts of progestagens during pregnancy (see Deanesly, 1966).

Removal of the ovaries before or after implantation usually interrupts pregnancy in the rat and the mouse. Substitutional treatment with

progesterone has been effective in maintaining viable foetuses until late pregnancy in the mouse (Hall and Newton, 1947; Smithberg and Runner, 1956; Rubenstein and Forbes, 1963; Poulson, Sullivan and Robson, 1965), rat (Lerner, Brennan, Yiacus, De Phillipo and Borkman, 1962), rabbit (Pickworth, 1963) and hamster (Harper, Prostkoff and Reeve, 1966). The proportion of implantation sites that are alive at any stage of pregnancy provide a more sensitive indicator of the effectiveness of the hormonal therapy than is provided by the number of mice with viable embryos. Oestrogen synergises with progesterone to maintain pregnancy in ovariectomised animals. (Courrier, 1950; Lerner et al., 1962, Harper et al., 1966).

There is some evidence that progesterone and oestrogen treatments do not always provide adequate support to allow normal foetal development in ovariectomised rats (Carpent, 1962). The time of ovariectomy, the doses of progesterone and oestrogen used and the strain of rats were found by Carpent to be important (see Chambon and Le Vève, 1966).

(c) Embryopathic effects associated with oestrogens and progestagens

Both gross and visceral foetal abnormalities have resulted from the artificial creation of endocrine imbalances during gestation.

Carpent (1962) describes gross malformations probably caused by the compression of the embryos or foetuses due to 'uterine hypertonicity', following ovariectomy and administration of progesterone and oestrogen to pregnant rats. In earlier work, Selye, Collip and Thomson (1935) and Zeiner (1943) believed that the uteri of ovariectomised rats contracted and that as a result the foetuses were compressed. Poulson, Robson and Sullivan (1965) noted that a minority of the foetuses carried by mice that were ovariectomised during embryogenesis and maintained on low levels of progesterone showed gross abnormalities.

Visceral malformations that were probably not secondary to uterine

changes have been found in rat foetuses by Carpent and Desclin (1967). Cardiovascular and ocular defects were seen in the few surviving foetuses from rats that were hypophysectomised and given a grafted pituitary gland early in pregnancy and then maintained with deficient hormonal substitution treatment.

Exogenous oestrogens are able to terminate pregnancy and induce modifications of the genitalia of both male and female foetuses in mice and rats (reviewed by Deanesly, 1966). The effective dose rate of oestrone needed to terminate pregnancy in the rat rises considerably after implantation (Edgen and Shipley, 1961). High levels of progesterone administered to pregnant mice were able to increase the prenatal mortality rate (Fowler and Edwards, 1960) and when given either subcutaneously or by intra-amniotic injection can cause foetal death (Petrelli and Forbes, 1964).

(d) Local uterine influences

The position, the number and the distances apart of foetuses in the uterine horns of the pregnant mice are reported to influence their growth and/or mortality rates. The body weight of foetuses closest to the oviducts was found to be significantly lighter than that of its neighbour (Hashima 1956; McLaren and Michie, 1959). Hollander and Strong (1950), investigated the mortality rate in relation to the number of foetuses per cornu and found that low and high numbers were associated with a higher mortality rate than were intermediate numbers. Placental fusion that followed close embryonic spacing was associated with the reduced growth rates of the surviving foetuses whose placentae were joined (McLaren and Michie, 1959).

SECTION I

THE OVARIAN HORMONAL REQUIREMENTS FOR OVA-IMPLANTATION

INTRODUCTION

The emergence of a workable hypothesis to explain the neuroendocrine control of ova-implantation in the rat (see Mayer, 1965; Psychoyos, 1966) has provided a basis on which to examine this problem in the mouse. The validity of the hypothesis rests on the release of oestrogen probably as a surge (Shelesnyak, 1959, 1960; Shelesnyak and Kraicer, 1963) late on day 3, which momentarily acting on a progesterone-dominated background is held to be essential for ova-implantation. Provided adequate progesterone is available, ovariectomy after the critical time of the oestrogen release permits subsequent implantation at the normal time, whereas ovariectomy before this time prevents implantation (Mayer, 1963). The experiments to be described were designed to investigate the possible existence of a similar critical time in the mouse. Mature pregnant mice were ovariectomised at different times post coitum, given graded dose levels of progesterone and later checked for implantation sites.

Earlier work in the mouse provided no indication of a critical time for ovariectomy during early pregnancy and gave conflicting evidence as to the requirements of oestrogen for ova-implantation after ovariectomy. Smithberg and Runner (1956) found that ovariectomy on day 1 of pregnancy followed by the administration of adequate progesterone allowed 100 per cent of the mice to implant ova. The results of work by Bloch, (1959) gave no indication of a critical time for ovariectomy in that the administration of daily progesterone to mice ovariectomised on days 1 and 3 of pregnancy allowed implantation of ova at the normal time in some mice.

However, the numbers of mice used by Bloch were small, and Smithberg and Runner used gonadatropin-stimulated prepubertal mice which may not accurately reflect the endocrine conditions of pregnant mature mice.

The exogenous hormonal requirements of ovariectomised pseudopregnant mice to form deciduomata were found by Finn (1965), to be dependent on the time of ovariectomy. By the use of intraluminal arachis oil as the stimulus to produce nontraumatic deciduomata formation (Finn and Keen, 1963; Finn and Hinchliffe, 1964, 1965) he found that ovariectomy on day 2, but not on day 3, prevented a positive response. Furthermore a single dose of oestrogen given after ovariectomy on day 2 also allowed the production of deciduomata in pseudopregnant mice. This work provided direct evidence for the existence of a critical time for ovariectomy in pseudopregnant mice and also indirect support for an oestrogen surge coinciding with this period of early pseudopregnancy.

The role of oestrogen in ova-implantation has been investigated when implantation was delayed either by lactation (Whitten, 1955, 1958; Bloch, 1958), or by ovariectomy without hormonal substitution treatment for several days (Smithberg and Runner, 1960). Whitten was able to shorten the expected delay to implantation in lactating mice by the administration of oestrogen during the delay. Bloch ovariectomised pregnant lactating mice during the period of blastocyst diapause and found that the ova would subsequently implant only if both oestrogen and progesterone were given simultaneously. Smithberg and Runner (1960), again using gonadatropin-stimulated prepubertal mice, noted that progesterone alone was relatively ineffective when compared with progesterone given with oestrogen in inducing blastocysts to implant after they had remained quiescent in the uteri of ovariectomised mice for variable lengths of time. These data suggest that oestrogen may play an important role during ova-implantation

in the mouse even though progesterone alone was apparently sufficient to permit implantation after ovariectomy in the experiments of Smithberg and Runner (1956) and Bloch (1959).

Macroscopic evidence of implantation after the administration of oestrogen to pregnant lactating mice was first seen 26 hours later (Whitten, 1958). This time interval is of the same order as the 30 hours found by Yasukawa and Meyer, (1966) to elapse between the administration of oestrogen to rats with blastocysts undergoing delayed implantation and early signs of implantation. Also when the 26 hour period is added to Finn's (1965) estimate of the critical time then it would place the time of implantation as occurring on the morning of day 4 of pregnancy. This estimate is in agreement with the results reported by Amoroso (1952), Snell (1956), Wilson (1963) and Dickson (1966) but differs from those of Enzmann et al. (1932) who considered that implantation occurred on day 6 of pregnancy. However, there are other discrepancies in the reported times of implantation during day 4, as well as those of Enzmann et al., which in part are due to the fact that different workers define the start of implantation in different ways. Strain differences (Snell, 1956) and environmental differences may alter the time of implantation and it was decided to make one's own estimate of the time of implantation with the strain of mice used.

The beginning of implantation was defined as the time at which the blastocyst first assumes a fixed position in relation to the uterus. Fixation is followed by a period of loose attachment and then by an invasive stage during which the developing embryo embeds within and is rapidly surrounded by the proliferating maternal tissue (Meyer, 1960; Snell, 1956).

MATERIALS AND METHODS

Two separate experiments, both in time and place, viz., Experiments A and B were performed in order to investigate the ovarian hormonal requirements for ova-implantation after ovariectomy at known different times during early pregnancy.

1. Experimental Conditions

(a) Animals

Randomly bred virgin albino female mice from the N O S (New Zealand Oestrogen Sensitive) inbred strain were used. Of the 400 females used for Experiment A, 220 were supplied from two colonies kept at Massey University and the remainder provided by Glaxo Laboratories, Palmerston North. The 170 females required for Experiment B were all from the Massey University Small Animal Research Unit (S A R U) colony.

Females were between 50 and 89 days of age (the mean ages and body weights with standard errors, for mice used in Experiments A and B were 66.5 ± 2.5 and 63.2 ± 2.1 days and 22.61 ± 0.13 and 20.89 ± 0.16 gm, respectively).

Twenty four and 12 fertile males of the N O S strain were used for experiments A and B, respectively.

(b) Feeding

During both experiments a pelleted diet (Table 1) and water were provided *ad lib*.

(c) Housing and environment

Experiment A was carried out in the Veterinary Faculty Small Animal Production Unit. Neither the ambient air temperature nor the humidity was controlled. The animals were exposed to natural changes in daylight over the period from the 9th of October to the 5th of December, 1966. Generally

TABLE 1

THE PELLETED DIET FED DURING EXPERIMENTS A, B AND C

(MASSEY MOUSE DIET NO. 2)

Wheat meal	800 lbs
Barley meal	200 lbs
Ground oats	80 lbs
Buttermilk powder	640 lbs
Wheatgerm meal	200 lbs
Lime	20 lbs
Salt	10 lbs
Blood meal	50 lbs

Vitamin concentrate containing A, B_2 and D mixed with blood meal and included in pellets fed during experiments B and C.

the temperature fluctuated between 18° and 24° C, although extremes of 14° and 31° C were recorded. Between weaning at about 3 weeks of age and allocation to Experiment A, females supplied by the Small Animal Research Unit (S A R U) were kept in groups of 14 per cage where the cages were 28.5 cm long, 21.5 cm wide and 10.5 cm deep. Mice in the Glaxo colony were raised in large crowded pens and at 56 days of age some had closed vaginal membranes. During Experiment A, 6 or less mice were kept on sawdust in standard S A R U cages (29.0 cm long, 13.0 cm wide and 11.5 cm deep). Experiment B was performed in the University S A R U. The mouse room was thermostatically controlled to keep the air temperature between 21° and 23° C, ephemeral extremes of 18° and 26° C were noted. Animals were again exposed to seasonal changes in daylight from the 27th of February to the 11th of April 1967. Relative humidity ranged between 41% and 86%. After weaning at about 3 weeks of age, mice were raised, 20 to 30 in cages measuring 40 cm long by 30 cm wide and 14 cm deep, in the same room as the experimental mice were kept. Apart from the use of wood shavings instead of sawdust in the cage, the experimental mice were caged as in Experiment A.

2. Detection of Stages of the Oestrous Cycle

Vaginal smears were taken daily for the duration of each of the two experiments. A small piece of cotton wool wound over the end of fine forceps served to transfer epithelial cells from the posterior vagina onto a glass slide. The vaginal smear was stained with dilute methylene blue and examined at x 100. Classification of the different stages of the oestrous cycle was after Snell (1956).

The day on the morning of which a vaginal plug was found was called day 0 of pregnancy. Some difficulty was encountered in deciding the day of mating for mice that did not have visible plugs. These animals had smears taken until sufficient leucocytes were seen to warrant inclusion into the

metoestrus - 1 stage, day 0 was estimated by counting back one day. Checks on the dating of the start of pregnancy showed that this method was sometimes in error, usually the dated start of pregnancy was one day late.

From the vaginal smear records the time taken for each female to mate (the mating delay) after being caged with a male was calculated.

Ovulation was assumed to take place at midnight and pregnancy was arbitarily taken as starting at 0200 hours.

3. Experimental designs

(a) Experiment A

The results of preliminary experiments demonstrated that mice ovariectomised during early pregnancy and treated with progesterone alone could still implant ova. This experiment was designed to test the effect of varying the time of ovariectomy together with the administration of different dose rates of progesterone (Text-fig.1).

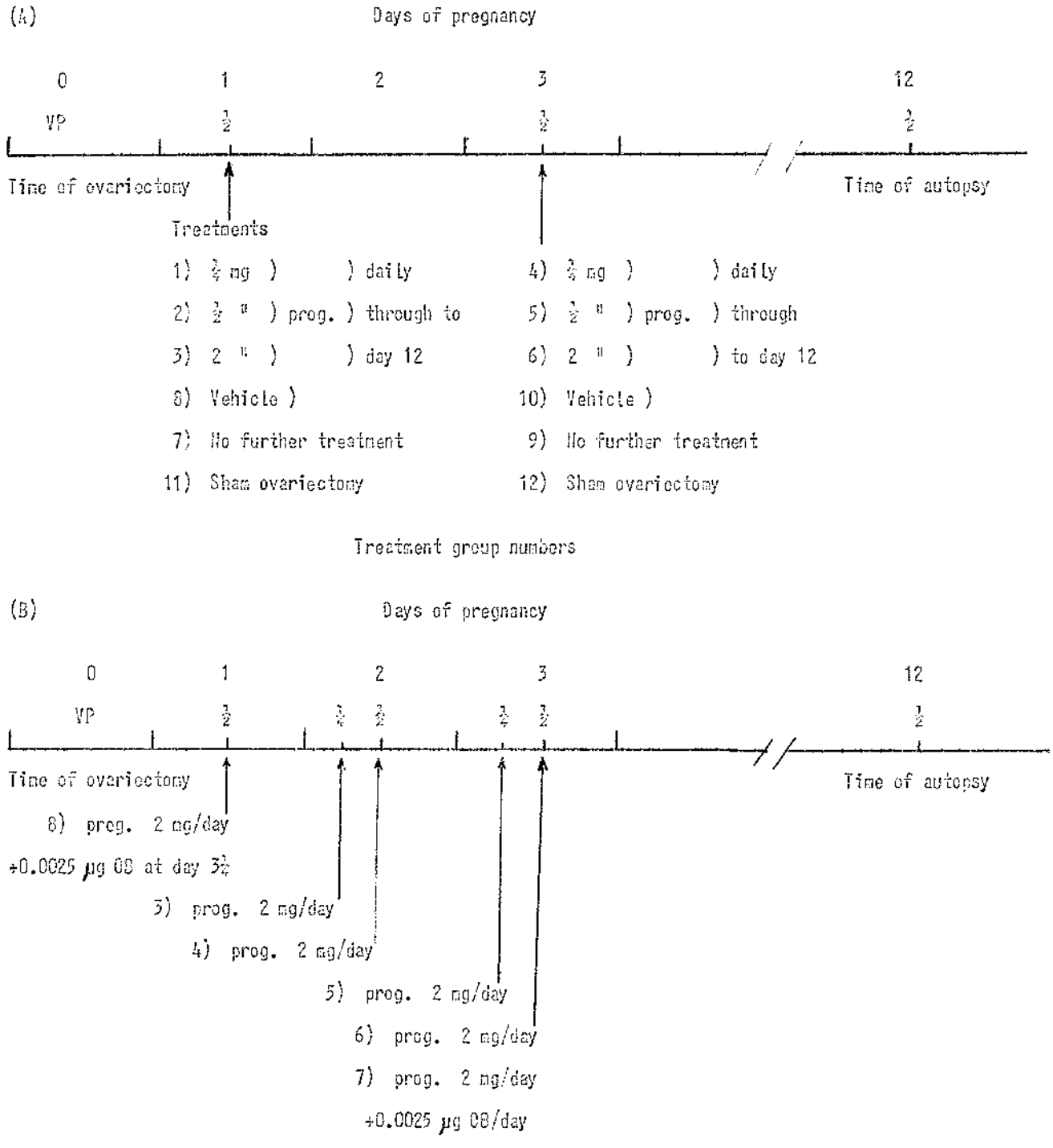
Thirteen different treatment groups each with 20 replicates were used. Untreated intact controls comprised one group (No. 13) and 6 different treatments were imposed both on mice ovariectomised at $1\frac{1}{2}$ days and at $3\frac{1}{2}$ days of pregnancy.

The experimental design could be treated as a 2 x 6 factorial with an additional (control) group or as a randomised block design with 13 treatments.

(b) Experiment B

This experiment was designed after Experiment A had been completed, and was concerned with ovariectomy and progesterone treatment alone between the times of $1\frac{1}{2}$ and $3\frac{1}{2}$ days of early pregnancy as well as with the effects of selected oestrogen treatments in combination with progesterone (Text-fig.1).

Eight treatment groups, each with 15 replicates, were used. Groups 1 and 2 were untreated controls and the remaining 6 consisted of:



TEXT - Fig. 1. (A) The design of Experiment A. (B) The design of Experiment B.
(Abbreviations; OB = oestradiol benzoate; prog. = progesterone;
VP = vaginal plug)

(3) ovariectomy at $2\frac{1}{4}$ days of pregnancy

(4) " " $2\frac{1}{2}$ " " "

(5) " " $3\frac{1}{4}$ " " "

(6) " " $3\frac{1}{2}$ " " " (by radical method)

(7) " " $3\frac{1}{2}$ " " " followed by daily injections 0.0025 µg of oestradiol benzoate (OB)

and (8) ovariectomy at $1\frac{1}{2}$ days of pregnancy and given a single injection of 0.0025 µg of OB at day $3\frac{1}{4}$ of pregnancy.

Groups 3 to 8 received about 2 mg of progesterone, absorbed daily from implant pellets placed subcutaneously at the time of ovariectomy.

4. Allocation of Mice to Treatment Groups

Female mice for both experiments were randomly assigned to all treatment groups at the time of joining with males. No deliberate attempt was made to place females with any particular male and a maximum of 3 cycling females were present with each male at any one time.

Each block of mice contained one animal for each treatment and additional mice (7 in Experiment A and 3 in Experiment B) to replace 'losses'. The 'spare' mice were allocated at random to treatments to replace mice removed from the experiments because they (1) had markedly irregular oestrous cycles and/or failed to mate within a given time (3 days after most of its contemporaries had mated) (2) died during or after surgery (3) lacked functional (bright red-coloured) corpora lutea at ovariectomy. An average of 3.8 and 1.5 'spares' per block were used in Experiments A and B, respectively.

The time intervals between the setting up of blocks ranged between 3 and 6 with a mode of 3 days during Experiment A. Three blocks were added at successive 6 day intervals for Experiment B.

5. Ovariectomy

Pentobarbitone sodium (Nembutal Abbott) given intraperitoneally at a

dose rate of about 1.0 mg per 10 gm body weight was used as an anaesthetic. When necessary, anaesthetic ether or ethylene chloride administered by inhalation was used to prolong the state of anaesthesia. Operational procedures were performed under clean but not antiseptic conditions.

Ovariectomy was performed by the dorsal route with the visual aid of a dissecting microscrope. The midline longtudinal skin incision was positioned so that when it was moved laterally, the peritoneal cavity could be entered immediately dorsal to either ovary. After exteriorization of the ovary the periovarian capsule was slit open with iris scissors and reflected back so that the ovary could be manipulated by grasping the ovarian stalk with fine curved forceps. The Fallopian tubes were then separated from the stalk containing the ovarian blood vessels and the latter cauterized through.

Throughout the operation, care was taken to ensure that all ovarian tissue was removed, that the exposed surface of the ovary was 'touched' by instruments as little as possible and that the Fallopian tubes suffered little damage. The number of Fallopian tubes that were obviously damaged as a result of the operation is shown in Table 11. To check on the completeness of the removal of ovarian tissue by the method described above, a more radical method of ovariectomy was used for one treatment group (No. 6) during Experiment B. For the radical operation performed at $3\frac{1}{2}$ days of pregnancy, the ovary in its intact capsule together with the greater part of the Fallopian tubes was removed.

The skin incision was closed with wound clips, the muscle layers of the abdominal wall were left unsutured.

The technique for sham ovariectomy differed from that described above in that after exteriorization, the ovaries were moved about by traction on the fat-pad and then without further interference replaced in the peritoneal

cavity.

Operations were, with few exceptions, performed within two hours of the times stated.

6. Hormones

For Experiment A, $\frac{1}{4}$, $\frac{1}{2}$ or 2 mg of progesterone were injected subcutaneously in 0.05 ml of vehicle. Crystalline progesterone (Mann Chemical Co., New York) was first dissolved in benzol alcohol and peanut oil added to contribute 85% of the total volume of the vehicle. Injections were given daily and started on the day of ovariectomy.

During Experiment B, progesterone was administered as two 70 - 110 mg implant pellets placed subcutaneously, one over each scapula, at the time of ovariectomy. The pellets were made singly, from crystalline progesterone with a hand-vice operated plunger and dye.

The amount of progesterone absorbed by each mouse was estimated by subtraction of the desiccator dried pellet weights after their removal at autopsy from their initial dry weights.

Oestradiol benzoate (Organon) at a dose rate of 0.0025 ug in 0.005 ml of peanut oil was given subcutaneously with an 'Alga' microsyringe to treatment groups 7 and 8 in Experiment B.

7. Experimental Parameters

Mice were killed by cervical dislocation at $12\frac{1}{2}$ days of pregnancy. At autopsy a) the number of mice with implantation sites and

b) the number of implantation sites per mouse were recorded (an implantation site was defined as visible evidence of ova-implantation).

Uteri of mice without implantation sites were flushed with 0.9 % saline in order to recover unimplanted blastocysts (for method see Section I (a)). The number of mice with blastocysts and the number of blastocysts

per mouse were recorded.

In early experiments, the Fallopian tubes of nonpregnant mice were occasionally distended with fluid. Numbers of ovariectomised nonpregnant mice in the present experiments that showed this characteristic are shown in Table 11.

SECTION I (a)

THE TIME OF OVA-IMPLANTATION

MATERIALS AND METHODS

Experiment C

This experiment was performed over the period from the 19th of April to the 10th of May 1967 (i.e., after the completion of experiments A and B). The design was essentially that employed by Dickson (1966). Ova were assumed to have implanted when they were no longer recoverable by flushing the uterine horns with physiological saline.

1. Animals

Virgin mice aged from 49 to 82 days from the S A R U N O S colony were used.

Feeding, housing, mating and caging procedures were the same as those used for Experiment B.

2. Experimental design

The stages of pregnancy were equated with the estimated developmental ages of ova in hours. Pregnancy was assumed to begin, as in the earlier experiments, at 0.200 hours on the day of the finding of a vaginal plug. To eliminate mice with uncertain mating histories, only those with visible vaginal plugs were included in the experiment.

Preliminary results, when mice were killed at 86, 94, 102, 110, 118, 126 and 132 hours of pregnancy, indicated that after 118 hours of pregnancy blastocysts were no longer recoverable by flushing the uteri. During the main experiment, mice were randomly assigned to one of the four killing times, viz., 94, 102, 110 or 118 hours of pregnancy.

3. Proceedures at Autopsy

At autopsy the uterus together with the cervix was removed and the broad ligaments and the tips of the uterine horns were cut away. The majority of uterine horns were then divided into oviducal and cervical halves. One ml of physiological saline was injected through each complete or each half uterine horn with a blunted 25 gauge hypodermic needle introduced through the cervix or the distal end of the oviducal half. The saline was collected in a watch glass and searched for ova at a magnification of x 18.

Ova together with a large drop of saline were drawn into a polythene tube with a one ml syringe and transferred to a glass slide. The length and width (diameter) of ova were measured to the nearest 5 μ with an eye piece micrometer at x 100.

Blastocysts were classified into four groups according to the stage of giant cell trophoblastic transformation, viz., as early blastocysts with no transformed cells, those with less than half of their cells transformed, those with more than half though less than all of their cells transformed and those whose trophoblast cells were completely transformed.

Variations in the shape and size of the blastocysts were noted.

STATISTICAL METHODS

General references, Goulden (1952) and Snedecor (1956).

Quantitative data were subjected to single or two way analyses of variance, and analyses of covariance. Observational results in the form of counts which included zero quantities were transformed using the $\sqrt{x + 1}$ transformation, where x represents the observation number.

In Section III analyses of covariance were performed when the two variables were both affected by the treatment. This was done to investigate the effect of the treatment on the relationship between the variables (see Cockran and Cox, 1957).

Between treatment comparisons were orthogonal where possible. Duncan's multiple range test (Duncan, 1955) was used to make nonorthogonal comparisons between block means.

Quantal data were analysed using 'Chi Square' techniques, by total Chi Square, by partitioning the total degrees of freedom, by construction of two by two contingency tables, or by the calculation of the exact probabilities by the method of Fisher (see Goulden, 1952).

Standard regression and correlation methods were used and orthogonal polynomial coefficients were employed to derive an equation to describe a curve obtained in the experiment to determine the time of ova-implantation.

The 5% level of significance was chosen ($P < 0.05$) i.e., if the probability of obtaining a particular result by chance alone was greater than 5% it was judged nonsignificant (N.S.).

SECTION I

RESULTS

1. Body Weight Changes

(a) Initial body weights

The mean values, with standard errors, for mice used in Experiments A and B were 22.61 ± 0.13 and 20.89 ± 0.16 gm respectively, at the time of placement with males (Table 2). Duncan's multiple range tests, incorporated in Table 2, showed that for both experiments there were significant between-block differences (tests made only at the 5% level of significance).

(b) Final body weights

The respective mean body weights for mice in Experiments A and B at the time of autopsy were 25.67 and 26.37 gm as shown in Table 2. From the summaries of statistical tests analysing body weight changes in Table 3, it may be seen that after adjustment of the treatment final weight means Y, by the inclusion of their initial weight means as the covariate x, in an analysis of covariance, pregnant mice were heavier than nonpregnant mice for Experiments A ($P < 0.001$) and B ($P < 0.01$). For both experiments, intact pregnant mice were heavier than ovariectomised pregnant mice ($P < 0.001$). Comparisons between pregnant intact mice and between pregnant ovariectomised mice showed that mice sham-ovariectomised at 3½ days and those that had received 2 mg of progesterone were heavier than those sham-ovariectomised at 1½ days ($P < 0.01$) of pregnancy and the pooled results of those given ¼ and ½ mg of progesterone daily ($P < 0.01$), respectively.

The final body weights of all nonpregnant mice (includes 3 mice with small implantation sites in Experiment B) were 24.45 ± 0.20 and 24.51 ± 0.29 gm for Experiments A and B, respectively.

TABLE 2

THE ACTUAL AND ADJUSTED BODY WEIGHT MEANS (IN GRAMS) FOR MICE USED IN EXPERIMENTS A AND B WITH STATISTICAL COMPARISONS

EXPERIMENT A

Block numbers	Initial block means ranked	Significant differences* ($P<0.05$)	Block numbers	Final block adjusted means (ranked)	Treatment numbers	Initial treat. means (ranked)
7	20.90		14	24.59	11	21.59
5	21.00		15	24.72	7	21.95
1	21.37		6	24.99	6	22.21
4	21.62		7	25.17	4	22.36
8	21.97		2	25.21	10	22.46
2	21.98		10	25.25	12	22.46
10	22.08		16	25.42	3	22.50
6	22.26		19	25.43	9	22.71
20	22.50		5	25.48	1	22.92
9	22.75		18	25.54	8	22.99
3	22.82		12	25.57	2	23.09
16	22.85		13	25.75	5	23.23
11	23.00		4	25.95	13	23.52
18	23.22		3	25.95		
14	23.28		17	25.95		
19	23.33		9	26.13		
12	23.55		8	26.31		
17	23.59		18	26.44		
15	23.97		11	26.82		
13	24.19		1	27.78		
General means	22.61 ± 0.13			25.67 ± 0.25		

EXPERIMENT B

No significant differences	Treatment numbers	Final treat. means (ranked)	Final treatment means adjusted+	Block numbers	Initial block means (ranked)	Significant differences ($P<0.5$)	Block numbers	Final block means (ranked)	Treatment numbers	Final treat means (ranked)	Final treatment means adjusted
	7	23.59	24.20	11	18.64		5	21.16	4	24.20	24.64
	9	23.67	23.66	15	19.13		7	23.50	3	24.35	24.35
	10	23.82	24.01	6	19.99		4	23.50	8	25.16	26.54
	4	24.73	24.96	13	20.03		2	23.55	5	26.07	25.46
	5	24.83	24.26	14	20.25		5	24.54	7	26.57	26.38
	3	24.85	24.95	12	20.69		8	25.19	6	27.27	24.65
	8	25.01	24.66	2	20.99		1	25.21	1	28.13	28.54
	1	25.03	24.74	1	21.04		14	25.87	2	29.22	29.19
	2	25.24	24.88	10	21.29		10	25.91			
	6	26.22	26.59	9	21.45		9	26.56			
	11	26.75	27.69	5	21.54		13	27.23			
	12	29.53	29.67	8	21.70		12	27.51			
	13	30.44	29.60	7	22.23		6	27.74			
				3	22.31		15	31.94			
				4	22.40		11	33.47			
			General means		20.89 ± 0.22			26.375 ± 0.47			

* Differences obtained from Duncan's multiple range test. Any two means not adjacent to the same line are significantly different. Any two means adjacent to the same line are not significantly different.

\+ Adjusted means from analysis of covariance.

TABLE 3

BODY WEIGHT CHANGES IN EXPERIMENTS A AND B

SUMMARY OF STATISTICAL TESTS

A. Analyses of Covariance

Source	df	Mean Squares	Variance Ratio	P
Experiment A				
Residual	227	4.532		
Difference for treatments	12	85.892	18.95	< 0.001
Difference for blocks	19	17.894	3.948	< 0.001
Experiment B				
Residual	97	9.075		
Difference for treatments	7	50.16	5.529	< 0.001
Difference for blocks	14	64.43	6.459	< 0.001

B.* Comparisons (between treatment means)

Experiment A

(i)	Pregnant (27.13^{+}) vs nonpregnant (24.43)	< 0.001
(ii)	" ovarx* (25.27) vs intact pregnant (28.99)	< 0.001
(iii)	" " $\frac{1}{2}$ mg (24.30) vs pregnant ovarx $\frac{1}{2}$ mg (24.26)	NS
(iv)	" " ($\frac{1}{2}$ + $\frac{1}{2}$) mg (24.61) vs pregnant ovarx 2 mg (26.59)	< 0.01
(v)	Sham ovarx $1\frac{1}{2}$ days (27.69) vs sham ovarx $3\frac{1}{2}$ days (29.67)	< 0.01
(vi)	" " ($1\frac{1}{2}$ + $3\frac{1}{2}$) days (28.60) vs intact controls (29.60)	NS

Experiment B

(i)	Pregnant (26.84) vs nonpregnant (25.18)	< 0.01
(ii)	" ovarx (25.50) vs intact pregnant (28.87)	< 0.001
(iii)	" " $3\frac{1}{2}$ days (25.46) vs pregnant ovarx $3\frac{1}{2}$ days (24.65)	NS
(iv)	" " $3\frac{1}{2}$ days $\overset{\pm}{\text{prog.}}$ (24.65) vs pregnant ovarx $3\frac{1}{2}$ days prog. + oestrogen (26.38)	NS

$^{+}$ adjusted means in grams

* ovarx = ovariectomised

$\pm$ prog. = progesterone

* Comparisons made using the method of Kramer (1957)

(c) Body weight and age

The mean ages (with ranges) of mice at the start of Experiments A and B were 66.4 ± 2.5 (50 to 89) and 63.2 ± 2.1 (51 to 72) days, respectively.

Significant correlations were found between the initial body weights and ages for the pooled data of the two experiments ($r = \pm 0.458$, $P < 0.01$) and for the mice used in Experiment B ($r = + 0.995$, $P < 0.001$). This correlation for the mice used in Experiment A was nonsignificant.

(d) The health of the mice used in experiments A and B

Mild diarrhoea was encountered in both experiments. Affected mice passed soft pale-coloured faeces and were less active. Overt scouring was unusual and the syndrome was obvious for from one to three days.

* 'Strinacin' (May and Baker) was fed mixed with buttermilk powder (2-4 mg/gm of feed) to all mice as soon as the condition appeared during Experiment B. Medication was not used during Experiment A, in the course of which however, only one affected mouse lost weight. Case histories showed that the course of pregnancy in affected mice was apparently unimpaired. Bacteriological cultures were uninformative and although the clinical signs were similar, it is not certain that the syndrome was the same for both experiments.

Of the 260 and 120 mice selected for Experiments A and B, 3 and 6 animals, respectively, died, were lost, or were deleted from the treatment groups and not replaced.

2. The Incidence of Vaginal Plugs

Two hundred and eight out of 260 (80%) and 93 out of 120 (77.5%) mice showed visible vaginal plugs in Experiments A and B, respectively, (Table 4).

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* Sulphapyridine 26.5% w/w; sulphamerazine 26.5% w/w; sulphadiazine 26.5% w/w and streptomycin 4.2% w/w.

TABLE A

THE INCIDENCE OF VAGINAL PLUGS DURING EXPERIMENTS A AND B

	Experiment A	Experiment B
Numbers of mice with vaginal plugs (VPs)	208	93
" " " without " "	52	27
" " " with VPs as % age of total	80	77.5

Summary of Chi Square tests

		X^2	df	P	X^2	df	P
Source (a)	Total between treatments	5.72	12	NS	2.29	7	NS
	" " blocks	43.38	19	< 0.001	20.42	14	NS
	Partitioning df for blocks						
	Glaxo colony mice	21.60	9	< 0.05			
	University colony mice	15.40	9	NS			
	Glaxo x University colony mice	6.98	1	< 0.01			
(b)	2 x 2 contingency tables						
	The effect of age						
	All mice; < 68 vs ≥ 68 days	0.216	1	NS			
	University colony mice; < 60 vs > 60 days	5.681	1	< 0.05	1.456	1	NS
	Glaxo colony mice; ≤ 70 vs > 70 days	1.525	1	NS			

Summary of Correlations tested

Between	r	df	
VPs and pregnancy	+ 0.556	18	< 0.05
VPs and not pregnant	- 0.556	18	< 0.05
Without VPs and pregnant	- 0.411	18	NS
Without VPs and not pregnant	+ 0.587	18	< 0.001
VPs and initial body weight	+ 0.377	18	NS
VPs and the mating delay	+ 0.00009	18	NS

Chi Square analyses showed no significant differences in the incidence of vaginal plug formation between treatment groups for either experiment or for between blocks in Experiment B.

(a) Factors influencing vaginal plug formation

There were significant between-block differences for Experiment A ($P < 0.001$, Table 4).

(i) Source of mice

Partitioning the degrees of freedom for the total Chi Square showed that the Glaxo mice used in Experiment A, displayed significant within treatment variation ($P < 0.05$). Mice from the University colonies showed no such variation and had a higher incidence of plug formation than Glaxo-bred mice ($P < 0.01$).

(ii) Age

Two by two contingency tables were set up in which the numbers of mice with and without plugs were tested for independence of age effects. Mice from the University colonies did show an age effect in that those below 60 days of age at the time of placement with males displayed a lower incidence of plug formation than those above 60 days ($P < 0.05$). Other age separations for Experiments A and B were nonsignificant.

(iii) Pregnancy

Mice considered were from treatment groups in Experiment A with no significant differences between the numbers of pregnant animals present (i.e., treatment group numbers 4, 5, 6, 11, 12 and 13).

The number of mice with vaginal plugs was positively correlated with the number pregnant ($r = + 0.558$, $P < 0.05$). Numbers of mice without vaginal plugs were correlated with numbers of nonpregnant

mice ($r = + 0.587$, $P < 0.001$).

Considering the numbers of mice with plugs and/or that were pregnant, 226 (85%) and 108 (88.9%) were known to have mated during Experiments A and B, respectively.

3. The Mating Delay

The delay to mating was measured in days, where day 0 was defined as the day females were placed with the males.

Data for each experiment were analysed using two way analyses of variance. Between treatment differences for both experiment were nonsignificant. However, within treatment differences for both experiments were highly significant (Table 5).

(a) The mating delay and age

The mating delay for mice, with the wider age range, in Experiment A was negatively correlated ($r = - 0.579$, $P < 0.05$) with their ages. That is the older mice mated more quickly than did the younger ones. This correlation was nonsignificant for data from Experiment B (Table 5).

4. Progesterone Pellet absorption

Possible factors influencing the absorption of progesterone from subcutaneous implant pellets are considered in Table 6.

The mean absorption rate per mouse for 82 mice was 19.88 ± 4.74 mg (or 9.94 ± 0.27 mg per pellet) for an overall average time of 9.9 days.

The amount of progesterone absorbed from 20 pellets with a flakey or crumbly surface consistency was greater than that absorbed by the other pellets ($P < 0.05$), Table 6). Also the 19 mice with one or two of these pellets absorbed significantly more progesterone than other mice ($P < 0.05$).

IMPLANTATION STUDIES

TABLE 5

DAYS ELAPSING FROM THE PLACEMENT OF THE FEMALES WITH THE MALES UNTIL MATING, SUMMARIES OF STATISTICAL TESTS

(a) Analyses of Variance

Experience 1

Source	df	Variance Ratio	P
Treatments	12	0.925	NS
Blocks	19	3.587	< 0.001
Residual	228		

Experiment 2

Treatments	7	1.383	NS
Blocks	14	5.317	< 0.001
Residual			

(b) Correlations between the mating delay and the age of females at the beginning of the experiments.

Source	r	df	P
Experiment A	-0.5079	19	< 0.05
Experiment B	-0.4142	13	NS
Experiment A and B combined	-0.211	33	NS

TABLE 6

THE ABSORPTION OF PROGESTERONE FROM SUBCUTANEOUS IMPLANT PELLETS DURING EXPERIMENT B

1. Amount of progesterone absorbed from all pellets.*
(Data available for 82 of 90 mice that received two 70-110 mg implant pellets)

mean weight progesterone used per mouse = 19.88 ± 4.74 mg

" " " lost per pellet = 9.94 ± 0.27 mg

2. The duration of the absorption period according to the time of ovariectomy and the mean amounts of progesterone used.

i.e.,	9	$9\frac{1}{4}$	10	$10\frac{1}{4}$	11 (days)
	20.27	18.26	20.09	19.68	20.96 (mg)

Summary of analysis of variance

	df	Mean Squares	Variance ratio	P
Treatments	4	14.285	<1	NS
Residual	76	21.947		

3. Absorption from 65 pellets used a second time.

Initial use 9.99 mg per pellet

Second " 10.25 " " "

(t 0.05, 64 df, NS)

4. Absorption from pregnant mice

37 pregnant mice 19.69 mg mean weight

all mice 19.88 " " "

(t 0.05, 36 and 81 df, NS)

5. Absorption in pregnant mice that received oestrogen

11 mice that received oestrogen + progesterone = 19.94 mg mean weight

13 " " " progesterone alone = 20.11 " " "

(t 0.05, 10 and 12 df, NS)

6. Absorption from pellets with a flakey or crumbly surface

a) 20 pellets with flakey or crumbly surfaces 12.33 mg mean wt

144 " " normal surfaces 9.61 " " "

(t = 2.77, 19 and 143 df, $P < 0.05$)

b) 19 mice with crumbly or flakey pellets 23.37 mg mean wt

63 " " normal pellets 18.83 " " "

(t = 3.99, 18 and 62 df, $P < 0.001$)

* Pellets were weighed to the nearest 0.1 mg.

1. The Number of Mice with Implantation Sites at Autopsy

Implantation sites ('sites') ranged in size from barely perceptable localizations of decidual tissue, or 'moles', up to large sites with live foetuses. Numbers of mice with implantation sites are given in Table 7, and a summary of statistical tests made, in Table 8.

Experiment A

Of the 59 and 60 mice ovariectomised at 1½ and 3½ days of pregnancy that received exogenous progesterone, 2 and 47 respectively, had implantation sites at autopsy.

Thirteen (out of 20), 17 (20) and 17 (20) mice had implantation sites after receiving ¼, ½ and 2 mg of progesterone per day, respectively, after ovariectomy at 3½ days of pregnancy. Sixteen (20), 15 (19) and 16 (20) mice had implantation sites after sham-ovariectomy at 1½ and 3½ days of pregnancy and for the control group, respectively. There were no significant differences in the numbers of mice that implanted ova for these six groups.

Mice that received vehicle or no treatment after ovariectomy, at either of the two times, did not implant ova.

Experiment B

Of the mice ovariectomised at 2¼, 2½, 3¼ and 3½ days of pregnancy, 1 (14), 2 (15), 11 (14) and 13 (14) respectively, had evidence of ova-implantation at autopsy.

Mice that were given 0.0025 ug of oestradiol daily after ovariectomy at 3½ days and those that were given a single injection of 0.0025 ug of estradiol on 3¼ days after ovariectomy at 1½ days of pregnancy, had 14 (14) and 0 (14) animals, respectively, with implantation sites.

Twenty nine of the 30 intact control mice implanted ova.

With mice ovariectomised at 1½ days excluded, Chi Square tests showed

TABLE 7

THE TOTAL NUMBER, SIZE AND SPACING OF IMPLANTATION SITES AT AUTOPSY FOR EXPERIMENTS A AND B

Treatment group numbers	Treatments	Mice with implantation sites (IS)	Without IS	Number with IS as percentage	IS per treatment group	Mean no. per mouse	Live IS per treatment group	Dead IS	No. live as percentage	Mean IS volume (mm^3)	Mean distances between sites (mm)	Mean lengths of uterine horns (mm)	Mean % age of horn lengths occupied with IS
Experiment A													
1	Ovarx 1½ days, ½ mg prog. daily	1	19	5.3	5	5.00							
2	" " " ½ " " "	1	19	5.6	7	7.00							
3	" " " 2 " " "	0	20	0									
4	" 3½ " ½ " " "	13	7	65.0	103	7.92	3	100	2.9	51.66	5.92	72.17	47.0
5	" " " ½ " " "	17	3	85.0	143	8.41	3	140	2.1	65.74	7.14	73.19	52.0
6	" " " 2 " " "	17	3	85.0	127	7.47	27	100	27.0	152.11	7.64	82.63	55.9
7	" 1½ " no treatment	0	19	0									
8	" " " vehicle daily	0	20	0									
9	" 5½ " no treatment	0	20	0									
10	" " " vehicle daily	0	20	0									
11	" 1½ " sham operation	16	4	80.0	137	8.56	125	12	91.2	228.10	12.42	139.85	72.5
12	" 3½ " " "	15	4	78.3	163	10.87	150	13	92.0	397.75	13.15	162.60	76.9
13	Intact controls	16	4	80.0	155	9.68	142	13	91.6	378.88	13.41	152.33	70.5
Experiment C													
1	Intact controls	14	1	93.3	127	9.07	119	8	93.7	318.92	11.72	130.33	73.9
2	" "	15	0	100.0	141	9.41	128	13	90.8	330.75	12.42	134.60	72.4
3	Ovarx 2½ days, ≃2 mg prog. daily	1	13	7.1	1	1.00	0	1					
4	" 3½ " " " " " "	2	13	15.4	2	1.00	1	1					
5	" 3½ " " " " " "	11	3	27.3	52	4.73	25	27	48.0	220.58	11.44	80.90	41.5
6	" 3½ " " " " " "	13	1	92.9	121	9.30	56	65	50.0	197.24	9.23	97.73	65.3
7	" " " " " " " + 0.0025 ug estradiol daily	14	0	100.0	136	9.71	101	20	83.4	291.24	9.34	115.15	68.1
8	" 1½ days ≃2 mg prog. daily + 0.0025 ug estradiol at day 3½ only	0	14	0	0	0							

TABLE 8

MICE WITH IMPLANTATION SITES FROM EXPERIMENTS A AND B, SUMMARY OF STATISTICAL TESTS

Experiment A

1. The number of mice with implantation sites (Chi Square tests)

Treatment groups considered 4, 5, 6, 11, 12 and 13

$X^2 = 2.75$ (5 df) P = NS

By inspection groups 1 and 2 had fewer mice with sites than the groups considered.

2. The number of implantation sites per mouse (Analyses of variance tests using the $\sqrt{X + 1}$ transformation, where X = the number of sites)

Source	df	Variance Ratio	P
Treatments	19	1.607	NS
Blocks	5	0.904	NS
Residual	95		

Experiment B

1, 2, 5, 6 and 7

$X^2 = 6.00$ (4 df) P = NS

Comparison: group 5 (11 with and 3 without sites) vs group 6 (13 with and 1 without sites)

By Fisher's exact method P = 0.09255

By inspection groups 3 and 4 had fewer mice with sites than groups considered.

Source	df	Variation Ratio	P
Treatments	14	8.76	< 0.001
Blocks	4	1.00	NS
Residual	56		

Comparisons

(i) Ovarx pregnant (2.749) vs intact controls (3.119) NS

(ii) Ovarx 3½ days (2.032) vs ovarx 3½ days (3.105) < 0.001

(iii) By inspection mice ovariectomised on day 2 had fewer sites than those ovariectomised on day 3.

that a higher proportion of the intact control had implantation sites than did the ovariectomised mice (Fisher's exact test P = .00001737). Ovariectomy at day 2 resulted in fewer mice with implantation sites than did this operation on day 3 ($P < 0.001$). The proportions of mice that responded by implantation after ovariectomy at $3\frac{1}{4}$, $3\frac{1}{2}$ days of pregnancy or after no treatment (the intact controls) were not significantly different.

2. The Number of Implantation Sites per Mouse

The mean number of implantation sites for mice in each treatment group and a summary of statistical tests made are given in Tables 7 and 8, respectively.

Experiment A

The mean number of implantation sites for the intact control, those sham ovariectomised at $1\frac{1}{2}$ and $3\frac{1}{2}$ days and mice ovariectomised at $3\frac{1}{2}$ days of pregnancy and given $\frac{1}{4}$, $\frac{1}{2}$ and 2 mg of progesterone daily were 9.68, 8.56, 10.87, 8.38, 8.41 and 7.47 respectively. After transformation of the individual mouse totals to $\sqrt{x + 1}$ values, analyses of variance showed no significant treatment, or block differences, for the numbers of implantation sites in these groups.

Experiment B

Mice ovariectomised on day 2 of pregnancy were judged by inspection (Table 7) to have fewer implantation sites than other groups with pregnant mice.

Data from treatment groups 1, 2, 5, 6 and 7 were transformed and analysed as for Experiment A. The mean numbers of implantation sites for mice ovariectomised at $3\frac{1}{4}$ days, viz., 4.73 was smaller than the mean value of 9.30 obtained for mice ovariectomised at $3\frac{1}{2}$ days of pregnancy and given progesterone alone ($P < 0.001$). The means for intact control mice and those ovariectomised on day $3\frac{1}{2}$, 9.24 and 9.52, respectively, were not

significantly different from one another.

3. Blastocysts recovered from 'Barren' Ovariectomised Mice

Numerical data and statistical results are given in Tables 9 and 10, respectively. 'Barren' mice were those pregnant at the time of ovariectomy but were without implantation sites at autopsy.

(a) The proportion of mice with blastocysts

Experiment A

Blastocysts were found in 20 of the 91 uteri flushed. The proportion of mice with recoverable blastocysts was higher after ovariectomy at $3\frac{1}{2}$ than at $1\frac{1}{2}$ days of pregnancy ($P < 0.05$). Other variables tested (Table 10) did not significantly influence the numbers of mice with flushable blastocysts.

Experiment B

Blastocysts were found in 12 of the 46 uteri flushed. None of the treatments significantly altered the proportions of mice with recoverable blastocysts.

(b) The number of blastocysts recovered per mouse

Experiment A

Neither the presence or absence of progesterone nor the time of ovariectomy caused significant variation in the numbers of blastocysts flushed.

Experiment B

Fewer blastocysts were recovered from mice ovariectomised at $1\frac{1}{2}$ and given estrogen at $3\frac{1}{2}$ days, than from mice ovariectomised on day 2 of pregnancy ($P < 0.001$).

4. 'Barren' Ovariectomised Mice with Damaged and/or Fluid Distended Fallopian Tubes

Both numerical and statistical data are given in Table 11.

(a) Fallopian tubes damaged during ovariectomy

TABLE 9

BLASTOCYSTS RECOVERED FROM 'BARREN'+ OVARIECTOMISED MICE DURING EXPERIMENTS A AND B

	Experiment A										Experiment B				
Ovarx during pregnancy at:-	1½ days					3½ days					2½	2¾	3¼	3½	1½ days
Treatments	¼ mg	½ mg	2 mg	8mg	Veh.	¼ mg	½ mg	2 mg	8 mg	Veh.					
Group numbers	1	2	3	7	8	4	5	6	9	10	3	4	5	6	7
No. uteri flushed	17	19	19	17	20	7	3	3	20	19	13	13	4	2	14
No. uteri with blastocysts	3	3	5	3	6	3	1	0	8	9	4	5	1*	1*	1
Total number of blastocysts flushed	5	11	19	10	8	6	4	0	36	27	20	22	4	2	1
Mean no. per mouse	1.66	3.66	3.80	3.33	1.33	2.00	4.00	0.00	4.50	3.00	5.00	4.20	4.00	2.00	1.00

* These mice were pregnant, having free blastocysts in one uterine horn and implantation sites in the other. Group Nos 5 and 6 were excluded from calculations.

\+ 'Barren' in the sense that they did not have implantation sites.

TABLE 10

BLASTOCYSTS RECOVERED FROM 'BARREN'+ OVARIECTOMISED MICE DURING EXPERIMENTS A AND B

SUMMARY OF STATISTICAL TESTS

1. The proportion of mice with blastocysts.

Experiment A (comparisons with 2 x 2 contingency tables)

	x^2	df	P
(i) All mice:- Day of ovariectomy ($1\frac{1}{2}$ vs $3\frac{1}{2}$)	4.619	1	<0.05
With progesterone vs without progesterone	1.905	1	NS
(ii) Day of ovariectomy with progesterone status			
prog. $1\frac{1}{2}$ days vs no prog. $1\frac{1}{2}$ days	0.0362	1	NS
" $3\frac{1}{2}$ " vs " " $3\frac{1}{2}$ "	0.265	1	NS
(iii) Between progesterone treated groups			
prog. $1\frac{1}{2}$ days vs prog. $3\frac{1}{2}$ days	0.199	1	NS
$\frac{1}{2}$ mg " " " vs $\frac{1}{2}$ mg " $1\frac{1}{2}$ "		1	0.33 *
$(\frac{1}{2} + \frac{1}{2})$ mg " " " vs 2 mg " " "		1	0.22 *
$\frac{1}{2}$ mg " $3\frac{1}{2}$ " vs $\frac{1}{2}$ mg " $3\frac{1}{2}$ "	0.500	1	
$(\frac{1}{2} + \frac{1}{2})$ mg " " " vs 2 mg " " "	0.329	1	NS
(iv) Between groups given no treatment (0) and/or vehicle (v)			
(0 + V) $1\frac{1}{2}$ days vs (0 + V) $3\frac{1}{2}$ days	0.000268	1	NS
V " " vs 0 $1\frac{1}{2}$ "	0.238	1	NS
V $3\frac{1}{2}$ " vs 0 $3\frac{1}{2}$ "	0.019	1	NS

Experiment B (total Chi Square for treatment groups 3, 4 and 8)

x^2 = 3.70 (2 df) P NS

2. The number of blastocysts recovered per mouse.

Summary of factorial analysis of variance.

Source	df	Variance Ratio	P
With and without progesterone	1	0.000689	NS
Time of ovariectomy ($1\frac{1}{2}$ and $3\frac{1}{2}$ days)	1	0.514	NS
Interaction	1	0.560	NS
Residual	37		

x^2 = 19.720 (2 df) P<0.001

Means

Group 3	5.0	blastocysts per mouse
" 4	4.2	" " "
" 8	1.0	" " "

Comparison

By inspection most of the variation is attributable to group 8, i.e., this mean is significantly lower than the means of groups 3 and 4.

* Probabilities calculated directly by Fisher's exact method.

+ 'Barren' in the sense that they had no implantation sites.

TABLE 11

'BARREN' MICE WITH FLUID DISTENDED AND OPERATIONALLY DAMAGED FALLOPIAN TUBES FROM EXPERIMENTS A AND B

		Experiment A												Experiment B					
1.	Damaged Fallopian (E) tubes	Ovarx 1½ days						Ovarx 3½ days						Day of Ovarx					
	Treatment	(0	¼	½	2) mg	Veh	Total	(0	¼	½	2) mg	Veh	Total	1½	2½	2¾	3½	3¾	Total
	Treatment group numbers	7	1	2	3	8		9	4	5	6	19		8	3	4	5	6	
	Total no. of horns flushed	36	34	38	36	40	184	40	14	6	6	38	104	28	26	26	6	2	88
	No. of partially immobilized E tubes	1	2	2	1	3	9							1	3		2		6
	" " totally " " "	2	2	1	1		6					1	1	2		3	1		6
	Total no. of damaged F. tubes	3	4	3	2	3	15					1	1	3	3	3	3		12
	" " " " " "																		
	as percentages	8.3	11.8	7.9	5.6	7.5	8.2					2.6	0.9	10.7	11.5	11.5	5.0	0.0	13.6
2.	Fluid distended Fallopian tubes																		
	Total no. of F. tubes distended	6	5	3	1	2	17		4		1	1	6		3	1	2		6
	" " " " "																		
	as percentages	16.7	14.7	7.9	2.8	5.0	9.2		10.0		16.7	2.6	5.6		11.5	3.8	33.0		6.8
3.	Fallopian tubes - both damaged and fluid distended																		
	Total no. of F. tubes affected	1		1								1	1	1	1	2			4

4. Summaries of Chi Square tests

	df	x^2	P
Distended F. tubes			
a) Total	9	10.038	NS
b) With prog. vs no prog.	1	0.000027	NS
c) Ovarx 1½ vs ovarx 3½	1	0.0517	NS
Damaged F. tubes			
a) Total	9	5.450	NS
b) Ovarx 1½ vs ovarx 3½	1	P = 0.0063228 (Fisher's exact test)	
		= <0.01	

Distended F. tubes			
a) Total x^2	4 df	3.627	NS
Damaged F. tubes			
a) Total x^2	4 df	0.229	NS

Sixteen of the 288 and 12 of the 88 uterine horns were damaged in Experiments A and B, respectively. More Fallopian tubes were damaged during ovariectomy at $1\frac{1}{2}$ than at $3\frac{1}{2}$ days of pregnancy during Experiment A ($P = 0.00652$).

(b) Fluid distended Fallopian tubes

Twenty two and 6 fluid distended tubes were observed at autopsy during Experiments A and B, respectively. Usually only the ampullae was dilated with a clear fluid, the proximal whorl of the isthmus being occasionally distended.

During Experiment A, 2 and during B, 4 Fallopian tubes were found to be both damaged and fluid distended.

SECTION I (a)

RESULTS

1. The Time of Ova-implantation in Intact Mice

The mean numbers of ova flushed from the uteri of mice killed at 94, 102, 110 or 118 hours of pregnancy were 8.1, 7.0, 6.8 and 2.2, respectively (Table 12).

The variance ratio for these means was significant and by the use of orthogonal polynomials highly significant linear and significant quadratic components were found (Table 12). Polynomial coefficients to the second degree were used to construct the curve shown in Text - fig. 2.

2. The Form of Ova recovered

The number, form and size recovered blastocysts are given in Table 12.

(a) Abnormal 'blastocysts'

During the experiment, 22 unclassified objects were counted (see Pl. V. figs. 2 and 3; Pl. VII, figs. 1, 2, 3 and 4). Nine blastocysts were not further classified.

(b) Nontransformed blastocysts

The majority of blastocysts recovered from mice killed at 94 hours showed no signs of transformation (Pl. III, fig. 2). However, these proportions were reversed for those collected at 102 hours of pregnancy. Fifty five of the 101 blastocysts recovered at these two earlier times were still in their zonae pellucidae (Pl. I, figs. 1 and 2) and 10 mice had zona-clad and zona-free blastocysts in their uteri.

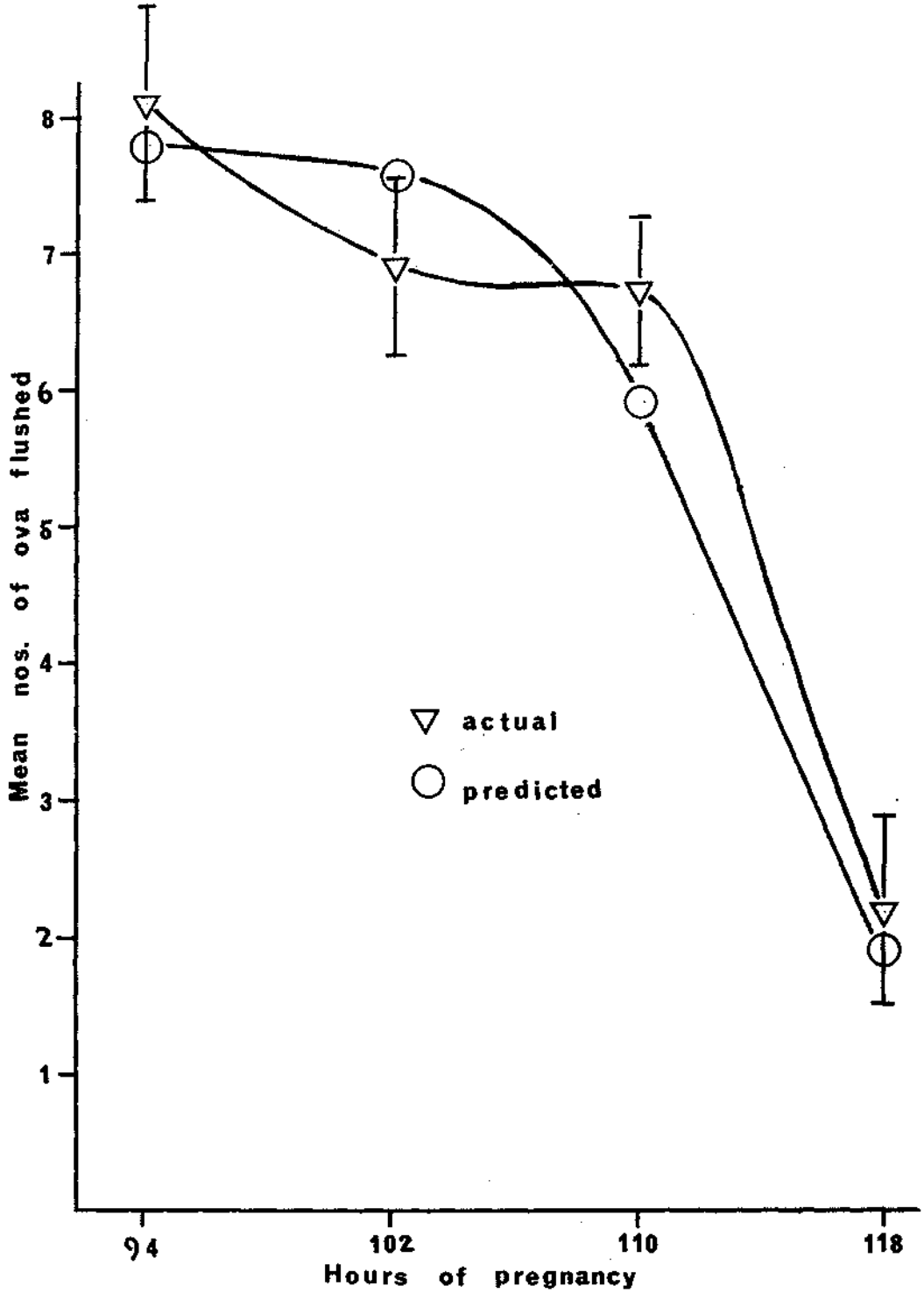
(c) Transformed blastocysts

Twenty seven blastocysts that were equal to or less than $\frac{1}{2}$ transformed ($\leq \frac{1}{2}$ T), (Pl. IV, fig. 1), and 35 blastocysts that were more than $\frac{1}{2}$

TABLE 12

THE NUMBER, STAGE OF DEVELOPMENT AND SIZE OF OVA RECOVERED DURING EXPERIMENT C

Stage of pregnancy (ova developmental age) in hours	The numbers of ova flushed						The numbers of ova at different stages of development							Size and shape of ova and the number of mice with uterine decidual swellings			
	Total nos. of ova flushed at autopsy	Mean nos. per mouse	Mice without recoverable ova	Unclassified objects	Ova flushed from oviduct horn halves	Ova flushed from cervical horn halves	Did not know	Within zonae pellucidae	Not transformed	Equal to or less than 2 transformed	More than 2 but not fully transformed	Fully transformed	Egg cylinders	Mean lengths (microns)	Mean widths (microns)	Ratio of mean length/mean width	No. of mice with measureable uterine swellings
94	89	8.1 ± 0.5		2	52	27	2	43	30	10	4			111.9	87.5	1.36	
102	76	7.0 ± 0.5		9	42	24	3	12	16	4	13	27		131.6	92.7	1.42	
110	74	6.8 ± 0.5		3	34	29	3			10	10	41	1	151.3	98.6	1.54	
118	24	2.2 ± 0.5	5	4			1					17	6	169.9	95.5	1.76	6
Totals	263		5	18	128	80	9	55	46	27	28	85	7				6
Numbers of mice	44		44	44	27	27	44	44	44	44	44	44	44	44	44		44



TEXT-FIG.2. The time of implantation. The actual means (± standard errors) obtained from raw data, refer to the no. of ova flushed per uterus. Predicted means were derived from the regression equation $(\hat{y} - \bar{y}) = b_1 \hat{c}_{1i} + b_2 \hat{c}_{2i}$; where $b_1 = -0.8954$ and $b_2 = -0.8049$

but less than fully transformed (<T) (Pl. IV, fig. 2), were recovered from mice pregnant 94, 102, and 110 hours. Mice pregnant 118 hours had 17 fully transformed blastocysts (T) (Pl. III, fig. 3), and 6 egg cylinders (EC) recovered from their uteri (Pl. III, fig. 4).

(d) The size of the recovered ova

Blastocysts aged 118 and 110 hours were each in turn significantly longer than the pooled values of younger blastocysts ($P < 0.001$) and 102 hour blastocysts were longer than 94 hour blastocysts ($P < 0.01$, Table 13).

(e) Further observation on the form of blastocysts

(i) General shape

There was considerable variation in the shape of blastocysts recovered from different mice at the same stage of pregnancy and even among those recovered from the same mice. Untransformed blastocysts were usually spheroidal shaped (Pl. III, fig. 2). While incompletely transformed blastocysts were often irregular in form, dented and partially flattened representatives were common. Transformed blastocysts were more consistently elongate in shape (Pl. III, fig. 3).

(ii) Size variation

Untransformed blastocysts, with or without their zonae pellucidae frequently varied markedly in size (Pl. I, figs. 1, 2, 3 and 4). Smaller blastocysts that looked contracted in form were found together with non-contracted blastocysts in the same mice.

(iii) Variations in the form of the abembryonic hemisphere

Giant cell transformation of the trophoblast begins at the abembryonic pole (Dickson, 1963) (Pl.I, fig. 6; Pl. IV, fig. 1).

TABLE 13

SUMMARIES OF ANALYSES OF VARIANCES CALCULATED FROM NUMBERS OF OVA RECOVERED AT 94, 102, 110 AND 118 HOURS OF PREGNANCY

Source of variation	df	Variance ratio	P
a) The number of ova collected			
Linear	1	32.43	< 0.01
Quadratic	1	5.72	< 0.05
Cubic	1	2.91	NS
Total	43		
b) The size of ova			
(i) Length			
Ova age	3	90.45	< 0.001*
Total	35		
(ii) Width			
Ova age	3		
Total	39	1.77	NS

* Comparisons

1. 118 hrs (168.0 μ) vs (94+102+110) hrs (131.6 μ) P < 0.001
2. 110 " (151.3 μ) " (94+102) " (122.8 μ) P < 0.001
3. 102 " (131.6 μ) " 94 " (119.9 μ) P < 0.01

Eight cone-shaped blastocysts (2 and 6 from mice 102 and 110 hours pregnant, respectively) with the giant cells at the abembryonic pole organized so that they formed a point were recovered (Pl. V, fig. 1). Two blastocysts with discrete peg like projections from the abembryonic pole were observed (Pl. I, fig. 5; Pl. II, fig. 1). Transformed and to a lesser extent incompletely transformed blastocysts often showed debris adhering to the lateral margins of the abembryonic hemisphere (Pl. I, fig. 2). These changes occurred independently of or together with protruding cellular processes that extended out from the surface of the abembryonic pole.

DISCUSSION

1. The Body Weight Changes and the Mating Behaviour

While discussing experiments in the rat concerned with an evaluation of the critical time for ovariectomy after which progesterone administration would support implantation at the expected time, Mayer (1963) emphasized that 'one should operate on animals of a well standardized colony as far as weight, food, temperature and light were concerned and see that the operations were carried out at a definitive time of the day'. Judged by these criteria, the conditions in neither experiment were ideal. No attempt was made to regulate the light-dark regimen and during experiment A the temperature fluctuated widely and mice from three different colonies were used. When it is also considered that the time of the start of pregnancy is subject to variation (see page 41) there is sufficient reason to check on the physical conditions and the mating behaviour of the mice used.

There were however, no significance between treatment differences present in either experiment for the initial body weights, the mating delay or the incidence of vaginal plugs. These findings then indicate that with regard to these parameters no sampling or other bias was introduced into the experiments.

Mice in all treatment groups in both experiments gained weight during the experiment periods. Pregnant mice in both experiments were heavier than nonpregnant mice. The additional weight of pregnant mice is due both to extra- and intrauterine changes. The increase accountable to extra-uterine components may exceed that contributed by the foetuses and placentae (see Deanesly, 1966) and Dewar (1957) found that progesterone administered to pregnant mice ovariectomised and hysterectomised between days 14 and 16 of pregnancy maintained the extra-uterine weight increase. The degree of

protection against weight loss and subsequent loss occurring after treatment were each proportional to the quantity of progesterone given (over the range 0.5 to 3.0 mg per day). Further 0.5 to 0.7 mg of progesterone daily given to nonpregnant or nonpregnant ovariectomised mice starting at the time of ovariectomy caused increases in body weight. The difference between the adjusted mean weights of pregnant and nonpregnant mice in Experiment A (2.70 gm) was greater than in Experiment B (1.66 gm). This may then, in part be due to the fact that all ovariectomised nonpregnant mice in the latter experiment were given progesterone whereas four of the seven ovariectomised nonpregnant treatment groups during Experiment A were not given this hormone. The dose-dependent weight-stimulating effect of progesterone together with the higher number of live foetuses carried by mice given 2 mg could explain why this group was heavier than the pooled results of those given $\frac{1}{4}$ and $\frac{1}{2}$ mg of progesterone daily, after ovariectomy at $3\frac{1}{2}$ day of pregnancy during Experiment A.

Intact pregnant mice were heavier than ovariectomised pregnant mice in both experiments. This difference reflects uterine (the length of the uterine horns) and intrauterine (the number and size of implantation sites) differences as these parameters were greater in the intact mice (Table 22).

A finding more difficult to explain is why mice sham-ovariectomised at $1\frac{1}{2}$ days were lighter than those sham operated at $3\frac{1}{2}$ days of pregnancy. There were no differences between the two groups in the numbers of implantation sites or the number of live foetuses at autopsy. However, both the uterine and intrauterine contributions were less for the mice sham-ovariectomised at $1\frac{1}{2}$ days. Their uterine horns were shorter and the size of their implantation sites smaller (Table 22). Delayed implantation after sham ovariectomy at $1\frac{1}{2}$ days was possibly responsible for these effects, as the foetuses from mice sham operated at $1\frac{1}{2}$ days had developmental ages and body weights

lower than those recovered from mice sham ovariectomised at 3½ days of pregnancy (Tables 29 and 32).

Between block differences allowed investigation of some factors that influenced the vaginal plug incidence during Experiment A. Plugging incidence was variable and lower for Glaxo bred mice than those from the University colonies (Table 4). This difference is thought to be due to the relative sexual immaturity of the Glaxo bred mice, some of which had closed vaginal membranes at 56 days of age.

Other tests indicated that within mice from the University colonies, mice over 60 days of age had a higher incidence of vaginal plugs than those below. This finding is complemented by the negative correlation ($r = -0.579$, $P < 0.05$) between the age of all mice used and the mating delay. The net result being that there is the suggestion that more older mice mated, and they did so more quickly than the younger mice. However, other age separations (Table 4) showed no correlations with the vaginal plug incidence and there was no correlation between the mating delay and age for mice used in Experiment B.

2. The Methods of administering Progesterone

By virtue of their continual presence progesterone implant pellets were considered to provide a more steady release of the hormone than was provided by daily subcutaneous injections of progesterone in peanut oil. There is a rapid disappearance of progesterone from oil after it has been injected intraperitoneally (Forbes, 1959) or subcutaneously (Cohen, 1959) in mice. In general the pellets were well tolerated, only two of the 164 pellets recovered were the foci of abcesses.

3. The Start of Pregnancy

Lewis and Wright (1935), Snell, Fekete, Hummel and Law (1940), Snell, Hummel and Abelmann (1944) and Dickson (1967) found that the majority of

mice mated between three and seven hours after the onset of darkness (usually between 2200 and 0100 hours) and that a minority of the mice mate after 0100 hours.

Ovulation in the mouse is spontaneous and the release of the eggs is probably about synchronous and sometimes not (Lewis and Wright, 1935) and occurs for the majority of mice within two to three hours after mating (Snell et al., 1940). The time of fertilization as determined by the emission of the second polar body, occurred within about two hours of copulation in the study by Lewis and Wright. The lack of precision regarding these estimates makes the dating of the start of pregnancy at 0200 hours approximate only.

4. The Time of Ova-implantation

Text-fig.2 shows that relatively few blastocysts were obtained by flushing the uteri at 118 hours of pregnancy. There was a progressive decline in the numbers of blastocysts recovered up to 118 hours which is represented by the significant linear component, and the superimposed accelerated decline between 110 and 118 hours accounted for the significant quadratic component in the equation for the recovery curve (Table 13).

The form of the recovered blastocysts or egg cylinders provides an indication of the stage of implantation achieved. Egg cylinders recovered at 118 hours with 'caps' of uterine tissue adhering to the abembryonic poles (Pl. II, fig. 4; Pl. III, fig. 4) together with the low recovery rate would suggest that some blastocysts attached well before this stage of pregnancy. The adherence of debris to the abembryonic hemisphere of some blastocysts at 102 and to more at 110 hours of pregnancy probably indicates that these blastocysts were fixed to the uterus at these times. Though, as blastocysts were recovered at 118 hours that did not show ragged peripheries or adherent debris, it is likely that not all attached

blastocysts showed visible evidence of early attachment. Enders and Schlafke (1967) were able to recover some blastocysts from rats that showed no damage at a time when other blastocysts were attached to the uterine epithelium of the implantation chambers by membrane to membrane adherence. The early stages of implantation in the rat and in the mouse (Potts, 1966) involve a series of changes which progress to a more intimate apposition between the blastocyst and the uterine epithelium. This means that blastocysts could be dislodged by flushing some time after primary attachment and certainly before the time at which they first assume a fixed position in relation to the uterus.

It is not known how the blastocyst first informs the uterus of its presence. An early change on the part of the uterus is the local increase in capillary permeability denoted by a leakage of intravenously injected Pontamine Sky Blue, a high molecular weight dye, in the region of the uterus surrounding the blastocyst. This change was first observed in the rat by Pyschoyos (1960) and has since been recorded in the hamster (Orsini, 1963) and in the mouse (McLaren, 1966). These workers found that the coloured regions indicate the conceptual area prior to decidualization, and in the rat, Enders and Schlafke (1967) found that the Pontamine Blue reaction was first seen at the time when the oedematous uterus was thought to 'clasp' and immobilize the blastocyst.

In a study of the temporal relationships of early changes in the uterus and blastocyst in the 'Q' strain of mice, Finn and McLaren (1967) found that the Pontamine Blue reaction was usually only positive for blastocysts that had lost their zonae pellucidae and was present in some mice as early as 93 hours post coitum (this corresponds to 92 hours of pregnancy in the present study but because of the inherent inaccuracy in dating the start of pregnancy, no correction will be made for this or any other

post coital time estimate) and consistently present at 95 hours p.c. These workers observed that the Pontamine Blue reaction preceded the appearance of localized uterine stromal oedema by 2 or more hours which suggests that some blastocysts may be stationary within the uterus before oedematous changes occur. They also noted a close temporal relationship between the giant cell trophoblastic transformation (Dickson, 1963, 1966a) and the emergence of Wilson's (1963) 'primary invasive cells' from the blastocyst. Wilson (1963) observed that the so-called invasive cells extended from the trophoblastic cells into the then intact uterine epithelial cells at about 100 hours p.c. Finn and McLaren observed the emergence of these cells after the Pontamine Blue reaction and before that of the stromal oedema i.e., at about 94 hours p.c.

As previously described by Dickson (1963, 1966a) trophoblastic giant cell transformation began at and extended from the abembryonic pole eventually to involve all the trophoblast cells excepting those positioned above the inner cell mass (Pl. I, fig. 6; Pl. IV, fig. 2 and Pl. III, fig. 3). Fourteen of the 89 blastocysts recovered at 94 hours, 44 of the 76 at 102 hours and all of the 74 at 110 hours showed signs of trophoblastic transformation and all the blastocysts recovered at 118 hours of pregnancy were completely transformed (Table 12). The time progress of giant cell transformation closely parallels that observed by Dickson (1966a) in Swiss Webster albino mice for which over 50 per cent of the blastocysts were completely transformed at about 108 hours p.c. If it is assumed that the onset of giant cell transformation coincides with the emergence of the 'primary invasive cells' and therefore with attachment of the blastocyst the uterus it can be seen (Table 12) that the stage of pregnancy at which these changes take place are for some blastocysts equivalent that observed by Finn and McLaren. However, there was marked asynchronism in the

progress of giant cell transformation in the present study. Dickson (1963, 1966a, 1967) has also commented on this asynchronism in blastocyst development for mice with the same mating histories from the T.T., Swiss Webster and C57BL strains of mice, respectively. Blastocysts from C57BL mice appeared to take several hours longer than those from Swiss Webster mice to reach a given stage of transformation and for the Swiss Webster strain at least, some of the retarded blastocysts were possibly due to late copulation (Dickson, 1967). Further there is often asynchrony in rate of blastocyst development in any one mouse (Dickson, 1966a; Finn and McLaren, 1967) or rat (Kraicer, 1967) and during the present study mice were found with nontransformed and blastocysts less than one half transformed together in their uteri.

The corollary of this asynchronism between and within mice of any one strain is that the time of implantation is probably variable both for blastocysts from different mice and from the same litter. There is other evidence from the present work and from the literature that does not depend on the morphology of the blastocysts to suggest that in any one mouse implantation is often asynchronous. Two mice, ovariectomised at $3\frac{1}{4}$ and $3\frac{1}{2}$ days of pregnancy during Experiment B and administered progesterone had implantation sites in one horn only and a free blastocyst present in the other uterine horn (see Table 9). These are examples of what Zeilmaker (1963) called 'incomplete implantation'. This author described this phenomenon in rats ovariectomised or hypophysectomised at the respective stages of early pregnancy after which the blastocysts would not implant in the absence of hormones from these two endocrine glands. Incomplete or asynchronous implantation is possibly a consequence of the curtailment of maternal support so that retarded blastocysts are denied systemic hormonal influences, by becoming receptive to these too late (see page 66). Further

while examining foetuses recovered after autopsy, a number were found that were retarded in development and smaller than others in the same uterine horn (see Gruneberg, 1943). An extreme example of such variation is shown on Plate XI figure 2, where a foetus of about 11 days was situated between two aged about 13 days. It is thought unlikely that local uterine influences would work to retard the development of some embryos after synchronous implantation (see page 99, Section II) and in view of the above described variation in blastocyst morphology and the occurrence of incomplete implantation it is concluded that blastocysts from the same litter often implant asynchronously.

In the present study then, working on the assumption that attachment of the blastocyst coincides with the commencement of giant cell transformation, some blastocysts were attached at 94 hours, over 50 per cent were attached at 102 hours and all blastocysts had attached to the uterus at 110 hours of pregnancy. While no precise estimate can be made as to the extent to which blastocysts in one mouse are retarded with respect to the most advanced blastocysts in other mice, there is at least 8 hours difference, as blastocysts with and without their zonae pellucidae, were recovered from the same mice both at 94 and 102 hours of pregnancy. Between mouse differences of about 17 hours were recorded by Dickson (1966a) with regard to the time period over which the loss of the zona pellucida was first seen to occur.

Presumably the blastocysts assumed a fixed position, and by definition began to implant, a few hours before they attached to the uterus. Thus the estimated time of implantation for most blastocysts was early on the day 4 of pregnancy. This time is in approximate agreement with those reported by Amoroso (1952), Snell (1956), Wilson (1963), Dickson (1966a) and Finn and McLaren (1967). Closer agreement is unlikely because of 1) the various definitions adopted concerning the start of implantation 2) the uncertainty

as to the time of fertilization 3) differences between strains of mice 4) differences in the prevailing environmental conditions, especially the light-dark regimens (see Bindon and Lamond, 1967) and 5) due to the inherent within and between mouse variation with regard to the time of blastocyst implantation.

5. The Form of the Blastocysts

(a) Size

The size of the blastocysts depended on their age and there was a progressive increase in length and in degree of giant cell transformation with advancing pregnancy. These changes are similar to those observed by Dickson (1966a). The volume would have provided a more accurate measure of the size of the blastocysts, however, because of the variable shape of recovered ova it was impracticable to calculate this parameter. The length is considered to be a valid estimate of the size as there was no change in the width with advancing developmental age and in such cases any increases in length would underestimate the corresponding changes in volume.

Dickson (1966a) took issue with the prevalent statement in the literature (see reviews by Austin, 1961; Blandau, 1961) that the size of the blastocyst at the time of implantation was the same as that of the tubal ova and produced evidence to show that blastocysts were almost twice as long at implantation. However, he defined implantation as beginning when uterine swelling was visible at the attachment of the mesometrium to the uterus at about 108 hours post coitum. The first measurable uterine swelling seen in the present work was at 110 hours and these were not regularly seen until 118 hours of pregnancy. Because the beginning of implantation was defined differently and was estimated to occur before 102 hours it is not known if the blastocysts were at this stage longer than tubal ova. It is probable that they were, as ova at 102

hours were longer ($P < 0.01$) than at 94 hours and it is reasonable to assume that there was a steady increase in size between these two times.

A number of what appeared to be 'contracted' blastocysts, either within or that had recently lost their zonae pellucidae, were recovered together with the more typical spheroidal forms from the same mouse at 94 and less often at 102 hours of pregnancy (Pl. I, figs. 3 and 4). Contracted blastocysts had a smaller blastocoele than usual and both the length and width of these forms were reduced. But for their small size blastocysts with contracted trophoblastic cells resembled partially transformed blastocysts. One nontransformed blastocyst, with one of its short diameters markedly reduced, expanded in order to assume an ellipsoid shape. There are two probable explanations for the existence of contracted blastocysts viz., that they are artefacts produced by in vitro conditions and secondly that they were contracted prior to their recovery. Dealing with the first alternative, it is known (from personal observations and see Borghese and Cassini, 1963) that blastocysts maintained in a number of in vitro culture mediums undergo permanent shrinkage after a certain time, perhaps in the present case, aided by the hypertonicity produced by evaporation from the saline drop. However, the smaller blastocysts in question were observed before shrinkage would normally have been expected. Also the more obvious case for rupture or other damage being inflicted during flushing is an unlikely explanation in that this would not be expected to affect blastocysts still within their zonae pellucidae.

Rather the second alternative offered is preferred, viz., that the blastocysts were contracted in utero. Borghese and Cassini (1963) and Cole and Paul (1965) observed that zona encased mouse blastocysts maintained in vitro displayed a 'rhythmic sequence of contraction and expansion'. Cole and Paul noted that these cycles continued after the blastocysts had

lost their zonae either by mechanical or natural means. Presumably the nature of these contractions was different from that of the aforementioned shrinkage in that they were reversible. These workers also observed that the period of expansion was always of a longer duration than the contraction phase which may be as short as 15 seconds. This could explain why sphercidal shaped nontransformed blastocysts are much more commonly seen in flushings, as they spend relatively more time in an expanded than in a contracted form.

(b) Abnormal blastocysts

Twenty seven abnormal blastocysts (Table 12) or 9.75 per cent of all the ova recovered were abnormal. While it is convenient to refer to these objects as abnormal blastocysts, it is by no means certain that they all were derived from blastocysts. Lewis and Wright (1935) divided abnormal mouse eggs they found into three classes viz., 1) those that were abnormal at the time of ovulation 2) unfertilized eggs that ultimately degenerated and 3) eggs that were injured by pathological conditions of the genital tract.

Considering the last class first, all the abnormal ova were found associated with normal ova from the same mice. And as reasoned by Huber (1915), this makes it likely that the ova themselves were abnormal, for if the genital tract was diseased it would be expected that the majority of the ova in each litter would be abnormal. Degenerate eggs within their zonae pellucidae were rarely encountered, one being recovered during Experiment A and one during Experiment B. These two eggs had finely-fragmented cytoplasm. Nine blastocysts with obscure cellular detail were observed and it is probable that the majority of these represented degenerate ova that had lost their zonae pellucidae. Huber (1915) found a small number of rat ova in which death and degeneration occurred at the end of

segmentation stages.

However, the majority of abnormal ova, or 18 unclassified objects, remain to be explained. Because the blastocysts were not seen until they had reached the uterus, it is not possible to know at what stage their form deviated from normal. This is relevant as it is probable that most of these objects were ova that had developed in a bizzare way, for example, none had zonae pellucidae. It is also possible that some were objects that resembled unusual blastocysts. With few exceptions the unclassified objects were smaller than normal blastocysts, in fact small size ($\leq$ 80 u long and $\leq$ 40 u wide) was one criterion of abnormality. The reasons for the existence of any of the forms are not known, though on morphological grounds explanations may have included pieces of ova, an alternative suggested by the small size of the unclassified objects. For example, the 'blastocyst' shown in Plate V, figure 2, perhaps the most normal looking of the unidentified objects, appears about to lose the knob of cells situated at what probably is the abembryonic pole and should this have occurred with other blastocysts it may explain the occurrence of some of the forms shown (Pl. VII, figs. 1, 2 and 3). Another way in which the size of the blastocysts can be reduced is by the loss or separation of blastomeres during cleavage. It is known that the experimental removal of the zonae pellucida mechanically or with 'pronase' from mouse ova cultivated in vitro allows blastomeres at the two and four cell stage, to separate after which they sometimes give rise to miniature blastocysts (Mulnard, 1965; and Gwatkin, 1963, respectively).

It is perhaps important that the majority of unclassified objects were recovered along with normal blastocysts both within their zonae pellucidae and those that had recently lost this membrane. This suggested that the premature loss of the zonae pellucidae may have been associated

with, if not the cause of, the appearance of most of these abnormal forms. Unusual forms developing from separated blastomeres would be more frequently encountered than miniature blastocysts (Gwatkin, 1963; Mulnard, 1965). There is, however, no guarantee that some of these unclassified objects may not have developed into viable blastocysts. Though due to their small size and the fact that a number of these forms are composed of numerous cells (Pl. V, fig. 3 and Pl. VII, fig. 4) which indicates that the mitosis was continuing although normal cavitation leading to blastula formation was not, suggests that the chance of these forms developing normally was remote. For example, the 'blastocyst' shown on Plate VII, figure 2, was flushed on day $12\frac{1}{2}$ from a mouse ovariectomised at, and given no further treatment from day $3\frac{1}{2}$ of pregnancy during Experiment A. By its appearance it would seem to be a possible derivative of the forms shown in Plate VII, figures 1 and 3 and it certainly was different from the more normal looking blastocysts collected on day $12\frac{1}{2}$ (Pl. IV, fig. 3 and Pl. VI, fig. 3).

Cavitation is apparently abnormal for the unclassified objects. Multiple cavitation as shown in an apparently normal egg (Pl. III, fig. 1) is probably common, the cavities soon becoming confluent and enlarging (Mintz, 1965). The difference between this and cavitation in the 'objects' is firstly, cavitation is normally completed while the ova are still within their zonae pellucidae and none of the ojbects was enclosed in this membrane, and secondly that the high incidence of 'objects' with cavities suggests that this process was prolonged or incomplete. While cavities are not shown in the 'objects' with large numbers of cells (Pl. V, fig. 3, Pl. VII, fig. 4) forms of this type were seen with small cavities.

Should the early loss of the zonae be a correct explanation it would also represent another casual factor contributing to embryonic loss.

Another explanation for the existence of some of the 'objects' is

that they represent pieces of normal blastocysts that resulted from damage inflicted during recovery. However, if this were the main cause, ruptured blastocysts should have been relatively common, and inbetween forms showing rents or tears should have been seen. Ruptured blastocysts were only occasionally encountered, and then only after implantation, and in such cases were clearly recognizable as such.

The last explanation offered is that they were not derived from germ cells at all and represented uterine tissue, secretions or debris. An example of this nature is shown (Pl. VI, fig. 2) which is probably composed of secretion droplets adhering to uterine debris. This is not considered to represent an unclassified object. Also when the uteri of nonpregnant uteri were forcibly flushed to provide control information, material identified as uterine epithelium did not resemble the objects in question, in that the strips of this tissue removed did not fold on themselves to give the solid appearance of the 'objects' in question.

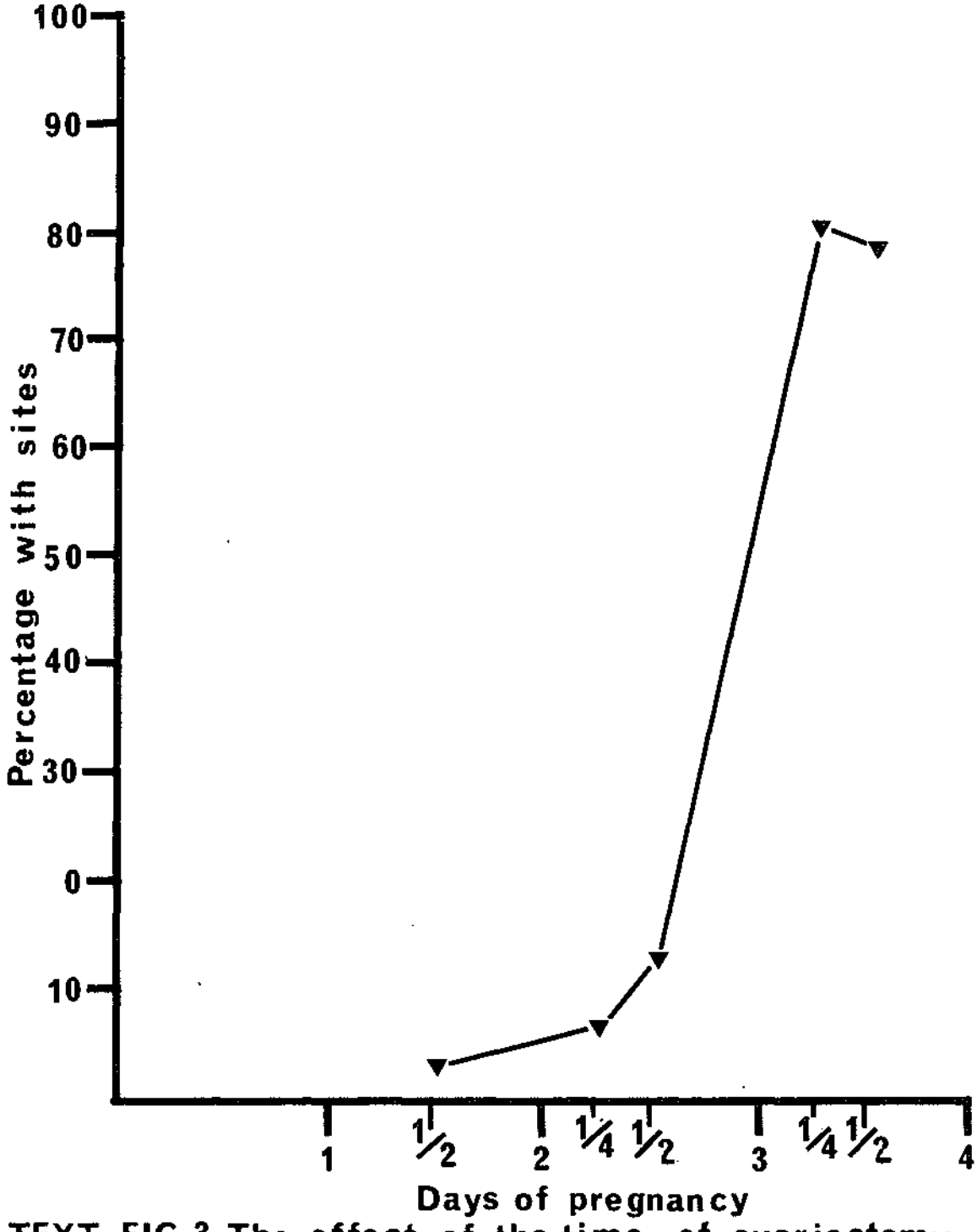
IMPLANTATION STUDIES

1. The Critical Time for Ovariectomy

Irrespective of the dose rate of progesterone used or its combination with oestrogen treatment on day 3½ (Group 8, Experiment B), ovariectomy at 1½ days of pregnancy usually inhibited ova-implantation. Ovariectomy on day 2 allowed only three of 26 mice to implant ova, this number being less than after this operation on day 3 of pregnancy when the numbers of mice with implantation sites were not different from their respective controls.

The number of implantation sites per mouse increased as the proportion of mice with implantation sites increased. Mice ovariectomised on day 2 had fewer sites than did those ovariectomised on day 3 and it is of significance that the removal of the ovaries on day 3½ was associated with fewer sites than at day 3½. The numbers of sites in mice ovariectomised at 3½ days whether given daily oestrogen (Group 7, Experiment B) or not, were not different from those found in their respective controls.

These data provide direct evidence for a time-dependent differential effect for ovariectomy with respect to implantation in a progesterone-treated mice (see Text-fig.3). The critical time for ovariectomy, after which immediate progesterone treatment permits mice to implant as many ova as do controls, is about 3½ days (84 hours) of pregnancy. If the criterion for this time was the proportion of mice implanting ova, then it would be 3½ days of pregnancy. However, the number of implantation sites is probably a more sensitive indicator of the hormonal requirements for implantation. During experiments where implantation was interrupted by the administration of oestrogens to mice (Martin, 1963; Stone and Emmens, 1964) or antiprogestagens to rats (Pincus, 1965d) reductions occurred first in the numbers of implantation sites in each mouse and then in the proportion of animals with



TEXT-FIG. 3. The effect of the time of ovariectomy during experiments A and B, on the percentage of progesterone treated mice with implantation sites.

sites. And during work to determine the optimal dose of oestrogen for implantation in ovariectomised progesterone-treated mice (Smith 1966), the parameters varied in the reverse order as the doses of oestrogen used were increased to the optimum. Presumably the only hormone of importance in relation to implantation missing from, or in short supply in the ovariectomised-progesterone treated mice was oestrogen. If ovariectomy before and after the critical time can be correlated with the absence and the presence, respectively, of oestrogen secretion from the ovary then the qualitative hormonal requirements for ova-implantation in the mouse would be similar to the rat. These data provide no direct evidence for the existence of an oestrogen surge. However, they could be interpreted as agreeing with the hypothesis that implantation is dependent on a release of oestrogen occurring immediately before the critical time. Though before the critical time can be ascribable to and considered dependent on the presence or absence of ovarian hormones per se, it is necessary to eliminate nonhormonal time-dependent effects of ovariectomy.

There is evidence from the present work to suggest that some ova from mice ovariectomised at 1½ days were prevented from reaching the uterus. Ova normally enter the uterus at about 72 hours post coitum (Lewis and Wright, 1935) or at about the beginning of day 3 of pregnancy, so that ovariectomy ½ and ½ a day later should not have obstructed transport through the Fallopian tubes. During Experiment A, more 'barren' mice had flushable ova after ovariectomy at 3½ days than after this operation at 1½ days (Table 10) and a relatively higher number of Fallopian tubes were damaged by ovariectomy at 1½ days of pregnancy (Table 11). During Experiment B, 'barren' mice ovariectomised at 1½ days had fewer ova per mouse than did those ovariectomised on day 2 of pregnancy. It is not known if the injection of 0.005 ug of oestradiol on day 3½ after ovariectomy

at 1½ days lessened the chances of recovering blastocysts. Acting alone this amount of oestrogen was probably too small to cause expulsion of ova (see page 64), though when acting synergistically with progesterone it may have lessened a blastocyst's chance of survival. Surgical interference at 1½ days then did lower the chances of blastocyst recovery when compared with any effects it may have had at day 3 of pregnancy.

The recovery rate of blastocysts from ovariectomised mice was low. Only 29.5 per cent of the 'barren' ovariectomised mice from both Experiments A and B had recoverable blastocysts at autopsy. Of which 21, 24.5 and 50 per cent were recovered after ovariectomy on day 1, 2 and 3, respectively, of pregnancy. The progressively higher percentage of mice with blastocysts when ovariectomy was performed later on in pregnancy provides support for the contention that this operation, whilst the ova are in the Fallopian tubes, especially after only one day, limits their chances of reaching the uterus. However, the variation in the recovery rate is probably not sufficient in itself to explain the variable implantation rates, especially when only 50 per cent of mice ovariectomised at day 3½ had recoverable blastocysts. Generally the overall recovery rate was lower than that recorded by Smithberg and Runner (1960) who recovered blastocysts from 54 per cent of mice between 11 and 15 days of pregnancy. Theirs, however, were intact prepubertal mice stimulated to mate and ovulate with gonadotropin treatment. Such mice did not implant ova because their corpora lutea failed to differentiate fully. After ovariectomy on days 0, 1 or 2 of pregnancy, 33 per cent of similarly prepared mice had flushable ova when autopsied between days 30 to 46 post coitum.

The occurrence of fluid distended Fallopian tubes warrants consideration as a possible contributing factor to the low blastocyst recovery rate. However, as only 7.6 and 6.8 per cent of the uterine horns from nonpregnant

mice in Experiments A and B, respectively, had distended tubes any contribution from this cause would be small. Fluid distension of the periovarian sac, noted in a few intact mice at the time of ovariectomy may be related to the present condition. Alden (1942b) found a small aperture in the sac that opened into the peritoneal cavity to be present in both rats and mice. This aperture was considered to allow fluid movement from the sac into the peritoneal cavity.

The fluid distension of the thin walled ampulla, and less often also that of the proximal whorl of the isthmus, was probably due to the closure of the anterior portion of the ampulla caused by operational damage.

Both uterine horns were flushed before ova were searched for in the saline, so data are not available to confirm the presence of blastocysts in horns with distended tubes.

Despite the apparent similarity between the distension of the periovarian sacs and that of the Fallopian tubes it is not at all certain that the fluid pressure built up in to tubes at the time of ova transport. Also, when Alden (1942b) artificially caused fluid distension of the periovarian sacs by closure of the aperture leading to the peritoneal cavity, a procedure that presumably increased the fluid pressure within the tubes, some ova were still able to reach the uterus in the rat. So it is unlikely that the fluid-distended Fallopian tubes adversely affected the recovery rate of blastocysts in the experiments reported here.

Presumably the majority of blastocysts from 'barren' ovariectomised mice in the present work, were either not seen or disintegrated before autopsy. It is unlikely that they were expelled through the vagina in the absence of ovarian or exogenous oestrogens. Originally it was thought that recovered blastocysts showed signs of degeneration, however, in view of the wide variation in form of 'normal' blastocysts recovered from intact mice,

it is improbable that even if some blastocysts were in the early stages of degeneration that this would be apparent from their outward appearance. Adams (1965) found that the morphological characteristics of cultured rabbit blastocysts were an unreliable criterion of their ability to implant after transfer to foster mothers.

It is of interest that neither the proportion of mice with blastocysts nor the mean numbers of blastocysts recovered from mice after ovariectomy in Experiment A, were significantly higher in mice that were given progesterone than in those that were not given this hormone (Table 11). Progesterone is usually accorded a protective role for blastocyst survival, though Smithberg and Runner (1960) also recovered similar numbers on ova from ovariectomised mice irrespective of whether or not they were given progesterone. It then appears probable that progesterone does not noticeably influence the number of blastocysts that remain in the uterus even though other evidence suggests that the hormone protects blastocysts that do remain in the uterus. Nutting and Meyer (1963) delayed implantation in the rat by ovariectomy on day 2 and attempted to initiate implantation by the administration of oestrogen together with progesterone on day 7 of pregnancy. From days 2 to 7, when the blastocysts remained unimplanted in the uterus, doses ranging from 125 to 4000 ug of progesterone daily were given. Regression analysis of the number of implantation sites against the doses of progesterone showed that the dose response slope was very highly significant with the highest dose of progesterone associated with the largest number of implantation sites.

2. <u>The Progesterone Requirements for Ova-implantation</u>

During the present experiments ovariectomised mice did not implant ova in the absence of exogenous progesterone. The hormonal needs for ova-implantation have been reviewed by Deanesly (1966) and there is general agreement that with the possible exception of the guinea pig and the

armadillo, progesterone is required at the time of implantation.

The quantitative requirements of progesterone were apparently met with the lowest dose used. Differences between the proportion of mice implanting ova and between the number of implantation sites per mouse for the different levels of progesterone (i.e., $\frac{1}{4}$, $\frac{1}{2}$ and 2 mg daily) used were not significant.

Although the assumption was made that implantation was complete with a $\frac{1}{4}$ mg of progesterone daily, this is difficult to confirm. However, the size of the dead implantation sites at autopsy for mice that received $\frac{1}{4}$ mg were not different from those that were given $\frac{1}{2}$ mg of progesterone (Table 16). That is, implantation proceeded to the same degree in both these groups. Smithberg and Runner (1956) found that 100 per cent of ovariectomised gonadotropin stimulated prepubertal mice in their work implanted ova when given $\frac{1}{2}$ mg of progesterone per day. Moreover, it is thought likely that incomplete implantation would have led to the resorption and disappearance of the sites by day $12\frac{1}{2}$ of pregnancy in the current experiments.

The threshold requirements of daily progesterone for implantation in the ovariectomised rat (Chambon, 1949b) and hamster (Orsini and Meyer, 1962) were about $\frac{1}{2}$ and $\frac{1}{4}$ mg, respectively. Oestrogen was not given in either of these experiments.

3. <u>The Influence of Ovarian Hormones on the Critical Time for Ovariectomy</u>

The critical time for ovariectomy cannot be adequately explained in terms of the physical effects of the operation itself. There are reasons to believe that it may owe its existence to the sequential release of ovarian hormones during the pre-implantation stage of pregnancy.

The importance of the third day in the present study agrees with work by Yoshinaga and Adams (1966). These workers ovariectomised mice on each

of the first five days of pregnancy and immediately instituted daily progesterone treatment. Ovariectomy on days 0, 1 and 2 usually inhibited implantation whereas this operation on days 3 and 4 of pregnancy was compatible with implantation at the expected time. It was notable though, that ovariectomy on days 0 and 1 allowed about 1/3 of the mice to implant ova which is a higher proportion than the two out of 49 that implanted ova after ovariectomy at $1\frac{1}{2}$ days (Experiment A) in the present study. Also implantation was delayed in these two mice (histological sections showed that the smaller than expected sites at autopsy contained live embryos) and one mouse had questionable ovarian remnants. Smith (1966) and Dickson (unpublished, Dickson and Araujo, 1966) ovariectomised mice on days 1 and 2, and 3 of pregnancy, respectively, and administered daily progesterone treatment from the day of the operation. Smith scored no implantation sites on day 9, while Dickson found that 2.5 mg of progesterone on day 3, allowed mice to implant ova. These results agree with a critical time falling on day 3 and those of Bindon and Lamond (1968) add further support.

Hypophysectomy at various timesduring early pregnancy of mice given adequate progesterone, showed that the role of the pituitary gland in initiating implantation was completed by about 4 am on day 3 (or about 76 hours) of pregnancy. The removal of the pituitary gland after, but not before this time, permitted most mice to implant ova when raised under a light regimen of 12 hours of light and 12 hours of darkness. These results are similar to those obtained by Zeilmaker (1963) for the rat, where pituitary involvement was completed by about 89 hours of pregnancy. The daily lighting schedule used by Zeilmaker was 14:10 hours of light: dark but his probably does not account for the differencesin timing between the two species. Comparisons between the times of the appearance of the first positive Pontamine Blue reaction in the rat (Enders and Schlafke, 1967) and in the mouse (Finn and

McLaren, 1967) suggests that implantation in the mouse occurs between 12 and 18 hours earlier than it does in the rat. The important point is that in the same study Zeilmaker found that ovarian involvement in implantation, as determined by ovariectomy and progesterone treatment was completed within about 97 hours of pregnancy or about 8 hours after the termination of pituitary involvement.

In the present study, the role of the ovary (apart from the supply of progestins) was completed by day $3\frac{1}{2}$ (or 84 hours) of pregnancy. If these results are combined with those of Bindon and Lamonds, then as found by Zeilmaker in the rat, the time interval between the completion of pituitary and ovarian involvement is also about 8 hours. The errors introduced by comparisons of this sort are likely to be considerable, however, of importance is the possibility that the pituitary-ovarian sequence of action in the mouse parallels that existing for the rat. It is then possible that the critical time for ovariectomy in the mouse can be explained in terms of the ovarian hormone, oestrogen. That is, oestrogen released during the morning of the day 3 of pregnancy is essential for implantation and the oestrogen release is completed by or not needed for implantation after day $3\frac{1}{2}$. This presupposes that oestrogen plays a predominant role in implantation. The strongest evidence against a critical time, and with this interpretation of an oestrogen release on day 3, was obtained by Smithberg and Runner (1956) where ovariectomy on day 1 allowed 100 per cent of mice to implant ova when sufficient progesterone was made available.

Other workers however, favour the view that as in the rat, oestrogen is essential for implantation and that it is needed after day 3 of pregnancy. Supporting evidence is available from work on 1) the hormonal control of the endometrial sensitivity to the induction of nontraumatic deciduomata (Finn, 1965, 1966a) 2) the implantation after a period of delay

(Whitten, 1955, 1958; Bloch, 1958, 1959; Smithberg and Runner, 1960; Yoshinaga and Adams, 1966) and 3) wholly experimental conditions, which are here defined as existing when neither the ova nor the ovarian hormones are produced by the foster mother implanting the blastocysts (Smith, 1966; Humphrey, 1967).

There are three main hormonal stages in the sensitization of the uterus to the decidual cell reaction of implantation in the rat (see Marcus and Shelesynak, 1967). Oestrogen secretions from the ovary at the time of oestrus and ovulation constitute the 'priming phase', this is followed by a period of progesterone dominance or the progestational phase and this is interrupted by a brief release ('surge') of oestrogen acting on the background on continuous progesterone secretion. Finn (1966a) confirmed the existence of these three phases in the ovariectomised mouse by the use of intrauterine arachis oil as the decidualizing stimulus. Both priming and 'surge' oestrogen were required for decidualization when acting with an optimal dose level of progesterone given for a definite time period.

Evidence supports the theory that the delay in implantation associated with lactation in the pregnant mouse and rat is primarily due to a deficiency of oestrogen secretion at this time.

The pituitary gonadotropin output in both species is decreased by suckling and in the mouse Whitten (1955, 1958) observed that lactation was associated with decrease in uterine weight and low activity of the ovarian interstitial tissue and notably that the delay to implantation could be prematurely terminated by the administration of serum gonadotropin. While in the pregnant nursing rat Weichert (1940) showed that the anterior pituitary extract 'Antuitrin S' accelerated implantation and more recently Minaguchi and Meites (1967) found that suckling caused a decrease both in the hypothalamic content of LH - RF and in the pituitary concentration of LH.

Lowered ovarian oestrogen secretion is a probable consequence of lowered gonadotropin output.

The administration of oestrogen to the lactating rat by subcutaneous injection (Weichert, 1942) or as a small local injection so as to supply a restricted area of the uterine horn (Yoshinaga, 1961) accelerated implantation in both horns and in the restricted area of the horn subjected to oestrogen influence, respectively. Systemic oestrogen treatment in the nursing mouse hastened implantation (Morel, cited Courrier, 1950; Whitten, 1955, 1958) and was necessary before implantation would occur after ovariectomy during the delay to implantation (Bloch, 1958, 1959). These results strongly indicate that the lowered output of gonadotropins is accompanied by a short supply of ovarian oestrogen during lactation.

Oestrogen has also been shown to be effective in causing the implantation of blastocysts that had remained free in the uterus for five days after ovariectomy and progesterone treatment in the nonlactating mouse (Yoshinaga and Adams, 1966). As in lactating mice (Whitten, 1955, 1958) a single dose of oestrogen was all that was needed to induce the implantation of diapausing blastocysts.

Experiments where neither the ova nor the ovarian hormones are supplied by the mice induced to implant blastocysts might have been expected to provide narrower limits for the qualitative hormonal requirements for implantation. The results however, are somewhat contradictory. Although Smith (1966) believed that oestrogen was definitely required for ova-implantation in the mouse, her results do not always appear to support this conclusion. Ova were cultivated from the two-cell to the blastocyst stage in vitro and then transferred to ovariectomised foster mothers given daily progesterone treatment. Oestrogen was administered subcutaneously to the mothers or the ova were cultured in a medium containing oestradiol. While

oestrogen treatment was beneficial, it was not essential for implantation. The proportion of mice that implanted ova, transferred one or two weeks after ovariectomy or five to six days after ovariectomy and adrenalectomy, was not significantly altered by the systemic oestrogen treatment. Only when ova were transferred two days after ovariectomy did both methods of oestrogen administration allow implantation in significantly more mice than in those given progesterone alone.

The results obtained by Humphrey (1967) however, showed that when ova (taken from donor mice on day 4 of pregnancy) were transferred to foster mothers ovariectomised 14 to 17 days previously that had received oestrogen priming and then daily progesterone, implantation only occurred when a further single dose of oestrogen was given during the period of progesterone treatment.

Smith's results then showed that oestrogen may affect the blastocyst directly by enhancing its ability to implant in a progesterone dominated uterus. Yasukawa and Meyer (1966) correlated shape changes of unimplanted blastocysts in the ovariectomised-progesterone treated rat with the administration of oestrogen. These changes in shape preceded implantation of the delayed blastocysts but may not have represented a direct effect of oestrogen on the ova. Smith argued in her studies, that the total amount of oestradiol introduced into the uterus by the ten transferred blastocysts (about 2.0 to 2.5 x 10^{-8} µg) was too small an amount to make it likely that the oestrogen's effect on the blastocyst was secondary to an effect on the uterus. Humphrey (1967) interpreted his results to mean that oestrogen was required before the endometrium would respond favourably to the presence of the blastocyst. Finn (1966a) had previously shown the oil-induced deciduomata required 'surge' as well as priming oestrogen, so it appears that Smith and Humphrey have emphasized different aspects of the action of oestrogen. However, Humphrey transferred four day old blastocysts to recipient mice

by which time it is possible that a release of oestrogen from the donor mice would have acted on the blastocysts. Should this be right, then Humphrey is perhaps astray in claiming that 'the prime effect of oestradiol appears to be on the endometrium'.

4. The Dose Rate and Temporal Relationships of Oestrogen Treatment to Ova-implantation

The main premise on which the role of oestrogen in ova-implantation resides is that this event can be induced more or less at will in mice and rats experiencing delayed implantation by the administration of a single dose of oestrogen. It is probable that this 'surge' dose of oestrogen both sensitizes the uterus and activates the blastocyst. A relevant question at this moment is why did the single injection of 0.0025 µg of oestradiol on day 3¼, fail to induce implantation following ovariectomy at, and progesterone treatment from day 1½ of pregnancy. Despite the low blastocyst recovery rate at autopsy it is unlikely that there were no blastocysts within the uterus at the time of the injection.

Theoretically the dose of oestrogen may have acted as an antifertility agent (e.g. caused expulsion of the blastocysts from the uterus or inhibited the decidual reaction) or it may have had no effect at all. As indicated by their strain name NOS (New Zealand Oestrogen Sensitive), the mice used are characteristically oestrogen sensitive. And taking as a measure of oestrogen response that of a comparable increase in mammary gland area in ovariectomised immature mice, animals from the NOS strain were about (from the results of Flux, 1954, 1957) three times as sensitive to oestrone as CFH mice. Notwithstanding any additional sensitivity to oestrogen is thought unlikely that 0.0025 µg of oestradiol acted to inhibit pregnancy. Preliminary work showed that oestrogen *per se*,

would not bring about the expulsion of or otherwise lessen the changes of blastocyst survival. Apparently 'normal' blastocysts were recovered after five (two mice) and eight days (one mouse) of oestradiol treatment (0.0025 µg per mouse per day) which started immediately after ovariectomy at day $3\frac{1}{2}$ of pregnancy. There is the possibility that in the treatment group of interest that the oestrogen may have acted synergistically with progesterone. Though this cannot be discounted the results of Stone and Emmens (1964) and Finn (1966a) allow another interpretation. Stone and Emmens found that a single dose of about 0.02 µg of oestradiol given at 72 hours of pseudopregnancy, a most sensitive period to the antifertility affect of oestrogens, was required before trauma induced deciduoma were inhibited. Further about 0.5 µg of oestradiol was required to inhibit pregnancy in the intact animal at this time. In ovariectomised oestrogen-primed progesterone treated mice Finn found that a dose of 0.0625 µg of oestradiol inhibited nontraumatic deciduoma formation. Even allowing for three times the present dose of 0.0025 or 0.0075 µg it is unlikely that this amount would inhibit the decidual reaction and it suggested that the dose had no effect at all.

Smith (1966) found that the minimum effective dose (MED) of oestradiol when given on day 4 to mice ovariectomised on, and given daily progesterone treatment from day 2 of pregnancy, was 0.0291 µg (with five per cent fiducial limits of 0.0176 and 0.0504 µg). Single doses of 0.024 and 0.05 µg of oestradiol were successful in initiating implantation in progesterone treated ovariectomised mice in the studies of Humphrey (1967) and Yoshinaga and Adams (1966), respectively. Smith's work in the mouse and that of Psychoyos (1961) in the rat have demonstrated that implantation is not an 'all or none' phenomenon, so it is probable that the dose used was below the optimal range.

With regard to the timing of the oestradiol surge dose of 0.0025 µg in the present study, there is close agreement with the results of Finn (1965)

who found that ovariectomy of pseudopregnant mice on day 2 (3-4 pm), but not at the same time on day 3, prevented the appearance of the decidual cell reaction after the intrauterine injection of oil on day 3 (3.30 - 4.30 pm). It could be predicted on the basis of the present work that if implantation had been the chosen parameter, parallel results would have ensued and of special interest was the observation that a single injection of 0.25 µg of oestradiol 7 hours before the decidual stimulus permitted a positive response after ovariectomy on day 2. Finn tried the injection of oestrogen at other times, viz., immediately after ovariectomy on day 2 or just before the intrauterine oil injection, however at these times the decidual cell reaction that resulted was poor. If it is assumed that the start of pseudo-pregnancy in Finn's study was at the same time as the start of pregnancy in the present work then the timing of his successful dose of oestradiol was given about 1 hour after that given during Experiment B.

Incomplete implantation has been and still is explainable in terms of the asynchronous development of the blastocysts from any one litter where the advanced members would be able to respond to the oestrogen released from the ovaries before their removal, while incompletely developed blastocysts would be unable to take advantage of the curtailed oestrogen release. It is not necessarily implied that the oestrogen has to act directly upon the blastocysts as Smith (1966) suggested; activation secondarily to that of the uterus is not excluded. The number of ova implanted by mice ovariectomised at day 3¼ was less than for mice ovariectomised at day 3½ and since the proportion of mice with implantation sites was not different for these two groups it is likely that ovariectomy at the earlier time produced incomplete implantation. This lag of the number of implantation sites behind the proportion of mice with sites was also obtained by Smith (1966) where lower doses of oestradiol than the MED were used to induce implantation in

ovariectomised progesterone-treated mice. The conclusion reached is that ovariectomy on day 3¼ before the completion of the nonprogesterone ovarian role in implantation is equivalent to attempting to induce implantation with a suboptimal dose of oestrogen. The corollary is that the release of oestrogen from the ovary during normal pregnancy would have to last for a time at least equal to the period spanned by the most and least advanced blastocyst in the litter. How long the release would last in any one mouse is largely a matter for conjecture though continual oestrogen as well as progesterone treatment after ovariectomy at day 3½ allowed a considerably higher proportion of implantation sites to survive than did treatment with progesterone alone (see page 94, Section II).

Alternative explanations to the surge hypothesis have been offered to explain the role of oestrogen in ova-implantation. Smith (1966) proposed a gradual build up in the amount of oestrogen secreted from ovaries during the pre-implantation period such that critical levels for decidualization were reached late on day 3 or on day 4 of pregnancy. She also suggested that the blastocysts became sensitive to the direct influence of oestrogen early on day 4. These suggestions do not satisfactorily explain the present results, as the ovaries were absent after day 3½, and it is unlikely that the oestrogen levels would continue to rise after their removal. Also if implantation is dependent on oestrogen directly effecting the blastocyst then this action would have to occur before day 3½ of pregnancy.

Oestrogen appears to have a delayed effect both on the blastocysts and on the uterus. Yasukawa and Meyer (1966) observed that blastocysts changed from a spheroidal to an elliptical shape in the normal intact rat at about 108 hours after fertilization (assumed to take place at 0400 hours) and noted that unimplanted blastocysts in the uteri of ovariectomised progesterone treated rats showed the equivalent change in shape 12 hours after the simultaneous administration of oestrone with progesterone. It was at least

another 18 hours before trophoblastic invasion of the endometrium began in both intact and ovariectomised rats, that is, the time interval between the administration of the 'surge' oestrogen to this early stage of implantation was in the order of 30 hours. Although strictly comparable results are not available for the mouse, the present work and that of Finn (1965) indicate that an oestrogen release probably occurred at about 82 hours of pregnancy and it was not until after 104 hours that mice regularly showed the first histological signs of decidualization (Finn and McLaren, 1967), which would precede trophoblastic invasion if the succession of events during implantation is the same as in the rat (Enders and Schlafke, 1967). This means that there was a delay of about 20 hours between the ovarian release of oestrogen and the start of implantation.

It could be argued that in view of the oestrogen-sensitive mice used in the current work, that ova implanted in ovariectomised progesterone-treated mice because residual oestrogen remained in or was made available to the uterus and/or ova after ovariectomy and that a surge of oestrogen was not necessary. It would then follow that mice ovariectomised on day 3 would be more likely to implant ova than those ovariectomised earlier on in pregnancy as residual levels would be higher prior to and at the time of implantation on day 4. The critical time would then be explainable as that time after which ovariectomy allowed adequate residual levels for implantation. The assumption inherent in this argument is that a threshold level of residual oestrogen is required after the critical time of day 3½ of pregnancy, perhaps as suggested by Smith (1966) at the time of implantation. Less oestrogen sensitive mice would be expected to have the critical time for ovariectomy moved closer to the time of implantation.

That oestrogen does remain in the body tissues is supported by the work of Takahaski (1961) who found that ovariectomised and adrenalectomised

rabbits continued to release oestrogen in their urine and that oestrogen extracted from several tissues showed decreases of only between 15 to 35 per cent of free, but not conjugated or protein bound oestrogens, when measurements were made before and 12 days after ovariectomy alone. Work in the mouse suggests that residual oestrogens would be effective for a shorter time after ovariectomy but the evidence is incomplete. Parkes and Deanesly (1966) have reviewed the effects of ovariectomy on the reproductive organs in rats and mice. They cite work by Brambell and Parkes (1927) that showed that the immediate effects of ovariectomy in the nonpregnant mouse depended on the day of the oestrous cycle on which the operation was performed. Ovariectomy immediately before oestrus did not inhibit subsequent oestrus changes in the uterus and vagina. Work by Stone, Baggett and Donnelly (1963) and Stone (1963), during which tritiated oestradiol and oestrone were administered to ovariectomised mice, by intravenous and subcutaneous routes, respectively, showed that although the uterus and the vagina incorporated higher levels than did nonreproductive tissues (with the exception of the liver), their relative rate of loss of oestrogenic activity was higher especially for the largest dose rate of 0.1 µg. There were still appreciable, and quantitatively similar, levels in the uterus 16 hours after the intravenous administration of 0.01, 0.33 and 0.1 µg of oestradiol. The middle dose of 0.03 µg is close to the MED of 0.0291 µg for implantation found by Smith (1966) and with this amount, the uterine levels of oestrogen remained fairly constant until between four and eight hours after administration when they fell by about 1/3 and after 16 hours they were about 1/4 of their highest level. If implantation were to begin some 20 hours after day $3\frac{1}{2}$ or on the beginning of day 4, then even if the mice used were more sensitive than those used by Stone et al., the oestrogen levels are likely to have been below an optimal range required for a full implantation response.

Certainly the levels after ovariectomy on day 3¼ would not have been thought high enough to allow the same proportion of mice to implant as after ovariectomy ¼ day later, as doses of oestrogen below optimum in the mouse (Smith, 1966) and in the rat (Psychoyos, 1961) are associated with reductions in the proportion of animals with implantation sites.

From this information it is unlikely that residual oestrogen levels would remain high enough for implantation. Other evidence however, does not fully support this assertion. Should the critical oestrogen levels for implantation coincide with a stage of pregnancy after the critical time of day 3½, then if the delay for the action of oestrogen, was constant implantation in ovariectomised-progesterone-treated mice would be delayed. Judged by their external developmental characteristics and body weights at autopsy (Tables 29 and 32), foetuses from mice ovariectomised at, and given progesterone from 3½ days during Experiment A were about 1 day younger than foetuses carried by intact control mice. Ovariectomy and progesterone substitution therapy did not delay implantation during Experiment B. The situation is complicated further by the finding that sham ovariectomy at 1½, but not at day 3½, also delayed implantation (by less than a day) during Experiment A. Mice in the treatment groups that showed delayed implantation either had delayed or nondelayed litters. This suggests that delayed implantation was an 'all or none' effect influencing the mother and not the result of asynchronous blastocyst development. For implantation to have been delayed in both ovariectomised and intact mice it seems unlikely that residual levels of oestrogen activating the blastocyst and/or uterus after day 3½ in the progesterone treated ovariectomised was the cause of the delay. The rejection of this explanation means that the concept of an optimal amount of oestrogen required for implantation can more readily be retained. Rather it is suggested that the time interval between the release

of oestrogen required for implantation and the start of implantation was prolonged.

These results may be explained by the presence of stressful stimuli peculiar to sham ovariectomy at $1\frac{1}{2}$ days and present only during Experiment A. All ovariectomised mice during the course of this experiment were handled daily to check their identification and the appropriate groups given injections. This means that mice sham ovariectomised at $1\frac{1}{2}$ days were handled two days earlier than those that underwent this operation at $3\frac{1}{2}$ days. Whereas during Experiment B ovariectomised progesterone treated mice were given progesterone in the form of implant pellets and were not handled daily. Moreover, environmental conditions were more favourable during Experiment B and mice with diarrhoea were given medication which was not the case during the first experiment.

The conclusion then is that although implantation was delayed in some mice in Experiment A, the possible intervention of stressful stimuli suggests that the performance of the strain of mice used may be better gauged from the results of Experiment B. The similar time relationships between ovariectomy and deciduomata formation (Finn, 1965) and implantation (Yoshinaga and Adams, 1966) to the results of the present study make it probable that the role of oestrogen in implantation is basically the same in oestrogen sensitive as in other mice.

Implantation has occurred in a minority of cases in both rats and mice under conditions where it is difficult to envisage that it was preceded by a discretely timed, optimal oestrogen release. Generally though, the prevailing hormonal conditions for these cases was difficult to define and ova that did implant did so in a less predictable manner than when implantation was induced by a single dose of oestrogen acting on a progesterone dominated background.

During early work rats ovariectomised before the now recognized time of the oestrogen surge and given daily progesterone implanted ova at the normal time (Chambon, 1949a, b; Canivenc, Laffargue and Mayer, 1956). Mayer (1963) attributed the occurrence of the so-called cases of 'spontaneous' implantation, in part to incomplete ovariectomy and found that its incidence could be reduced by ensuring the complete removal of the ovaries and the use of chemically pure progesterone. With these results in mind, it was decided to decrease the likelihood of incomplete ovariectomy by removing the ovaries without opening the periovarian sacs in one treatment group (No. 6, experiment B) instead of shelling the ovaries from their sacs as was done for other groups. The more radical operation allowed a full implantation response, indicating that complete ovariectomy was of little consequence for implantation.

Ovarian remnants were found by inspection at ×7.50 in one of the two mice that implanted ova after ovariectomy at day 1½. Implantation in both these mice and in two of the mice with implantation sites following ovariectomy on day 2 was delayed some four to five days. Ovarian remnants were not seen in these or in a third mouse that had one implantation site, that was only two days younger than expected at autopsy, after ovariectomy on day 2 of pregnancy. The inconsistent delay period to implantation, and the small number of sites in mice ovariectomised on day 2 (one in each mouse) compared with five and seven for mice ovariectomised on day 1, together with the failure to find ovarian remnants in all mice and the small numbers of animals involved, makes it difficult to draw any conclusions. Nevertheless, the failure of the majority of mice to implant ova after ovariectomy on days 1 and 2 and the delayed implantation in those that did, again suggests that incomplete ovariectomy does not explain the results obtained after ovariectomy on day 3.

The adrenal cortex is a potential source of extra-ovarian oestrogens

(see review by Parkes and Deanesly, 1966) and after the incubation of mouse adrenal glands with progesterone, Vinson and Chester Jones (1964) were able to identify two oestrogens, viz., 16-oxooestrone and 17 - epioestriol, each of which accounted for less than one per cent of the total progesterone substrate. The role of adrenal gland is complex, adrenal hormones, presumably adrenocorticosteroids secreted in response to stressful conditions, inhibited implantation in the rat (see page 11). Mayer (1963) reported the occurrence of spontaneous implantations in ovariectomised and adrenalectomised rats and adrenalectomy failed to prevent unexplained implantation in Smith's (1966) experiments. Smith obtained implantation in 80 per cent of mice after the transfer of blastocysts cultured in vitro to recipients ovariectomised one or two weeks prior to ova transfer. Ovariectomy and adrenalectomy five to six days before the transfer of ova still allowed 73 per cent of mice to implant ova. Smith suggested that extra-adrenal tissue may have been responsible for the production of oestrogen. However, the mice used in the present work have been used for the assay of plant oestrogenic activity and vaginal cornification has not occurred in non-treated ovariectomised animals (Munford pers. comm.). Owing to the paucity of information about adrenal hormones playing a constructive role in ova-implantation no recourse for an explanation of the present results will be made.

Smithberg and Runner (1960) believed that progesterone alone was able to cause implantation in ovariectomised mice whose uteri retained some effects of 'recent oestrogen stimulation'. They took this stand to reconcile apparently contradictory results. During earlier work (Smithberg and Runner, 1956) they showed that 100 per cent of mice ovariectomised on day 1 implanted ova when given 0.5 or 1.0 mg of progesterone daily starting on day 2, and further that implantation could be started on day 18 of pregnancy by giving three to four daily injections of 1.0 mg of progesterone. Their results in

their later study are different in that daily progesterone alone, given after variable periods of delay, to mice ovariectomised on days 0, 1 and 2, was relatively ineffective in causing implantation when compared with continuous simultaneous oestrogen and progesterone treatment. They suggested (see Runner, 1963) that the oestrogenic effect persisted until about 14 to 18 days post coitum, and that during this time the ability of progesterone to initiate implantation on its own progressively diminished to zero. It is not clear as to what is meant by 'recent oestrogenic stimulation'; was residual oestrogen present or can there be an oestrogenic effect in the absence of oestrogen? During their earlier work, implantation occurred on days 5 or 6 post coitum and the possibility of residual oestrogen being present is rather high, while it is much less sure that oestrogen per se (unless from extra-ovarian sources) would still be available at 14 days post coitum. There is, evidence however, that with regard to some parameters that an oestrogenic effect is possible in the absence of oestrogen.

Prior oestrogen stimulation in ovariectomised rats and rabbits (Courrier, 1950) and in ovariectomised monkeys (Hisaw and Hisaw, 1961) allowed progesterone to be more effective in the production of a proliferative progestational type of endometrium. This was interpreted by Courrier to mean that acting successively, oestrogen and progesterone acted synergistically. Whether or not these hormones act in this manner to induce implantation in the mouse is another question. In the rat there is evidence that when acting simultaneously, oestrogen and progesterone act synergistically to initiate implantation (Meyer, 1959; Yoshinaga, 1961; Psychoyos, 1966). But there is little support for successive synergism being important in this species. Ovariectomy before the expected time of the oestrogen surge and continuous progesterone administration for five days (Nutting and Meyer, 1963, 1964; Meyer and Nutting, 1964) or six days (Psychoyos, 1961) or

up to 45 days (Cochrane and Meyer, 1957) did not result in predictable or regular implantation, which in each case was effected by supplying oestrogen as well as progesterone. In fact, these are the conditions required to delay implantations in this species (Mayer, 1963). Further, in the pregnant lactating mouse or rat why is implantation delayed if progesterone is available with low levels of oestrogen? (see page 61). Smithberg and Runner's hypothesis is not suited to explain the current results, as if all that is required of oestrogen is its recent stimulation, then why did mice ovariectomised on days 1 and 2 fail to implant? But the converse is equally true, the surge hypothesis does not explain how 100 per cent of mice ovariectomised on day 1 would implant ova. Although Smithberg and Runner used prepubertal mice that were stimulated to mate and ovulate with gonadotropin treatment it is difficult to know why, in the absence of excessive stimulation, the hormonal conditions that allow implantation should be different than in adult mice.

The ability of the blastocyst itself to be able to synthesize or carry out biotransformation of steroids has been demonstrated for rabbit blastocysts (Huff and Eik-Nes, 1966). Chambon (cited Mayer, 1960) suggested that the rabbit blastocyst produced oestrogen and that this was necessary for implantation. It has been shown that the induction of traumatic deciduoma in the ovariectomised rabbit required both progesterone and oestrogen (Kehl and Chambon, 1950; cited Deanesly, 1963) whereas it is known that in this species ova implant in ovariectomised animals given progesterone or some of its derivatives (Rennie and Davies, 1965; Pickworth, 1963).

Also there is the possibility suggested by Huff and Eik-Nes who found that LH, and to a lesser extent ACTH, when added to the culture medium speeded blastocyst differentiation; that the pituitary gonadotropins have a direct tropic effect on the blastocysts. However, as gonadotropin release

during normal pregnancy is followed by ovarian steroid secretion it is not possible to decide whether or not the gonadotropins directly affect the blastocysts and during experiments where gonadotropins have induced implantation in the hypohysectomised rat (Macdonald, Armstrong and Greep, 1967) and mouse (Bindon and Lammond, 1967) the ovaries have been present.

SUMMARY AND CONCLUSIONS

By definition implantation began when ova assumed a fixed position with respect to the uterus. Blastocysts were able to be flushed from uteri after implantation had begun. The time of implantation for the majority of blastocysts in intact mice was early on day 4 of pregnancy. There was considerable variation in the stage of development reached at a fixed time during pregnancy by ova from different mice with the same mating histories. Also, ova from any one mouse did not always develop in phase with one another. Asynchronous development of ova would imply that implantation in any one mouse would take place over a period of hours.

The size of the blastocysts increased as implantation and giant cell trophoblastic transformation proceeded.

The morphology of some of the nontransformed blastocysts recovered suggested that they may have undergone contraction in utero. It was concluded that blastocysts may rhythmically expand and contract while in the uterus as they have been observed to do in vitro (Borghese and Cassini, 1963; Cole and Paul, 1965).

A number of abnormal blastocysts were recovered from intact mice. These were smaller than normal, often irregularly shaped, showed no obvious inner cell mass and displayed abnormal cavitation. It was concluded that they resulted from abnormal cleavage due to or associated with the premature loss of the zonae pellucidae.

The critical time for ovariectomy after which progesterone alone permitted a full implantation response was day 3½ of pregnancy. Ovariectomy at day 3¼ allowed the same proportion of mice to implant ova but the number of implantation sites per mouse was reduced. The critical time was interpreted as coinciding with the stage of pregnancy when sufficient ovarian oestrogen had been released to allow implantation. Thus the ovarian hormonal

requirements for ova-implantation in the mouse, of an oestrogen release during the period of progesterone dominance, were thought to be the same as postulated for the rat (Shelenyak, 1960; Meyer, 1963). However, while oestrogen released by day 3½ may be sufficient for implantation, continued oestrogen secretion would seem to favour embryonic survival (see page 94, Section II).

Implantation was delayed in some mice after sham ovariectomy at day 1½ of pregnancy and after ovariectomy at and progesterone treatment from day 3½ during Experiment A. The reason for the delay to implantation in some mice from Experiment A but not in similarly treated mice in Experiment B is not known. It was suggested that stresses associated with daily handling of mice may have been responsible.

SECTION II

THE OVARIAN HORMONAL REQUIREMENTS FOR POST-IMPLANTATION SURVIVAL

INTRODUCTION

Ablation studies have demonstrated that the ovaries are required for maintenance of the greater part of pregnancy in mice and rats (Harris, 1927; Robson, 1936; Selye, Collip and Thomson, 1935; Zeiner, 1943; Carpent, 1962; and see Amoroso, 1955; Deanesly, 1966 for reviews).

Although progesterone has maintained pregnancy in mice ovariectomised after ova-implantation (Hall and Newton, 1947; Rubenstein and Forbes, 1963; Poulson et al., 1965), little is known about its ability to maintain normal pregnancy in mice ovariectomised prior to implantation. While Smithberg and Runner (1956) found progesterone was sufficient for foetal survival until term, they experimented with gonadotropin-stimulated prepubertal mice. These animals did not show evidence of a critical time for ovariectomy with regard to implantation and in this respect, may differ from adult mice (see Section I).

There is also reason to question the efficacy of progesterone and progesterone-oestrogen substitution treatment following ovariectomy after implantation. Gross foetal abnormalities were present in some foetuses maintained with progesterone in the mouse (Poulson et al., 1965) and with progesterone and oestrogen in the rat (Carpent, 1962).

The establishment of implantation in mice ovariectomised while ova were free in the uterus (Experiments A and B, Section I) allowed the effectiveness of graded doses of progesterone in the maintenance of pregnancy to be gauged. The treatment group in which oestrogen was given simultaneously with progesterone (group 7, Experiment B) afforded a comparison.

Emphasis was also placed on the stage during pregnancy of embryonic death and on the distribution of dead sites within the uterine horns.

MATERIALS AND METHODS

EMBRYONIC SURVIVAL

1. Pre-implantation Loss

An estimate of this loss was made by relating the number of implantation sites to the number of corpora lutea counted by inspection at autopsy for intact control mice during Experiment B. The assumption being that each corpus luteum represented an ovulated ovum.

2. Post-implantation Loss

Death was assessed at autopsy during Experiments A and B by:-

(i) The size and colour of implantation sites usually provided an indication of their viability. Small blue-black or very pale-coloured sites were invariably dead. Changes in the appearance of the uterus such as obliteration of between-site constrictions and blood coloured exudate free in the lumen were associated with dead implantation sites.

(ii) Dissection of all uteri from pregnant mice (with the exception of 15 control and sham ovariectomised mice from Experiment A and 8 histologically sectioned uteri) at autopsy.

(iii) Histological sections were made from 2 uteri with sites classified as dead and from 6 uteri about which no decision could be made, by appearance alone.

3. The Distribution of Dead Implantation Sites within the Uterus

Implantation sites in uterine horns were numbered from the cervix towards the oviduct. The number of the position of a dead implantation site was considered as the quantitative variable Y, in an analysis of covariance. The independent variable or covariate X, was the total number of foetuses

in the uterine horn. The analysis was performed on the data from the intact and ovariectomised pregnant mice from Experiment B (i.e., treatment groups 1, 2, 5, 6 and 7) after the use of the transformations $x = \sqrt{X + 1}$ and $y = \sqrt{Y + 1}$.

RESULTS

1. Pre-implantation and Total Embryonic Losses

Experiment B (Intact control mice)

At 12½ days of pregnancy, the 29 control mice had 289 corpora lutea and 268 implantation sites. The mean number of corpora lutea and implantation sites were 9.96±0.20 and 9.24±0.25, respectively.

This means that 7.2% of the eggs ovulated did not implant.

From these 29 mice a total of 247 live foetuses were recovered, which represented a post-implantation loss of 7.8% (the 76 intact mice from both Experiments A and B had a post-implantation mortality rate of 7.0%).

The total loss up to day 12½ of pregnancy was equivalent to 17.4% of the eggs ovulated.

2. Post-implantation Losses

The numbers of surviving foetuses in each treatment group for both experiments are shown in Table 7. Death signs are summarised in Table 14.

Experiment A

Analysis with 2 x 2 contingency tables (Table 15) showed that collectively, the progesterone-maintained ovariectomised mice had a considerably lower proportion of live embryos (0.08) than that found in intact groups (0.92, $P < 0.001$). Two mg of progesterone was associated with a significantly higher survival rate than that shown from the pooled results of groups given ¼ and ½ mg daily ($P < 0.001$).

Sham ovariectomy did not influence the mortality rate when compared with the intact control mice.

Experiment B

Intact control mice had a higher survival rate (0.92), than did mice ovariectomised on day 3 of pregnancy (0.62, $P < 0.001$). Of mice ovariectomised at 3½ days, those given daily oestrogen had a higher proportion of live

TABLE 14

SIGNS OF EMBRYONIC (OR FOETAL) DEATH

AT AUTOPSY

1. External signs in mice with all members of their litters dead

Experiment A

Treatments	Ovariectomised at $3\frac{1}{2}$ days				Sham ovariectomised	
	$\frac{1}{4}$	$\frac{1}{2}$	2 mgs	progesterone daily	$1\frac{1}{2}$	$3\frac{1}{2}$ days
Number resorbing*	6	8	6			
" aborting+	3	6	3		1	
" with vaginal exudate‡	1	3	1		2	1
Totals	10	17	10		3	1

Grand total 40

Experiment B

Treatment	Ovariectomised $3\frac{1}{4}$ days about 2 mg progesterone daily
Number resorbing	2
" aborting	
" with vaginal exudate	
Total	2

Grand total 2

2. Appearance of the uteri

a) The size of implantation sites:- dead sites were smaller than live sites (exceptions occurred when implantation was delayed or when foetal death occurred shortly before autopsy).

b) Colour of the sites:- blue-black or very pale sites were dead. Ecchymotic or larger haemorrhages were often present on and between implantation sites in ovariectomised progesterone treated mice uteri. However, haemorrhagic changes were not always associated with dead foetuses.

c) Between site constrictions were often absent (Pl. VIII, fig. 3(a)) especially in mice aborting foetuses.

3. Histological signs of embryonic death

a) The absence of the embryo in serial sections that included the widest diameter of the implantation site.

b) Localized areas of necrosis (pyknosis and karyorrhexis of nuclei, absence of cellular outline and often the presence of polymorphic leucocytes) frequently surrounded by viable decidual and epithelial cells.

* Mice with closed cervices

\+ " " open "

‡ Blood coloured exudate in the vagina but no other signs of abortion

TABLE 15

THE PROPORTION OF LIVE IMPLANTATION SITES IN EACH TREATMENT GROUP FOR EXPERIMENTS A AND B, SUMMARY OF CHI SQUARE TESTS

	df	X^2	P
Experiment A			
1. All mice			
Intact (0.923) vs ovarx 3½ days (0.085)	1	587.1	<0.001
2. Between groups ovariectomised at 3½ days of pregnancy			
¼ mg prog.+ (0.018) vs ½ mg prog. (0.020)	1	0.946	NS
(¼+½) mg prog. (0.020) vs 2 mg prog. (0.212)	1	37.0	<0.001
3. Between intact mice			
Sham ovarx 1½ days (0.914) vs sham ovarx 3½ days (0.921)	1	0.0199	NS
Sham ovarx (1½+3½) days (0.917) vs intact controls (0.916)	1	0.0080	NS
Experiment B			
1. All mice			
Intact (0.923) vs ovarx (0.619)	1	70.15	<0.001
2. Between ovariectomised mice that received approx. 2 mg progesterone daily			
ovarx 3½ days (0.481) vs ovarx 3½ days (0.463)	1	0.0259	NS
ovarx 3½ days (0.463) vs ovarx 3½ days + OB* (0.834)	1	35.11	<0.001

\+ progesterone

* 0.0025 ug of oestradiol benzoate daily

sites (0.83) than did mice given progesterone alone (0.46, $P < 0.001$).

3. The relative Time of Death

The size of the dead implantation sites and a summary of statistical comparisons made are shown in Table 16. The size (volume) of the dead sites was positively related to the duration of embryonic survival.

Experiment A

Ovariectomised pregnant mice had dead sites that were not different in size from that those carried by intact mice (73.83 cf. 61.68 mm^3). Ovariectomised pregnant mice given 2 mg progesterone daily had larger dead sites than the average for mice given $\frac{1}{4}$ and $\frac{1}{2}$ mg of progesterone daily (119.68 cf. 53.49 mm^3, $P < 0.001$).

Experiment B

The mean volume of dead sites was greater in ovariectomised pregnant mice than in intact controls (92.12 cf. 41.43, $P < 0.05$). Mice ovariectomised at $3\frac{1}{4}$ days had larger dead sites than those ovariectomised at day $3\frac{1}{2}$ of pregnancy (155.30 cf. 58.50 mm^3, $P < 0.001$).

4. The Distribution of dead Implantation Sites within the Uterine Horns

The adjusted mean positions in the uterine horns for the dead implantation sites are given in Table 17. Differences between the treatment means were significant. Intact control animals had the mean position of their dead sites located closer to the cervix than did ovariectomised pregnant mice ($P < 0.001$). There were significant differences between the means of the two control groups ($P < 0.01$). Comparisons between pregnant mice ovariectomised at $3\frac{1}{2}$ days of pregnancy showed that those given oestrogen as well as progesterone had dead sites further away from the cervix than those given only progesterone after ovariectomy ($P < 0.01$).

TABLE 16

THE SIZE OF THE DEAD IMPLANTATION SITES IN EXPERIMENTS A AND B

EXPERIMENT A

GROUP NOS.	TREATMENTS	MEAN VOLUME OF SITES (MM^3)
4 Ovarx	3½ days, ½ mg prog. daily	47.01
5 "	" " ½ " " "	53.38
6 "	" " 2 " " "	119.63
11 Sham Ovarx 1½"		59.23
12 " " 3½"		86.43
13 Intact controls		42.46

EXPERIMENT B

GROUP NOS.	TREATMENTS	MEAN VOLUME OF SITES (MM^3)
1	Intact controls	46.71
2	" "	32.12
5	Ovarx 3½ days, controls 2 mg prog. daily	155.39
6	" 3½ " " " " " "	58.50
7	" " " " " " " "	62.55

+ 0.0025 mg oestradiol daily

SUMMARIES OF ANALYSES OF VARIANCE

EXPERIMENT A

Source	df	Variance Ratio	P
Treatments	3	30.31	<0.001
Residual	373		

EXPERIMENT B

Source	df	Variance Ratio	P
Treatments	3	8.54	<0.001
Residual	114		

COMPARISONS						P
(i)	Grp Nos.	4+5+6 (73.83)	vs	11+12+13	(61.68)	NS
(ii)	" "	4 (47.01)	vs	5	(53.38)	NS
(iii)	" "	4+5 (53.49)	vs	6	(119.68)	<0.001
(i)	" "	5+6+7 (92.12)	vs	1+2	(41.43)	<0.05
(ii)	" "	5 (155.30)	vs	6	(58.50)	<0.001
(iii)	" "	7 (62.55)	vs	6	(58.50)	NS

TABLE 17

THE DISTRIBUTION OF DEAD IMPLANTATION SITES WITHIN THE UTERINE HORNS
(TREATMENT MEANS AND SUMMARY OF ANALYSIS OF COVARIANCE*)

Treatment group Nos.	No. dead sites per group	The mean positions in the uterine horns of the dead implantation sites (dependent variable)			The mean number of implantation sites per uterine horn (independent variable)
		Transformed‡ (y)	Adjusted (y)	Detransformed* (Y)	Detransformed (X)
1, Expt. B	8(127)+	1.8513	1.9226	3.72	4.62
2, " "	13(141)	1.3223	1.3313	1.77	5.08
5, " "	27 (52)	1.6078	1.8277	3.34	3.81
6 " "	65(121)	2.0170	1.9327	3.74	5.73
7 " "	20(121)	2.0880	2.0322	4.13	5.53
				Y	X
12&13 " A	26(318)			2.85	5.127

Summary of Analysis of Covariance

Source	df	Mean Squares	Variance Ratio	P
Residual	128	0.1567		
Difference for treatments	4	1.2244	7.814	<0.001

Reduction in error mean square = $1 - \frac{20.0547}{35.4295}$

= 43.4%

Comparisons between the adjusted y means

(i)	ovarx ($3\frac{1}{4}$‡ + $3\frac{1}{2}$) (1.9252)	vs	intact controls (1.5592)	<0.001
(ii)	" $3\frac{1}{4}$ (1.8277)	vs	ovarx $3\frac{1}{2}$, prog. (1.9327)	NS
(iii)	" $3\frac{1}{2}$, prog. + OB (2.0322)	vs	" $3\frac{1}{2}$, prog. (1.9327)	< 0.01
(iv)	intact control No. 1 (1.9226)	vs	intact control No. 2 (1.3313)	< 0.01

‡ Data transformed: $y = \sqrt{Y + 1}$ and $x = \sqrt{X + 1}$ where X and Y are the raw data observations.

* Detransformed X means are the raw data means and detransformed Y means are the raw data means ± adjustments.

\+ Bracketed numbers refer to the total number of implantation sites per treatment group.

‡ $3\frac{1}{4}$ = ovariectomy at day $3\frac{1}{4}$ of pregnancy.

5. The Influence of the Number of Implantation Sites in the Uterine Horns on Foetal Survival

The foetal survival rate for intact mice did not appear to be influenced by the number of implantation sites in the uterine horns (Table 18, Text-fig. 4). However, in ovariectomised progesterone treated mice from Experiment B (groups 5 and 6) a higher percentage of sites in the uterine horns were dead when there were from one to three sites than in horns with from three to eight sites.

6. Single surviving Foetuses

There were a relatively high number of single surviving implantation sites (in uterine horns with both live and dead sites) found in mice with large dead implantation sites (group 6, Experiment A and group 5, Experiment B, Table 19).

TABLE 18

THE SURVIVAL RATE IN THE UTERINE HORNS ACCORDING TO THE NUMBER OF SITES IN THE HORNS

	INTACT MICE									OVARIECTOMISED MICE										
NUMBER OF IMPLANTATION	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	10	11
SITES IN A HORN																				
Treatment Groups										Treatment Groups										
Experiment A										Experiment B										
Groups 12 & 13*										Group 5										
(a) Total number of sites	2	6	27	44	65	48	147	8	19	(a)	8	6	6	8	-	24				
(b) % age of sites alive	0.0	83.4	77.8	90.9	87.6	89.7	92.5	100.0	100.0	(b)	37.5	33.3	66.7	50.0	-	50.0				
(c) Number of Horns	2	3	9	11	13	8	21	1	2	(c)	9	3	2	2	-	4				
Experiment B										Group 6										
Group 1																				
(a)	4	2	24	28	20	18				(a)	2	4	6	16	25	24	7	8	18	- 11
(b)	100.0	100.0	90.8	86.7	100.0	100.0				(b)	0.0	0.0	16.7	62.5	36.8	62.5	71.4	75.0	21.2	- 54.5
(c)	4	1	8	7	4	3				(c)	2	2	2	4	5	4	1	1	2	- 1
Group 2										Group 7										
(a)	2	6	24	16	45	18	7			(a)	2	4	15	12	20	36	-	32		
(b)	75.0	80.9	90.9	90.0	94.5	85.8	87.5			(b)	100.0	50.0	93.4	91.5	85.0	77.8	-	84.2		
(c)	2	3	8	4	9	3	1			(c)	2	2	5	3	4	6	-	4		
Groups 1 & 2																				
(a)	6	8	48	44	65	36	7													
(b)	91.5	91.5	90.8	88.0	96.2	92.9	87.5													
(c)	6	4	16	11	13	6	1													

* Includes some mice from a second control group carried part of the way through Experiment A.

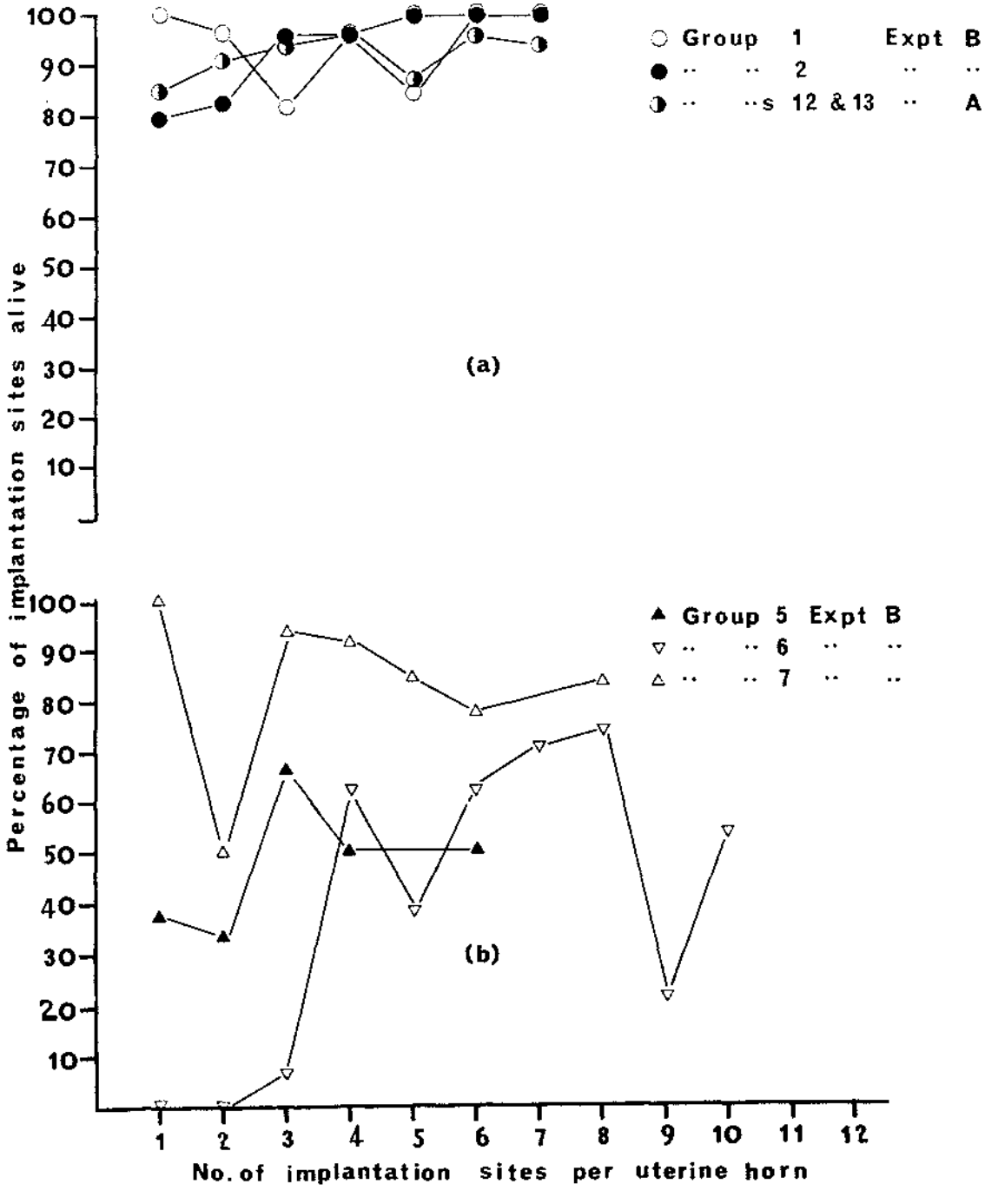
TABLE 19

VIABILITY CLASSIFICATION OF THE IMPLANTATION SITES IN THE UTERINE HORNS

Uterine horns with sites	all alive	all dead	alive and dead	with one site alive
Treatment groups				
Experiment A				
Ovarx 3½ days, ¼ mg prog.* (group 4)	0	24	2	1
Ovarx 3½ days, ½ mg prog. (group 5)	0	32	1	0
Ovarx 3½ days, 2 mg prog. (group 6)	1	23	8	4
Intact controls (group 13)	17	3	11	1
Experiment B+				
Ovarx 3¼ days (group 5)	6	7	6	5
Ovarx 3½ days (group 6)	0	7	17	4
Ovarx 3½ days, OB daily (group 7)	15	1	10	0
Intact controls (groups 1 and 2)	18	0	11	1

* ¼ mg of progesterone daily.

\+ Ovariectomised mice given about 2 mg of progesterone daily and group 7 given 0.0025 ug of oestradiol benzoate (OB) daily.



TEXT-FIG. 4. The effect of the no. of implantation sites in the uterine horns on their survival rate: (a) = intact mice and (b) = ovariectomised hormone treated mice.

DISCUSSION

Differences between the numbers of corpora lutea and implantation sites counted in the intact mice at 12½ days of pregnancy, showed that 7.2 per cent of the eggs did not implant. This compares reasonably well with the estimate of 9.8 per cent of recovered blastocysts that were classified as abnormal (page 49, Section I). However, it is likely that 7.2 per cent is a minimal estimate. Serial histological sections of ovaries removed at 3½ days of pregnancy showed some corpora lutea to be very close together, so that macroscopically two corpora lutea looked like one large one. It is possible that errors of this kind were made.

The percentage of pre-implantation losses was considerably lower than the 26 per cent found by Danforth and de Aberle (1928) for mice of various ages and strains.

Hollander and Strong (1950) found that the post-implantation mortality rate, measured from 1080 pregnancies (which included 10 outbred, 6 inbred and 10 mongrel strains of mice), was 15 per cent when there were four to ten embryos in a horn.

The total embryonic loss up to 12½ days of pregnancy, equivalent to 17.4 per cent of ova ovulated, was considerably lower than the equivalent estimate of between 25 and 35 per cent found by Danforth and de Aberle (1928). Although post-implantation deaths can occur at all stages of pregnancy, the peak mortality incidence has been reported by Hollander and Strong (1950) and Boshier (1968) to occur before day 12. Thus the present estimate of 17.4 per cent would not be expected to be much higher at term.

Strain differences (Boshier, 1968) and probably the age and reproductive histories of the mice (Krohn, 1965) affect the mortality rate. Even if allowances were made for the shortcomings of the current estimates, the efficiency of reproduction in the mice used was comparatively high.

It is apparent from Table 7 that there was only a small number of live foetuses at autopsy in the ovariectomised progesterone treated mice from Experiment A. Some deaths can be explained by the administration of insufficient progesterone to maintain pregnancy. Those that received 2 mg had a higher proportion of live foetuses (27 per cent) than those given $\frac{1}{4}$ and $\frac{1}{2}$ mg of progesterone daily (2.1 and 2.8 per cent). These figures are low relative to the survival rates of mice ovariectomised at $3\frac{1}{4}$ and $3\frac{1}{2}$ days of pregnancy and given about 2 mg of progesterone daily during Experiment B, of 47 and 46 per cent, respectively.

The difference between the results of the two experiments may be related to the different methods of administering progesterone. It does not appear to be the direct result of the comparatively less suitable environmental conditions encountered during Experiment A, as intact control mice in both experiments had post-implantation survival rates of over 90 per cent.

During Experiment A injections of progesterone in an oil solution were given daily, while in Experiment B subcutaneous progesterone implant pellets were inserted at the time of ovariectomy. As well as any deleterious affect that may have accrued from daily handling of the mice the daily injections of progesterone may not have provided an even supply of this hormone. Forbes (1959) and Cohen (1959) estimated, by the use of a bioassay technique for total progestagens, the levels in the oil vehicle and in the plasma after the injection of progesterone intraperitoneally and subcutaneously, respectively, in ovariectomised mice. In both cases there was rapid loss of progestagen from the oil vehicle after injection and 'free' plasma progestagen levels varied inconsistently with the dose rate after the intraperitoneal injection, and decreased rapidly after five minutes post injection. 'Bound' levels were often not detected. The free plasma levels after subcutaneous administration decreased quickly and were low 10 minutes post injection.

It is probable that a more steady rate of release of progesterone followed its administration in pellet form and this may have been an important contributing factor for the higher survival rates obtained in Experiment B.

More mice with all members of their litter dead were found in Experiment A than in Experiment B (Table 14). Further, only during the former experiment were mice seen to be in the process of aborting dead embryos. The signs of death and the methods used to establish the viability of implantation sites are reviewed in Table 14. It is probable that mice in Experiment A with a sanguinous exudate in the vagina were in the early stages of abortion. Blood present in the vagina on days 12 or 13 of pregnancy as a 'sign of pregnancy' (Eckstein and Zuckerman, 1956) is a normally expected occurrence. However, in the mice under consideration, it was present in large amounts and all members of the litters were dead, so it is not thought to have been confused with the normal sign of pregnancy. Harris (1927) reported that ovariectomy of pregnant mice between days 3 and 15 of pregnancy lead to the resorption of embryos or foetuses, while this operation after day 15 was associated with abortion. Twelve progesterone treated ovariectomised mice in Experiment A aborted dead embryos, some of which estimated by the size of the dead sites, died on days 9 or 10 of pregnancy.

Despite the lower embryonic survival rate in ovariectomised pregnant than in intact mice, there is little reason to suspect that 2 mg of progesterone daily, was too small a dose. Smithberg and Runner (1956) ovariectomised gonadotropin-stimulated mice on day 1 and found that 0.5 mg and 2.0 mg of progesterone daily maintained pregnancy to term in 35 and 88 per cent of mice respectively. Similarly Poulson et al. (1965) found that when mice were ovariectomised on day 6, the administration of 0.25, 0.5-0.75, 1.0 and 2.0 mg of progesterone daily from days 5 to 9 and 2 mg from days 10 to 18, that 0, 26.7, 55.2 and 69.6 per cent of the mice respectively, carried live

foetuses to day 18 of pregnancy. Also Rubenstein and Forbes (1963) and Hall and Newton (1947) reported that after ovariectomy on days 10, 11, 14, 16 and 15, respectively, pregnancy was maintained in a high proportion of mice with less than or 1 mg of progesterone daily.

It is also unlikely that the daily dose rate of 2 mg was too high. Petrelli and Forbes (1964) found that 3.5 mg of progesterone contained in 0.5 ml of sesame oil when injected intra-amniotically on day 14 or subcutaneously on days 14, 15 and 16 of pregnancy in intact mice of the Brown Belt strain was toxic and caused the death of over 60 per cent of the two most anterior situated foetuses in the uterine horns. Moreover, this strain of mice were more economical in their utilization of progesterone than CHI mice in the maintenance of pregnancy after ovariectomy (Rubenstein and Forbes, 1963).

Seemingly the quantitative threshold of progesterone required for implantation, in mice ovariectomised at $3\frac{1}{2}$ days of pregnancy, was lower than that required for the maintenance of embryonic survival. This conclusion agrees with the results of Smithberg and Runner (1956). During Experiment A, implantation was considered to have been completed (page 58, Section I) in mice given $\frac{1}{4}$ and $\frac{1}{2}$ mg of progesterone daily. However, the present results and reports in the literature indicate that these amounts were too low for continued embryonic survival.

The inclusion of oestrogen in the supportive treatment that followed ovariectomy at $3\frac{1}{2}$ days in Experiment B was associated with a higher survival rate than with progesterone alone (83 cf. 50 per cent). Indeed Humphrey (1967), reported that the development of viable foetuses after ova transfer to ovariectomised mice required both progesterone and oestrogen treatments. Mice resorbed embryos after day 6 of pregnancy when given progesterone alone. The ovaries and/or the placentae probably produce small amounts of oestrogen throughout pregnancy. Huff and Eik-Nes (1967) detected plasma oestrogen

in the pregnant rat but were unable, by the use of a sensitive chemical technique, to measure levels accurately. And although Harkness, McLaren and Roy (1964) were unable, by the use of a chemical method, to detect oestrogen with certainty in mouse placentae, there is biological evidence that placentae do produce some oestrogen during pregnancy (Amoroso, 1955; Deanesly, 1966). Whatever its source, oestrogen acting with relaxin is required for pelvic dilation in the pregnant mouse (Hall and Newton, 1947). Thus oestrogen and progesterone would have been expected to simulate more closely to the physiological hormonal situation during gestation than provided by progesterone alone.

With the exception of infrequent cases of delayed implantation, the size of the dead implantation site was inversely related to the duration of survival of the embryo. From the size of the dead sites in Experiment A, the majority of deaths in both ovariectomised progesterone-treated and intact mice occurred at about day 7 of pregnancy. However, of the ovariectomised pregnant mice, those that received 2 mg maintained embryos in a viable state for a longer period of time than did mice given $\frac{1}{4}$ and $\frac{1}{2}$ mg of progesterone daily. Although ovariectomised hormone-treated mice had larger dead sites than those found in intact mice in Experiment B, this difference was probably due to the incidence of very large dead sites in animals ovariectomised at $3\frac{3}{4}$ days of pregnancy. Dead sites in this group (5) were considerably larger than in mice ovariectomised at $3\frac{1}{2}$ days and a number of foetuses that showed external characteristics consistent with the twelfth day of development and contained in cloudy amniotic fluid were found.

The majority of deaths in both intact and ovariectomised pregnant mice occurred at about the same time as found by Hollander and Strong (1950) who reported that about 72 per cent of post-implantational deaths in intact mice occurred between days 6 and 8. Days 6 to 9 of pregnancy coincide with

the period of embryogenesis during which Poulson et al. (1965) found that the quantitative requirements of progesterone were especially critical for continued embryonic survival. Also Nelson and Evans (1954) reported that 90-100 per cent of the embryonic deaths in pregnant rats fed a protein deficient diet occurred at 8 or 9 days of gestation. This effect was probably the result of a lowered output of gonadotropins (Ershoff, 1952; Kinzey and Srebnik, 1963) as either gonadotropins or progesterone and oestrogen administration (Nelson and Evans, 1954; Berg, Sigg and Greengard, 1967) allowed maintenance of pregnancy in protein deficient rats. Thus it appears that the period of embryogenesis is a critical time for the developing embryo during which it is susceptible to endocrine deficiencies or imbalances, as imposed in the present work.

Not all deaths were confined to this early period of embryogenesis. The size of the dead sites, in mice ovariectomised at $3\frac{1}{2}$ days (group 6, Experiment A and at $3\frac{1}{4}$ days (group 5, Experiment B) and given about 2 mg of progesterone daily, suggested that deaths occurred later on in pregnancy (Table 16). It is possible that the low and/or irregular plasma levels of progesterone, that were considered to have been instrumental in producing the higher mortality rate in Experiment A than in Experiment B, became inadequate for embryonic survival at different times during pregnancy in different mice. Twenty two of the 100 dead sites were over 200 mm^3 in volume and 8 of these were over 300 mm^3. Between days 11 and 13 of pregnancy in the rat Fajer and Barraclough (1967) found that there was a sharp increase in the output of ovarian progestagens and should there be a comparable increase in the mouse, and if this did represent increased requirements for progesterone at this stage of pregnancy, then its absence in ovariectomised mice may explain some of the late deaths that occurred.

A hazard that growing foetuses had to contend with in ovariectomised

progesterone treated mice was intrauterine space confinement, that occasionally resulted in pressure induced external deformities (Pl. IX, figs. 1 and 2). Carpent (1962), who described a similar condition produced by ovariectomy in the rat, considered external deformities to result from pressure exerted on the foetuses by a hypertonic uterus. Carpent found that the administration of oestrogen and progesterone to rats ovariectomised on day 3 of pregnancy did not prevent this condition.

Evaluation of the increased intrauterine pressure as a cause for embryonic death is not straight forward. This is in part, because the endocrine conditions causing the uterine hypertonicity may have had an independent adverse affect on the survival of the embryos. The failure of the uterus to grow and distend undoubtedly was responsible for the death of the few foetuses that died after expulsion through a rupture in the uterine wall into the peritoneal cavity (page 123, Section III). While Selye et al. (1935) believed that foetal death in the ovariectomised rat was due to increased pressure caused by the partial involution of the uterus, this explanation is not appropriate for the current results. The increased intrauterine pressure was interpreted to have resulted from a reluctance of the uterus to distend rather than from positive involution. It is perhaps misleading to implicate increased pressure per se as being responsible for the early embryonic deaths that occurred in ovariectomised mice. It is not certain that the uteri were hypertonic at this time and most deaths occur at this stage of pregnancy in intact mice for unknown reasons. Only in those groups where the dead implantation sites were larger than those of their respective intact control animals (i.e., group 6, Experiment A and group 5, Experiment B) will increased pressure be considered as a possible cause of death.

It appears reasonable to assume that the pressure on each foetus would

be positively related to the number of foetuses in a uterine horn. There is some evidence to suggest that this was so. Uterine horns or segments of horns that contained foetuses orientated so that their heads pointed at right angles to the direction of the horns, were always crowded (Pl. VIII, fig. 1, page 122, Section III). However, there was no consistent evidence to show that the mortality rate was positively related to the number of implantation sites in the uterine horns (Text-fig.4, Table 18). For example, of the ovariectomised mice in Experiment B, those in group 6 where the number of implantation sites per mouse was higher than in group 5, actually showed an increase in the proportion of embryo surviving as the number of sites increased from five to eight per uterine horn. Although the number of horns was small there is little reason to believe that in other groups (viz., 5 and 7) that the survival rate varies inversely with the number of sites. Moreover, there is justification for believing that the converse applied, that is, the proportion of embryos surviving was lowest when there were few sites in the horns of ovariectomised mice given progesterone alone.

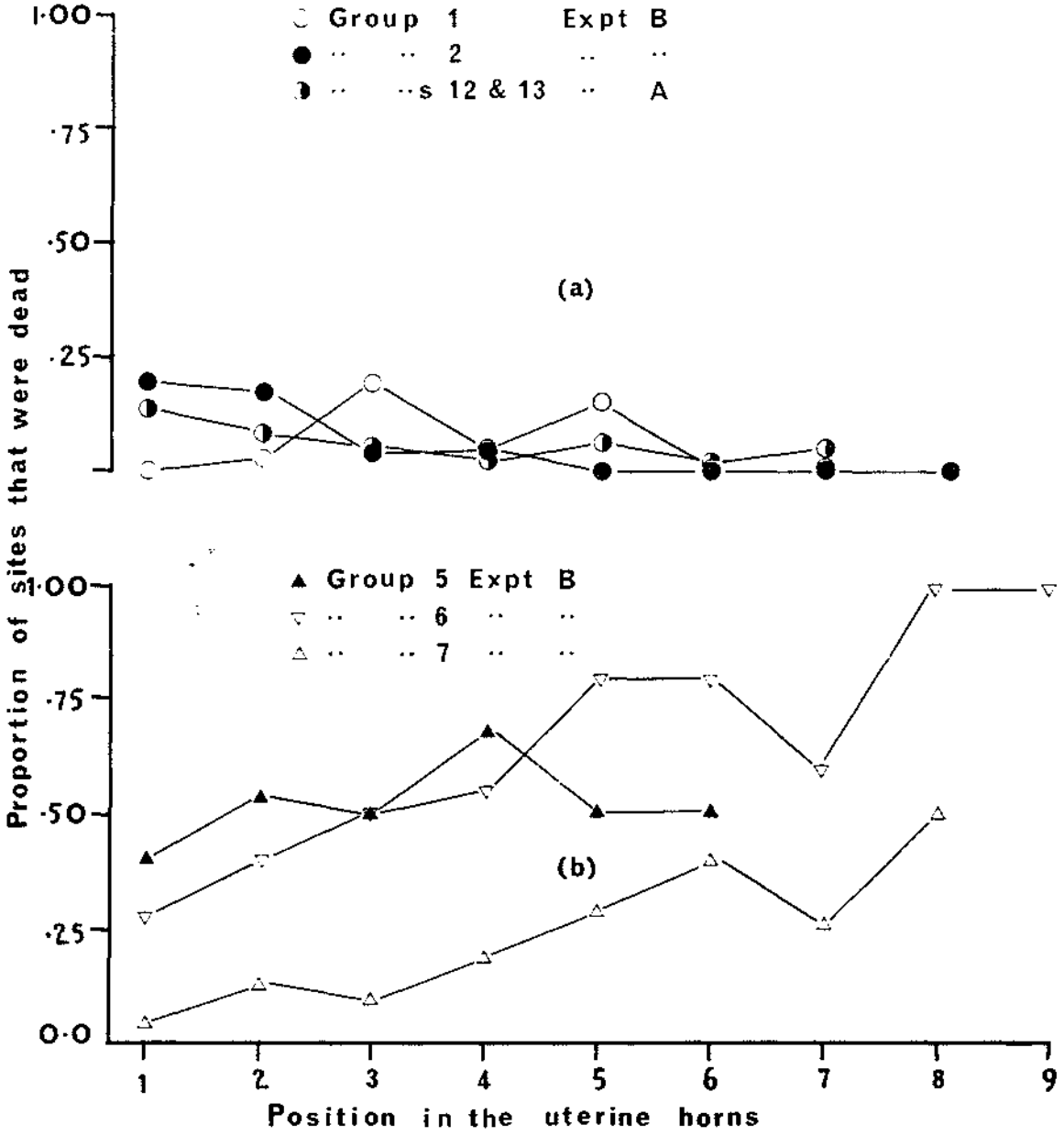
A further objection to the view that pressure alone killed the embryos or foetuses is that there was no constant time of death. It would have been expected that intrauterine confinement would have become more serious as the rate of foetal growth increased. However, 27 and 47 per cent of the foetuses from groups 6 (Experiment A) and 5 (Experiment B), respectively, were alive at autopsy and of those that died, there was a lack of uniformity in the size of the sites. Only a few died on days 11 or 12 and the others died at times before this stage of pregnancy. Thus, the susceptibility of the embryos and foetuses to pressure would have had to have been variable.

There was a surprisingly high number of single surviving implantation sites in horns with both live and dead sites in the groups with the relatively

large dead sites (Table 19). Of the single surviving sites, three out of four in group 6, and four out of five in group 5 occupied position 1 (that nearest the cervix) in the uterine horns. With regard to the proportion of sites dead in each position, number one has lowest death rate of all positions in ovariectomised mice (see Text-fig.5). One explanation is that the uterus is progressively less suitable for foetal survival as the foetuses are positoned further away from the cervix. It is also possible that as lone surviving sites were often present with large dead sites, that they derived some benefit from the presence of the latter. There is a chance that some of these dead sites contained live placentae. Selye et al. (1935) found that although ovariectomy at mid-pregnancy in rats killed the foetuses, the placentae were still alive five days after the operation. However, for the survival of the lone foetuses to depend on secretions from other placentae it would mean that their own placentae were deficient.

Carpent (1962) observed that the placentae of foetuses, from rats ovariectomised on day 3 and given oestrogen and progesterone treatments from days 9 to 25, were often hypertrophied. These placentae showed trophoblastic proliferation with blood stasis in the placental labyrinth. Although placental morphology was not studied in the present work, the similarity between the two experiments with regard to the presence of uterine hypertonicity, deformed foetuses and closer spacing (see Section III) suggests that the placentae in the current experiment may not have been normal. And if the placentae were endocrinologically incompetent, it is conceivable that the combined secretions of several may have been required for the survival of one foetus and the most likely foetus to survive would be the one in the most favourable region of the uterus.

The highest rate of foetal survival was obtained in ovariectomised mice given progesterone and oestrogen. However, as it was about 9 per cent below



TEXT-FIG 5. The distribution of dead implantation sites within the uterine horns of intact (a) and ovariectomised hormone treated (b) mice.

that recorded for intact mice, it is likely that some degree of endocrine imbalance remained. The embryonic survival rate in mice after ovariectomy at 3½ days of pregnancy in Experiment B, was considerably higher in group 7 that received both oestrogen and progesterone treatments than in mice given progesterone alone (group 6) (Tables7 and 15). This suggests that oestrogen was definitely beneficial for survival and as the average time of death in both groups was about day 7, oestrogen was effective in reducing deaths during this period of embryogenesis. In Section I (page 67) it was proposed that an oestrogen release, required for ova-implantation, was completed on day 3½ of pregnancy. However, in view of the effectiveness of oestrogen in reducing early post-implantational deaths oestrogen may be continually released after day 3½.

Although various progesterone and oestrogen combinations were not explored in the present study there is inconclusive evidence that these hormones maintain normal pregnancy in ovariectomised rats. Zeiner (1943) was unable to prolong the survival time of foetuses in ovariectomised rats with various combinations of oestrogen and progesterone. However, Lerner et al. (1962) claimed that progesterone and some of its derivatives were able to maintain pregnancy in up to 100 per cent of ovariectomised rats. Oestrogen synergized with progesterone so as to lower its minimum effective dose. Although Carpent (1962) found that a high percentage of embryos survived after the administration of oestrogen and progesterone to ovariectomised rats, a higher than normal percentage of these showed visceral abnormalities and regardless of the combination of ovarian hormones administered, a minority of foetuses showed external deformities that resulted from uterine hypertonicity. The latter defect appears to be due to ovariectomy and incomplete or imbalanced supportive treatment as hypophysectomised rats with grafted pituitary glands and/or progesterone and oestrogen therapy

do not have hypertonic uteri (Carpent, 1963; Carpent and Desclin, 1967). Surviving foetuses in these animals, however, still showed visceral abnormalities which were thought to result from an endocrine imbalance operating during embryogenesis.

The limited success of progesterone and oestrogen treatment in the maintenance of pregnancy and in the establishment of uterine changes adequate for normal foetal development in the ovariectomised mice and in ovariectomised rats (Carpent, 1962) may mean that the ovary secretes other essential hormones during pregnancy.

It is possible that relaxin is important for the growth and distension of the uterus during pregnancy. This hormone is known to inhibit uterine motility in oestrogen-primed mice and rats and is concerned with the relaxation of the pubic symphysis and cervices in these and other species (reviewed by Zarrow, 1961). McClintock and Zarrow (1967) have developed an antibody effective in blocking the activity of exogenous and endogenous relaxin in pregnant mice. Localization of this antibody occurred in the ovaries, placentae and, in relatively smaller amounts, in the uterus of pregnant mice (Zarrow and McClintock, 1967). Although there is some doubt about the site(s) of synthesis of relaxin, it is fairly certain that the ovaries are essential for its production. While pubic relaxation fails to occur in ovariectomised progesterone-oestrogen treated mice, it can be brought about by the simultaneous administration of relaxin (Hall and Newton, 1947).

Evidence for a role of relaxin in uterine growth and distension during gestation, then is indirect. It is, in part, based on the similarity of the proposed action to the known effects of relaxin in producing pelvic and cervical changes throughout the latter part of pregnancy. Further, the localization of relaxin in the pregnant uterus may be related to its known

function of inhibiting uterine motility. It will be recalled that uterine hypertonicity only occurred in ovariectomised and not in hypophysectomised hormone-treated rats (Carpent, 1962, 1963; Carpent and Desclin, 1967).

The mortality rate in uterine horns from intact mice was calculated according to the number of sites in the uterine horns (Table 18, Text-fig.4). There was no reason to believe that for intact mice from Experiment B, that the mortality rate was dependent on the number of sites present in the horns. The survival rate of embryos from ovariectomised mice in Experiment B was more variable. Uterine horns with few sites had a higher proportion of deaths than in horns with more sites. The effect of this would be to move the mean position for deaths in the horns of the ovariectomised mice a little closer to the cervix.

Hollander and Strong (1950) found that the mortality rate may depend on the number of sites in a horn; 15 per cent of the embryos died when there were between four and 10 per uterine horn and the mortality rate rose to 17 and 20 per cent when there were three and two sites per horn, respectively. They also recorded a sharp increase in the death rate over 15 per cent when there were more than 10 embryos in a horn.

The distribution of the dead implantation sites in the uterine horns for the different treatment groups in Experiment B was examined to see if local uterine influences adversely affected embryonic survival. The regions of the uterine horns were identified and equated with the positions of the implantation sites, when these were numbered from the cervix towards the oviduct. The number of a dead site was then treated as the dependent variable Y, in the analysis of covariance. A difficulty encountered when equating regions of the horns with site numbers, is that the position in the uterus occupied by a particular embryo is probably dependent on the number of embryos in the uterine horn (Tables 27 and 28, Section III). Some

measure of control over this variation was gained by including the total number of sites in a horn as the independent variable X, in the analysis.

Comparisons between the mean positions occupied by the dead sites showed that on average dead embryos were found closer to the oviduct in ovariectomised hormone-treated mice than in intact mice (Table 17). Of the mice ovariectomised at 3½ days, those given oestrogen as well as progesterone had the mean location of their dead sites closer to the oviduct than in mice given progesterone alone. An interesting result was the different mean distribution for the dead sites between the two intact control groups. The numbers of dead sites in these groups were small and for comparison the mean distribution (derived from the raw data) of dead sites in intact control and mice sham ovariectomised at 3½ days of pregnancy from Experiment A was calculated. The result of 2.85 was not very different from the mean position of all intact mice from Experiment B of 2.42 and suggests that the comparison between intact and ovariectomised mice from Experiment B may have been meaningful. Further, reference to Text-fig.4, shows that for intact mice from both experiments, there were two peak incidences of dead sites corresponding to two different regions of the uterine horns. Deaths were more likely to occur from positions one to three and at position five than elsewhere. The different mean distribution of dead sites found in two groups of intact mice from Experiment B is explained from the fact that whereas in group 2 (mean 1.77), the peak incidence of deaths occurred at positions 1 and 2, there were two peaks, one each at site positions 3 and 5, for group 1 (3.72).

There was a higher mortality rate in all positions for mice ovariectomised and given progesterone than in the control animals (Text-fig.4). The mortality rate for the first three positions in ovariectomised mice given oestrogen and progesterone treatments were of the same order as that found

in intact mice. Thus while the administration of oestrogen with the progesterone-substitution therapy was associated with a reduction in the death rate in all positions, it was relatively more effective for positions closer to the cervix. Hence the mean position for dead sites of 4.13 in this group is located closer to the cervix than in any other group. However, the distribution of dead sites in the three ovariectomised groups followed a similar pattern. The peak incidence of deaths occurred nearer the oviduct than the cervix in all groups. The increase in the proportion of sites dead for each position as they approached the utero-tubal junction was relatively steady in groups 6 and 7. However, while there is only a suggestion of a plateau in these groups, there was a definite plateau at position 2 in group 5 and the peak positional incidence of dead sites occurred closer to the cervix than in groups 6 and 7. There is reason to believe that the region of the uterus associated with the peak positional incidence of death was similar for all three groups. There was an inverse relationship between the number of sites in a horn and the distance at which they were spaced apart (Tables 27 and 28, Section III). And because there were less implantation sites per mouse in group 5 than in the other groups, position 4 is relatively closer to the oviduct in this group. The high proportion of deaths at position 8 in groups 6 and 7 is of doubtful significance because the number of sites involved was small.

To summarize, some areas of the uterus were less favourable for embryonic survival than others. Thus in intact mice, the central regions of the uterine horns were the most favourable for survival. While in ovariectomised hormone-treated mice, the position closest to the cervix was the safest. Probably the most significant result is that positions 3, 4, 5 and 6 in these groups were the least favourable for survival.

The oviducal region of these uteri may have been less suitable for

embryonic survival because of the disruption of its blood supply at ovariectomy. During this operation the uterine-ovarian vessels were severed thereby necessitating that the oviducal region of the horn receive its blood supply from the uterine vessels running from the direction of the cervix.

Dead implantation sites were not distributed in the present work in the same way as in the superovulated mice used by McLaren and Michie (1959). These workers found that deaths were more frequent in the central than in the oviducal or cervical regions. However, the uterine horns in superovulated mice would be more crowded with foetuses than they were in the experiments described. For this reason, the results are probably not strictly comparable.

Foetuses that did survive to day $12\frac{1}{2}$ of pregnancy in the oviducal regions of the uterine horns in ovariectomised mice, were neither lighter nor younger than those in other positions of the horns (Tables 29 and 32, Section III). This is interpreted as evidence that adverse local uterine conditions do not retard embryonic development after implantation.

SUMMARY AND CONCLUSIONS

It is concluded that progesterone was only partially successful in maintaining pregnancy to day 12½ of pregnancy in mice ovariectomised prior to implantation. Embryonic death in these mice was attributed to insufficient progesterone, an irregular supply of progesterone, to the absence of exogenous oestrogen in the presence of adequate progesterone and a minority due to increased intrauterine pressure *per se*, a consequence of uterine hypertonicity accompanying progesterone treatment. As only one dose combination of oestrogen and progesterone was used, an imbalance of these hormones may have been responsible for a small percentage of embryonic deaths. Uterine hypertonicity was also present to a lesser extent, when both oestrogen and progesterone were given simultaneously. It is suggested that relaxin may have been required for uterine growth and distension during pregnancy.

The most critical period for embryonic survival was during the stage of embryogenesis, and the majority of deaths in intact and ovariectomised mice occurred at about day 7 of pregnancy. In two ovariectomised progesterone treated groups the average time of death was later on in pregnancy, and some foetuses died on days 11 and 12. Single surviving foetuses in uterine horns, along with large dead implantation sites, may have owed their survival to secretions from living placentae whose own foetuses were dead.

The distribution of dead implantation sites in the uterine horns of intact mice was variable. Adverse local uterine effects were present in ovariectomised hormone treated mice with the oviducal region of the horns being less favourable for embryonic survival.

SECTION III

THE OVARIAN HORMONAL REQUIREMENTS FOR EMBRYONIC SPACING WITHIN THE UTERUS

INTRODUCTION

Blastocyst spacing in the mouse, as in other polytocous species, is preceded by a number of little understood, though well ordered events. Ova are distributed throughout the lumen of the uterine horns and orientated in a definite way prior to implantation. Implantation occurs in a crypt located on the antimesometrial side of the uterine lumen in such a way that the blastocysts are orientated with their abembryonic poles directed antimesometrially (Mossman, 1937; Bloch, 1939, 1966; Boyd and Hamilton, 1952; Amoroso, 1952; Snell, 1956).

Ova travel through the Fallopian tubes in a discontinuous way, showing back and forth movement (Harper, 1966; Bennett and Kendle, 1967) and in the mouse are said to enter the uterus in a 'clump' (McLaren and Michie, 1959) at about 72 hours post coitum (Lewis and Wright, 1935). On average about equal numbers of ova enter either uterine horn (Danforth and de Aberle, 1928; Hollander and Strong, 1950; Hashima, 1956; McLaren and Michie, 1956) and in the rabbit they are dispersed throughout each horn by myometrial contractions (Markee, 1944; Böving, 1956; Reynolds, 1965). The contractility of the uterus varies throughout the oestrous cycle (Keye, 1923; Markee, 1944; Reynolds, 1965) in such a way that the amplitude of the contractions is greatest and the frequency least, when oestrogen is the dominant ovarian hormone and vice versa under conditions of progesterone dominance.

According to Snell (1956) and Wilson (1963) ova in the mouse are spread throughout the horn shortly after their entry into the uterus. Snell believed that they were more or less evenly spaced while Wilson reported that they were randomly spread along the uterine horns and between 84 and 96

hours post coitum, settled in a uterine crypt lost their zonae pellucidae and became orientated. Mossman (1937) postulated that in polytocous species ova implanted serially, that is, the first embryo attached nearest to the oviduct, the next a little further down and so on. This was offered as a theoretical explanation for the observation that there was a progressive decrease in size of the early embryos, 'in an ordinary bicornuate uterus as in the pig', as they were positioned further away from the oviduct. McLaren and Michie (1959) weighed foetuses contained in each sixth of the uterine horns of mice at 18½ days post coitum and finding no ordered progression for foetal weights as they were positioned away from the oviduct, rejected Mossman's concept. However, the question cannot be regarded as settled as much could happen between the time of implantation and 18½ days post coitum. For instance, though it seems reasonable to assume that the weight of embryos during early pregnancy would be constantly related to their age and therefore to the time of implantation, it is another matter as to whether or not this relationship continues during the accelerated growth phase of the foetus that occurs during the second half of gestation.

In species that implant one or two ova, such as the ewe, cow, Elephantulus, man and monkey, there appear to be predetermined areas in the uterus where implantation usually occurs (Mossman, 1937; Mayer, 1960). In polytocous species it is thought unlikely that the equivalent preformed regions exist (Mayer, 1960; McLaren and Michie, 1959) as Bloch (1939) proposed for the mouse. However, in the parous mouse Orsini (1963) has observed that implantation does not occur on placental scars left from the previous pregnancy.

There has been disagreement as to the regularity of embryonic spacing in the uterine horns. Earlier workers believed that the ova and implantation sites were evenly spaced along the horns (Mossman, 1937; and for references pertaining to the mouse see Bloch, 1939). More recently, others have

questioned this (Hollander and Strong, 1950; McLaren and Michie, 1959; Bloch, 1966), and the only species in which the spacing of blastocysts has been shown to be more even than random is the rabbit (Böving, 1956; reviewed by Blandau, 1961).

Two main problems were investigated, the first was that of finding the time during normal pregnancy at which blastocysts became spaced prior to implantation and secondly, the spacing of implantation sites in ovariectomised progesterone treated mice was compared with that in intact mice at 12½ days of pregnancy.

During Experiment C (Section I (a)) oviducal and cervical uterine horn halves were flushed separately to determine if there was a stage of pregnancy when equal numbers of ova could be recovered from each of the horn halves. This was done to see if ova were spaced shortly after their entry into the uterus as claimed by Snell (1956) and Wilson (1963). Also by observing the developmental status of the blastocysts at different times during pregnancy, it would be discernable if implantation occurred first in the oviducal horn half as would be expected if Mossman's (1937) concept of serial implantation was correct. As a further check on the positional order of implantation in the uterine horns the age and weight of foetuses was measured at autopsy. The assumption made was that the older foetuses in each litter would have developed from blastocysts that implanted earliest.

In preliminary experiments during which ova implanted in ovariectomised mice given progesterone, the implantation sites appeared to be more closely spaced together than in intact mice. So it was of interest to measure the spacing distances between implantation sites at 12½ days of pregnancy in intact and ovariectomised hormone treated mice.

MATERIALS AND METHODS

1. The Spacing of Ova prior to Implantation

During Experiment C, which was designed to determine the time of ova-implantation in intact mice, 54 uterine horns (18 at each of the times; 94, 102 and 110 hours of pregnancy) were cut into cervical and oviducal halves that were flushed separately with physiological saline.

The ova recovered from each half of the horn were counted and examined as described (Section I (a)).

2. The Spacing of Implantation Sites

The uteri, of mice killed at an estimated day $12\frac{1}{2}$ of pregnancy, were measured to find;

(i) the distance from cervix to uterotubal junction after each uterine horn was stretched taut and pinned on cardboard

(ii) the distance between adjacent implantation sites

(iii) the mesometrial-antimesometrial diameter for each implantation site

(iv) the length (parallel to the long axis of the horn) of each site.

Each site was assumed to have a simple spheroidal shape and its volume was calculated from the formula:

$$v = \frac{\pi l_1 l_2^2}{6}$$

v = the volume in mm^3

l_1 = the length of the site

l_2 = the diameter of the site.

The assumption of spheroidal shape means that the volumes obtained

were approximate.

3. Observations made on Foetuses at Autopsy

Foetuses were dissected from uteri, that were preserved in 8% buffered formalin. There were 32 uteri from Experiment A and 19 from Experiment B.

(a) Developmental ages

Ages were estimated to the nearest half day from the stage of differentiation of some external features, in particular the shape of the footplates (Gruneburg, 1943).

(b) Foetal weights

Weights were measured to the nearest mg on a torsion balance after excess amniotic fluid had been removed with blotting paper.

(c) The orientation of the foetuses

Note was made of the direction in which the foetal heads pointed.

RESULTS

EMBRYONIC SPACING

1. The Spacing of Ova prior to Implantation

The total numbers, and the numbers of ova recovered at each stage of development, from the oviducal and cervical segments of the uterine horns are shown in Table 20.

More ova were recovered from the oviducal than the cervical segments (Table 21, $P < 0.01$).

With time as the independent variable, there was a significant negative regression for the numbers of ova recovered from the oviducal segments and the curves for ova recovered from the different uterine segments showed significant nonlinearity (Table 21, Text-fig.6).

Of the total number of blastocysts showing no evidence of giant cell trophoblastic transformation, more (59) were recovered from the oviducal than the cervical halves (25) ($P < 0.01$). There was no significant difference between the total numbers of blastocysts partially or completely transformed recovered from either half (Table 20).

2. The Spacing of Implantation Sites

Numerical and statistical data are presented in Tables 7 and 22, respectively.

(a) The mean distances between implantation sites (intervals)

Ovariectomised pregnant mice had implantation sites spaced closer together than did intact pregnant mice in Experiments A ($P < 0.001$) and B ($P < 0.001$).

Comparisons between ovariectomised pregnant mice from Experiment A showed that those that were given $\frac{1}{4}$ had smaller intervals than those given $\frac{1}{2}$ mg of progesterone daily ($P < 0.01$).

TABLE 20

THE NUMBER AND DEVELOPMENTAL STATUS OF THE BLASTOCYSTS

RECOVERED FROM THE OVIDUCAL AND CERVICAL UTERINE HORN HALVES

Hours of pregnancy	94		102		110		
Uterine horn half*	ov	c	ov	c	ov	c	
(a) Stage of Development							Totals
Within zonae pellucidae (ZP)	21	16	6	5	0	0	48
Nontransformed (NT)	20	4	12	0	0	0	36
$< \frac{1}{2}$ T	6	3	10	5	6	6	36
< T	5	3	4	8	9	9	38
T	0	0	10	6	19	14	49
Totals	52	26	42	24	34	29	207

(b) Totals in each uterine horn half

	ov	c
(i) Total number of early blastocysts (ZP + NT)	59	25
(ii) Total number of partially or completely transformed blastocysts ($< \frac{1}{2}$T + < T + T)	69	54

Summary of Chi Square analyses

Source	df	X^2	P
(i)	1	12.80	< 0.001
(ii)	1	2.04	~ 0.20 (NS)

* ov = oviducal half

c = cervical half

TABLE 21

OVA SPACING PRIOR TO IMPLANTATION

A. The numbers of ova recovered from cervical and oviducal uterine segments at 94, 102 and 110 hours of pregnancy: Summary of analysis of variance (as 2 x 3 treatment assay).

Source of variation	df	Variance Ratio	P
Segments	1	19.45	<0.01
Common linearity	1	2.73	NS
Deviations from linearity	1	5.37	<0.05
Common quadratic	1	0.33	NS
Deviation from quadratic	1	0.10	NS
Total	53		

B. Summary of regression analyses for the number of ova recovered from each segment with time.

(a) Oviducal Segments

Linear regression	1	6.275	<0.05
Error	25		
Total	26		

$b_{y.x} = -0.1248 \pm 0.0483$

(b) Cervical Segments

Linear regression	1	1.74	NS
Error	25		
Total	26		

$b_{y.x} = 0.0208 \pm 0.0967$

TABLE 22

THE SPACING OF IMPLANTATIONS SITES, SUMMARIES OF STATISTICAL TESTS

The mean linear intervals between implantation sites (mm)

Summaries of analysis of variance				Comparisons for Experiment A					Comparisons for Experiment B					Summaries of analysis of covariance		
Source	df	Variance ratio	P					P					P	Experiment B		
Experiment A				(i) ovarx $3\frac{1}{2}$ (6.90) vs intact mice (12.99)				<0.001	(i) ovarx ($3\frac{1}{2}$+$3\frac{1}{2}$),(9.66) vs intact controls (12.07)				<0.001	Source	df	Means square
Treatments	5	21.170	<0.001	(ii) " " $\frac{1}{2}$ mg(5.92) vs ovarx $3\frac{1}{2}$, $\frac{1}{2}$ mg(7.14)				<0.01	(ii) ovarx $3\frac{1}{2}$, (11.44) vs ovarx $3\frac{1}{2}$ prog. (8.23)				<0.05	(means spacing intervals = Y		
Residual	79			(iii) " ($\frac{1}{2}$+$\frac{1}{2}$mg) (6.53) vs " " 2 mg(7.64)				NS	(iii) ovarx $3\frac{1}{2}$, prog + ob (9.34) vs " " " (8.23)				NS	mean volumes = X independant variable)		
Experiment B				(iv) Sham ovarx $1\frac{1}{2}$ (12.42) vs Sham ovarx $3\frac{1}{2}$ (13.14)				NS								
Treatments	4	4.079	<0.01	(v) Sham ovarx ($1\frac{1}{2}$+$3\frac{1}{2}$) (12.78) vs intact contols (13.40)				NS						Residual	52	8.550
Residual	54													Treatment and resid.	56	
Experiment A				(i)	90.811	vs	359.240	<0.001	(i)	233.02	vs	324.78	<0.001	Difference	4	18.925
Treatments	5	40.123	<0.001	(ii)	51.663	vs	65.742	NS	(ii)	220.59	vs	187.24	NS	Reduction in EMS = $1 - \frac{8.558}{445.02}$		
Residual	86			(iii)	59.669	vs	152.108	<0.01	(iii)	291.24	vs	187.24	<0.001	= 98.1%		
Experiment B				(iv)	298.396	vs	387.750	<0.05						(Proportion of uterus occupied with sites = Y		
Treatments	4	45.715	<0.001	(v)	348.275	vs	378.877	NS						Mean volumes = X)		
Residual	59													Residual	56	0.02356

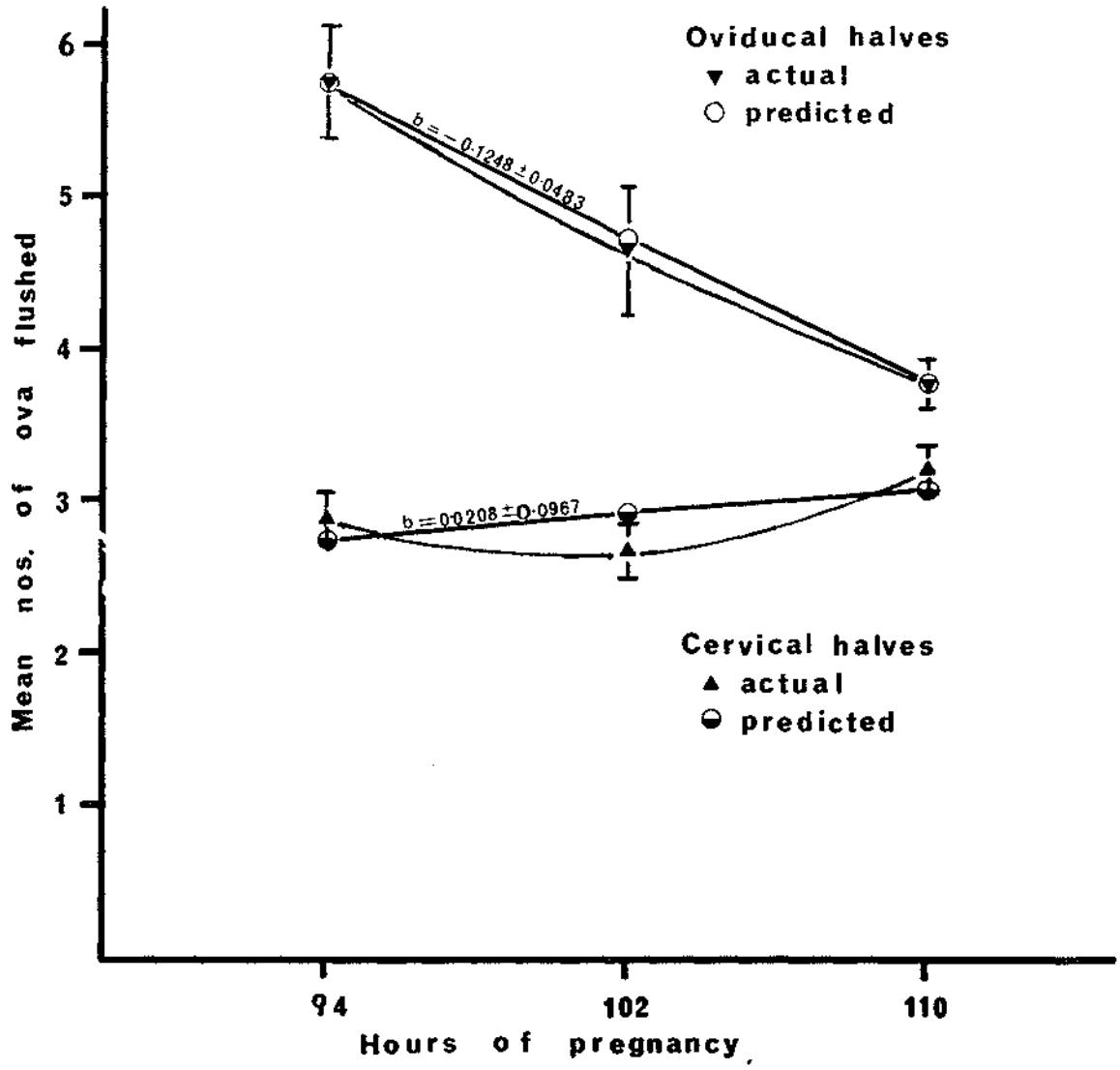
The mean volume of all implantation sites (mm^3)

Experiment A				(i)	75.99	vs	147.93	<0.001	(i)	97.93	vs	132.47	<0.001	Treatment and resid.	60	
Treatments	5	40.343	<0.001	(ii)	72.17	vs	73.19	NS	(ii)	80.99	vs	97.73	<0.05	Difference	4	0.1347
Residual	83			(iii)	72.60	vs	82.63	NS	(iii)	115.54	vs	97.73	<0.05	Reduction under EMS = $1 - \frac{0.02356}{1.3195}$		
Experiment B				(iv)	128.85	vs	162.60	<0.001						= 98.2%		
Treatments	4	17.353	<0.001	(v)	145.73	vs	152.33	NS						Comparisons		
Residual	56													(i) 0.69 vs 0.61		NS
														(ii) 0.45 vs 0.71		<0.001
Experiment A				(i)	0.510	vs	0.730	<0.001	(i)	0.583	vs	0.737	<0.001	(iii) 0.60 vs 0.71		NS
Treatments	5	5.577	<0.001	(ii)	0.470	vs	0.525	NS	(ii)	0.415	vs	0.653	<0.05			
Residual	84			(iii)	0.496	vs	0.556	NS	(iii)	0.681	vs	0.653	NS			
Experiment B				(iv)	0.725	vs	0.760	NS								
Treatments	4	6.557	<0.001	(v)	0.724	vs	0.705	NS								
Residual	57															

Proportion of uterine lengths occupied with implantation sites

+ Ovarx $3\frac{1}{2}$ = ovariectomy at day $3\frac{1}{2}$ of pregnancy

* $\frac{1}{2}$ mg = $\frac{1}{2}$ mg of progesterone daily



TEXT-FIG.6. The spacing of ova prior to implantation. Ova were flushed from the oviducal and cervical halves of the uterine horns. The actual means were derived from raw data and the predicted means from the regression equations.

(b) The mean size (volume) of implantation sites

Ovariectomised pregnant mice had smaller sites than intact pregnant mice in Experiments A ($P < 0.001$) and B ($P < 0.001$).

Experiment A

Of the ovariectomised pregnant mice, those given $\frac{1}{4}$ and $\frac{1}{2}$ mg when considered together, had smaller sites than those given 2 mg of progesterone daily ($P < 0.01$).

Of the intact pregnant mice those sham-ovariectomised at $1\frac{1}{2}$ had smaller sites than those that underwent this operation at $3\frac{1}{2}$ days of pregnancy ($P < 0.05$).

Experiment B

Of the mice ovariectomised at $3\frac{1}{2}$ days of pregnancy, those given progesterone alone had smaller sites than those that received oestrogen as well as progesterone ($P < 0.001$).

(c) The effect of the size of the implantation sites on the size of their spacing intervals

The mean intervals Y, were adjusted after the inclusion of their mean volume estimations as the covariate X, in an analysis of covariance for data from Experiment B. The resultant variance ratio was nonsignificant (Table 22) indicating that differences in spacing distances between treatments could be accounted for by variation in the size of implantation sites.

(d) Uterine length

The mean lengths of the uteri of ovariectomised pregnant mice were less than those of intact pregnant mice in Experiments A ($P < 0.001$) and B ($P < 0.001$).

Experiment A

Of the intact pregnant mice those sham-ovariectomised at $1\frac{1}{2}$ had shorter uteri than those that underwent the same operation at $3\frac{1}{2}$ days of pregnancy ($P < 0.001$).

Experiment B

Of the mice ovariectomised at $3\frac{1}{2}$ days, those given progesterone alone had shorter uteri than those given progesterone and oestrogen ($P < 0.05$).

(e) The proportion of the uterine length occupied with implantation sites

A smaller proportion of the uterine length was occupied with sites in ovariectomised pregnant mice than in intact pregnant mice in both Experiments A ($P < 0.001$) and B ($P < 0.001$).

Experiment B

Mice ovariectomised at $3\frac{1}{2}$ days had a smaller proportion of their horns occupied with sites than did those ovariectomised at $3\frac{1}{2}$ days of pregnancy and given progesterone alone ($P < 0.05$).

(f) The effect of the size of the implantation sites on the proportion of the uterine length they occupied

The mean proportions of the lengths of horns occupied with sites Y, were adjusted after the inclusion of mean volume estimates as the covariate X, for data from Experiment B, in an analysis of ovariance. The variance ratio remained significant ($P < 0.001$) and the mice ovariectomised at $3\frac{1}{2}$ still had a smaller proportion of the uteri occupied with sites than did mice ovariectomised at $3\frac{1}{2}$ days and given progesterone alone (adjusted means; 0.45 cf. 0.71, $P < 0.001$).

3. Foetal Orientation at Autopsy

Numerical and statistical data are presented in Tables 23 and 24, respectively.

Experiment A

Of the total of 246 foetuses whose direction was recorded 114, 124 and 8 had their heads pointing towards the oviduct, cervix and at right angles to the direction of the uterine horns, respectively.

Foetuses pointing at right angles to the long axis of the uterine horns were found only in ovariectomised mice.

Experiment B

Eleven of the 73 foetuses from ovariectomised hormone-treated mice pointed at right angles to the direction of the uterine horn.

FACTORS AFFECTING THE SIZE OF THE SPACING INTERVALS

1. The Size of the Intervals according to their Position in the Uterine Horns

The mean spacing distances are given in Table 25 and the statistical comparisons made are summarised in Table 26.

With the exception of group 5 Experiment B, two-way (intervals with regard to position and horns) analysis of variance tests were performed on each treatment group from both experiments. This type of analysis was used so that the variation between the horns, which in all cases was significant ($P \leq 0.01$ or $P \leq 0.001$), would not mask variation in interval length. Due to the fact that there were not equal numbers of intervals for each position, this was an approximate analysis only.

Reference to Table 25 shows that in general, for every treatment group there is a progressive decrease in interval size as the intervals approach the oviduct. However, as discussed below this effect is due to the

TABLE 23

THE LONGITUDINAL AXIS ORIENTATION OF FOETUSES[+]

(Numbers refer to numbers of foetuses unless otherwise specified)

Foetal head directed towards:-		oviduct	cervix	right angles*		oviduct	cervix	right angles*
Experiment A					B			
a) Total number of mice	33			2	19			2
" " " foetuses	246	114	124	8	101	47	43	11
" " " " in left horns	129	62	65	2	46	19	22	5
" " " " " right horns	117	52	59	6	55	28	21	6
b) Treatment and orientation								
Intact control mice	108	53	55		29	14	15	
Sham ovariectomised at 1½ days	51	25	26					
" " " 3½ days	52	22	30					
Ovariectomised progesterone-treated	30	11	11	8	39	18	13	8
" prog. and oestrogen treated					34	16	15	3

c) Position in uterine horn and orientation (positions numbered from the cervix towards the oviduct)

Position	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Foetal head directed towards: oviduct	25	24	14	22	13	9	5	0	11	8	8	5	8	4	2	1
" " " " cervix	21	23	31	15	14	10	2	2	16	11	5	8	1	1	1	0

[+] Umbilical cords were not twisted for any of the foetuses recovered.

* Foetuses orientated at right angles to the long axis of the uterine horn

TABLE 24

THE LONGITUDINAL AXIS ORIENTATION OF FOETUSES

SUMMARY OF ' STUDENT t ' TEST

Experiment A (all foetuses)

Sources	df	t	P
Direction of orientation (cervix vs oviduct)	32	0.666 (2.042)	NS ($P < 0.05$) *

Experiment B (all foetuses)

By inspection 't' test judged NS

* Required for significance at the 5% level.

TABLE 25

THE MEAN SPACING INTERVALS IN MILLIMETRES (WITH STANDARD ERRORS+) WITH REGARD TO POSITION BETWEEN IMPLANTATION SITES ACCORDING TO TREATMENT FOR EXPERIMENTS A AND B

Experiment A						B				
Intervals*	1	2	3	4	5	1	2	3	4	5
Treatment groups										
Intact controls	14.0 ± 0.9 (26)	13.2 ± 0.8 (25)	13.7 ± 0.5 (21)	12.4 ± 0.6 (18)	11.7 ± 0.3 (25)	13.2 ± 0.4 (53)	12.3 ± 0.5 (48)	11.4 ± 0.4 (44)	10.7 ± 0.5 (27)	10.7 ± 0.6 (23)
Sham ovariectomised 1½ days	13.0 ± 0.9 (29)	13.2 ± 0.8 (27)	11.2 ± 0.9 (19)	11.0 ± 1.0 (12)	9.2 ± 0.6 (13)					
" " 3½ "	13.5 ± 0.7 (30)	13.4 ± 0.7 (28)	12.7 ± 0.5 (25)	11.8 ± 0.6 (21)	11.8 ± 0.4 (29)					
Ovarx 3½ days ½ mg progesterone daily	6.7 ± 0.7 (22)	6.2 ± 0.7 (22)	4.5 ± 0.7 (15)	4.2 ± 0.9 (9)	4.6 ± 1.2 (9)					
" " " ½ " " "	7.5 ± 0.9 (26)	7.9 ± 1.0 (22)	4.3 ± 0.5 (17)	3.5 ± 0.4 (13)	3.8 ± 0.5 (17)					
" " " 2 " " "	8.6 ± 0.7 (28)	6.5 ± 0.6 (25)	7.0 ± 0.8 (21)	6.0 ± 0.8 (13)	6.3 ± 1.0 (7)					
" 3½ " " 2 " " "						10.3 ± 1.9 (10)	7.1 ± 0.8 (8)	6.6 ± 0.6 (5)	8.0 ± 0.6 (5)	13.5 ± 1.9 (4)
" 3½ " " 2 " " "						8.6 ± 1.1 (22)	7.8 ± 1.1 (22)	7.0 ± 0.8 (19)	5.5 ± 0.4 (15)	6.1 ± 0.5 (26)
" " " " 2 " " daily	+ 0.0025 OB					10.4 ± 0.6 (23)	10.6 ± 0.8 (21)	10.6 ± 1.4 (16)	8.2 ± 0.9 (13)	8.7 ± 0.8 (19)

+ Standard errors calculated from individual group variances.

* The first interval (1) is between the first and second implantation sites and so on up to the fifth interval (5) which included interval numbers greater than 5.

TABLE 26

THE MEAN SPACING INTERVALS[+] (IN MILLIMETRES) WITH REGARD TO POSITION BETWEEN IMPLANTATION SITES ACCORDING TO TREATMENT FOR EXPERIMENTS A AND B

SUMMARIES OF ANALYSES OF VARIANCE

Experiment A	Source	df	Variance Ratio	P	Comparisons *								
					I	P	II	P	III	P	IV	P
Intact controls	Interval (I)	4	4.718	<0.01	14.0 vs 13.2	NS	13.6 vs 13.7	NS	13.6 vs 12.4	<0.05	13.4 vs 11.7	<0.001
	Horns (H)	25	6.065	<0.001								
	Residual (R)	100										
Sham ovariectomised 1½ days	I	4	19.828	<0.001	13.0 vs 13.2	NS	13.1 vs 11.2	<0.001	12.6 vs 11.0	<0.01	12.4 vs 9.2	<0.001
	H	28	17.576	<0.001								
	R	112										
" " 3½ "	I	4	4.461	<0.01	13.5 vs 13.4	NS	13.4 vs 12.7	NS	13.2 vs 11.8	<0.01	12.9 vs 11.8	<0.05
	H	29	6.065	<0.001								
	R	116										
Ovarx 3½ days ½ mg progesterone daily	I	4	11.059	<0.001	6.7 vs 6.2	NS	6.4 vs 4.5	<0.001	5.9 vs 4.2	<0.001	5.7 vs 4.0	<0.05
	H	21	13.440	<0.001								
	R	84										
" " " 1 " " "	I	4	6.510	<0.001	7.5 vs 7.0	NS	7.3 vs 4.3	<0.01	6.5 vs 3.5	<0.01	6.0 vs 3.8	<0.05
	H	27	3.347	<0.001								
	R	108										
" " " 2 " " "	I	4	4.264	<0.01	8.6 vs 6.5	<0.01	7.6 vs 7.0	NS	7.4 vs 6.0	NS	7.2 vs 6.3	NS
	H	27	2.747	<0.01								
	R	108										
Experiment B												
Intact controls	I	4	16.826	<0.001	13.2 vs 12.3	<0.01	12.5 vs 13.4	<0.001	12.3 vs 10.7	<0.001	12.1 vs 10.7	<0.001
	H	52	8.510	<0.001								
	R	139										
Ovarx 3½ days ≈ 2 mg progesterone daily	I	4	2.364	NS								
	H											
	R	25										

TABLE 26 (Contd)

Experiment 5	Source	df	Variance Ratio	P	Comparisons * I	P	II	P	III	P	IV	P
Ovarx 3½ days + 2 ug progesterone daily	I	4	3.190	<0.05	8.6 vs 7.9	NS	7.3 vs 7.1	<0.05	7.9 vs 5.5	<0.05	7.4 vs 6.1	<0.05
	H	22	3.583	<0.01								
	R	77										
" " " " " "	I	4	3.707	<0.01	10.4 vs 10.6	NS	10.5 vs 10.6	NS	10.5 vs 8.2	<0.01	10.1 vs 8.7	<0.05
+ 0.0025 ug oestradiol ben. daily	H	22	6.057	<0.001								
	R	88										

+ The first interval (1) was between the first and second implantation sites and so on up to the fifth interval (5) which included interval numbers greater than 5.

* Comparisons: I = Interval 1 versus interval 2
II = " " plus " " versus interval 3
III = " " " " " plus " " versus interval 4
IV = " " " " " " " " plus " " versus interval 5

comparison of intervals from horns with different numbers of implantation sites. Inspection indicated that ovariectomy followed by hormone substitution therapy, had no apparent consistent effect on interval size within the horns when compared with intact animals and for this reason between group comparisons were not made.

2. The Mean Size of All Intervals according to the Number of Implantation Sites in the Uterine Horns

This analysis was performed on data from intact control mice used in Experiment B (groups 1 and 2) and demonstrated that the fewer the implantation sites the greater the distance between them (Tables 27 and 28).

3. The Mean Size of the Intervals according to their Position and the number of Implantation Sites in the Uterine Horns

Data were from groups 1 and 2, Experiment B (Table 27).

Intervals within a horn were compared only when the horns contained the same numbers of sites. The intervals within horns containing 2-3, 4 or 5 or more sites were not significantly different (Table 28).

The progressive decrease in length of the intervals with their positioning away from the cervix (Tables 25 and 26) is explained from the fact that the size of an interval is inversely related to the number of sites in the horn and the larger intervals are those closest to the cervix.

4. The Mean Size of the Intervals according to the Viability of the Implantation Sites bordering the Interval

This analysis was performed on data from mice ovariectomised at $3\frac{1}{2}$ days and given about 2 mg of progesterone daily (group 6, Experiment B) as there were about equal numbers of dead and live sites in this group. The results are given in Table 27 and the statistical tests made shown in Table 28. The intervals between live sites were not significantly different from those between live and dead sites. However, the mean

TABLE 27

THE EFFECT OF THE NUMBER[+] OF IMPLANTATION SITES PER UTERINE HORN AND THEIR VIABILITY[‡] ON THE SPACING INTERVAL(S) (IN MILLIMETRES)

a) The mean size of all spacing intervals (with standard errors)** according to the number of sites per horn

Number of sites	1-3	4	≥5
Spacing intervals (means with standard errors)**	15.5±0.8	12.8±0.5	11.3±0.2
Total number of intervals	(13)	(48)	(131)

b) The mean spacing intervals (with standard errors)** according to their position and the number of sites in the horns.

Intervals*	1	2	3	4	5
Number of sites					
2-3	16.1±0.8 (9)	14.3±1.4 (4)			
4	13.9±0.6 (16)	12.4±0.9 (16)	12.2±0.9 (16)		
5	11.9±0.5 (27)	11.9±0.6 (27)	11.3±0.5 (27)	10.7±0.5 (27)	10.7±0.6 (23)

c) The mean spacing intervals (with standard errors)** according to the viability of the sites on either side of the interval

Interval between:-	Two live sites	One dead and one live site	Two dead sites
Spacing interval	8.0±0.7 (33)	8.5±1.0 (29)	5.2±0.5 (35)

+ Data from intact control mice from Experiment B.

‡ Data from mice ovariectomised at and given about 2 mg of progesterone daily from day 3½ during Experiment B.

* The first interval was between the first and second implantation sites and so on. The fifth interval (5) includes interval numbers greater than 5.

** Standard errors calculated from individual group variances.

TABLE 28

THE EFFECT OF THE NUMBER OF IMPLANTATION SITES PER UTERINE HORN AND THEIR VIABILITY ON THE MEAN SIZE OF THE SPACING INTERVAL (IN MILLIMETRES)
(SUMMARY OF STATISTICAL TESTS)

Source of variation (for analyses of variance and one "t test")	df	Variance Ratio	P
1. The mean size of all intervals according to the number of sites per horn	2	15.580	<0.001
Residual	189		
2. The mean size of the intervals according to their position and the number of sites per horn			
a) 4 sites per horn	2 45	1.217	NS
b) 5 or greater sites per horn	4 126	1.280	NS
c) 2 and 3 sites per horn	11	t = 0.051	NS
3. The mean size of the intervals according to the viability of sites on either side of the interval	2 94	6.452	<0.001

Comparisons

1. Intervals for horns with 2 + 3 (15.5 mm) vs those with 4 sites (12.8 mm) P < 0.01
   " " " " 2 + 3 + 4 " vs those with 5 sites P < 0.001

2. Intervals between two live (8.0 mm) vs those between one live and one dead site (8.5 mm) P = NS
   " " two live + those between one live and one dead (8.2 mm) vs those between two dead sites (5.6 mm) P < 0.001

interval between two dead sites was smaller than the combined means of the other two groups ($P < 0.001$).

OBSERVATIONS MADE ON FOETUSES AT AUTOPSY

1. Foetal Developmental Age

(a) The effect of treatment on the developmental ages of foetuses recovered at day $12\frac{1}{2}$ of pregnancy

Foetuses from pregnant mice that did not show vaginal plugs were excluded (i.e., those from mice with uncertain mating histories).

Experiment A

Foetuses from intact pregnant mice were older than those from ovariectomised mice ($P < 0.001$) (Table 29).

Of the intact mice those sham ovariectomised at day $3\frac{1}{2}$ had older foetuses than those that underwent this operation at day $1\frac{1}{2}$ of pregnancy ($P < 0.001$).

Experiment B

There were no significant treatment effects on foetal development age.

Foetuses from Experiment B were younger than those from Experiment A (Table 29).

(b) The effect of position in the uterine horns on the developmental ages of all foetuses

Foetuses were included from mice with uncertain mating histories.

Experiment A

Judged by inspection there was no positional effect on foetal age nor were there reckoned to be any position-treatment interactions (Table 30).

TABLE 29

THE EFFECT OF TREATMENT ON THE DEVELOPMENTAL AGES OF FOETUSES RECOVERED ON DAY 12½ OF PREGNANCY *

Experiment	A		B	
	Number of foetuses	Developmental age in days (means ± standard errors)	Number of foetuses	Developmental age
Treatment groups				
Intact control mice	88	12.98 ± 0.01	34	12.60 ± 1.13
Sham ovariectomised at 1½ days	34	12.78 ± 0.02		
" " " 3½ "	61	13.16 ± 0.02		
Ovariectomised-progesterone treated	27	11.94 ± 0.07	23	12.04 ± 1.38
" " + oestrogen treated			43	12.44 ± 1.01
Mean age for foetuses from each experiment		12.86 ± 0.03		12.42 ± 0.7

Summaries of analyses of variance - Source	df	Variance Ratio	P	Source	df	Variance Ratio	P
Treatment	3	70.75	< 0.001	Treatment	2	0.0511	NS
Residual	206			Residual	97		

Comparisons

Intact mice (12.98) vs ovarx-prog. treat. (11.94)

< 0.001

Sham ovarx 1½ (12.78) vs sham-ovarx 3½ days (13.16)

< 0.001

Sham ovarx (1½ + 3½) (13.03) vs intact controls (12.98)

NS

* Foetuses from pregnant mice that did not show vaginal plugs were excluded.

TABLE 30

THE EFFECT OF POSITION IN THE UTERINE HORNS ON THE MEAN DEVELOPMENTAL AGES OF ALL** FOETUSES (IN DAYS)

Position in uterine horn (numbered from the cervix)	1	2	3	4	5	6	7	Last*
Experiment A								
Intact control mice	12.98(26)‡	13.12(26)	13.06(25)	13.10(25)	13.13(17)	13.23(13)	13.15(10)+	
Sham ovariectomised at 1½ days	12.68(12)	12.77(13)	12.58(12)	12.72 (9)	12.50(10)+			
" " " 3½ days	13.21(14)	13.21(14)	13.28(14)	13.30(13)	13.22 (9)	13.25 (8)	13.25 (8)+	
Ovarx-prog. treated	11.94 (8)	11.88 (6)	12.30 (5)	11.75 (8)				
All foetuses; means ± S.E.++	12.83±0.10	12.94±0.11	12.95±0.10	13.02±0.09	12.98±0.13	13.06±0.13	13.00±0.18	12.98±0.11
No. of foetuses in each position	60	59	56	50	34	26	21	53
Experiment B								
Intact control mice	12.57 (7)	12.57 (7)	12.80 (5)	12.57 (7)	12.58 (6)+			
Ovarx-prog. treated	12.06(15)	12.65(10)	12.43 (8)	12.33 (6)	13.00 (5)+			
Ovarx-prog. + oestrogen	12.41(11)	12.33 (7)	12.50 (7)	12.68 (6)	12.30 (5)	12.38 (4)	12.75 (2)	12.00 (2)
All foetuses; means with S.E.+++	12.29±1.21	12.52±1.40	12.65±1.56	12.50±1.65	12.58±2.02	12.50±1.87+		12.46±2.02
No. of foetuses in each position	33	25	20	18	12	14		12

‡ Bracketed figures are the numbers of mice from which each mean is derived.

* Last: represents the last position in a horn (with 2 or more sites) if the site contained a live foetus.

\+ Means from combining positions closer to the oviduct.

++ Standard errors derived from individual treatment group variances.

+++ " " " " the Error Mean Square, or estimate of total within variance for Experiment B.

** Foetal weights and developmental ages indicated that not all mice were killed at 12½ days of pregnancy. Some foetuses were collected from a second intact control group in Experiment which was later discarded because of insufficient numbers.

Experiment B

The position of the foetuses in the uterine horns did not have any significant effect on their developmental ages (Table 31), and by inspection of the means Table 30, there were no position-treatment interactions.

2. Foetal Weight

(a) The effect of treatment on the weights of foetuses recovered at day 12½ of pregnancy

Experiment A

Foetuses from intact pregnant mice were heavier than those from ovariectomised pregnant mice ($P < 0.001$, Table 32).

Of the intact mice foetuses from those sham ovariectomised at day 3½ were heavier than those from mice that underwent this operation at day 1½ of pregnancy ($P < 0.001$).

Experiment B

Treatments did not significantly effect the weights of foetuses.

(b) The effect of position in the uterine horns on the weights of foetuses

In neither experiment did the position of foetuses significantly influence their weight (Table 33).

3. The Mean Weights of all Foetuses according to their Developmental Ages

In general the older the foetuses, the heavier they were; the only exception was that during Experiment B. Foetuses reckoned to be 11½ were lighter than those 11 days old (Table 34).

TABLE 31

THE EFFECT OF POSITION IN THE UTERINE HORNS

ON THE MEAN DEVELOPMENTAL AGES OF ALL FOETUSES

(IN DAYS)

SUMMARY OF ANALYSIS OF VARIANCE

Experiment B (all foetuses)

Source	df	Variance Ratio	P	
Position	6	0.688 (2.17)*	NS	(<0.05*)
Residual	127			

Experiment A (all foetuses)

By inspection. Variance ratio judged by NS.

* Required for significance at the 5% level.

TABLE 32

THE EFFECT OF TREATMENT ON THE WEIGHTS OF FOETUSES RECOVERED ON DAY $12\frac{1}{2}$ OF PREGNANCY*

Experiment	A		B	
	Number of foetuses	Mean weights (mg) (with standard errors)	Number of foetuses	Mean weights
Treatment groups				
Intact control mice	103	88.9±2.4	34	64.4±2.7
Sham ovariectomised at $1\frac{1}{2}$ days	48	76.0±3.4		
" " " $3\frac{1}{2}$ days	71	93.2±2.4		
Ovariectomised-progesterone treated	26	63.9±4.6	22	68.7±3.4
" " + oestrogen treated			43	70.4±2.4
The mean weights for each experiment	248	85.0±1.6	99	68.6±1.6

Summaries of analyses of variance	Source	df	Variance Ratio	P
A	Treatment	3	13.171	0.001
	Residual	244		
B	Treatment	2	1.377	NS
	Residual	96		

Comparisons				
	Intact mice (88.9)	vs	ovarx prog. treat. (63.9)	< 0.001
	Sham ovarx $1\frac{1}{2}$ (76.0)	vs	sham ovarx $3\frac{1}{2}$ (93.2)	< 0.001
	Sham ovarx ($1\frac{1}{2}$ + $3\frac{1}{2}$) (86.2)	vs	controls (88.6)	NS

* Foetuses from pregnant mice that did not show vaginal plugs were excluded.

TABLE 33

THE EFFECT OF POSITION IN UTERINE HORNS ON FOETAL WEIGHTS (ALL‡ FOETUSES)

Position in uterine horn	1	2	3	4	5	6	7+	Last*
Experiment A								
Number of foetuses	56	54	58	50	35	27	20	58
Mean weights (with standard errors) in mgs	94.64±4.91	101.50±4.97	99.36±5.05	99.50±5.34	106.86±6.44	101.78±7.32	100.80±10.35	101.41±5.22
Experiment B								
Number of foetuses	32	25	20	18	12	11		16
Mean weights	65.31±2.93	71.36±3.32	75.25±3.71	70.39±3.91	71.00±4.78	65.73+±5.01		68.25±4.15

Summaries of analyses of variance	Source	df	Variance Ratio	P
Experiment A	Treatments	7	0.349	NS
	Residual	350		
Experiment B	Treatments	6	0.926	NS
	Residual	127		

+ Position 7 in Experiment A represents the combined positions of 7, 8 and 9
" 6 in Experiment B " " " " " 6, 7 and 8

* Last represents the last position in a horn (with 2 or more sites) if the site contained a live foetus.

‡ Foetal weights and developmental ages indicated that not all mice were killed at 12½ days of pregnancy.

TABLE 34

THE MEAN WEIGHTS (WITH STANDARD ERRORS[+]) OF ALL FOETUSES ACCORDING TO THEIR DEVELOPMENTAL AGES

Developmental age in days	11	11½	12	12½	13	13½	14
Experiment A							
Number of foetuses	18	10	18	10	137	32	45
Mean weights of foetuses (in mg)	35.2±1.8	46.5±1.6	66.7±1.9	75.5±3.0	86.5±1.5	132.7±2.8	158.9±2.6
Experiment B							
Number of foetuses	13	5	23	9	67		
Mean weights of foetuses (in mg)	47.5±1.5	45.0±4.5	57.2±1.8	67.3±2.2	81.0±1.2		

[+] Standard errors derived from individual group variances.

DISCUSSION

1. The Spacing of Ova prior to Implantation

Overall, more ova were flushed from the oviducal than from the cervical uterine horn halves (Table 20). This is interpreted to mean that there were a greater number of ova present in the oviducal halves and not that they were more difficult to recover from the cervical halves. Differences in numbers of ova flushed from the two halves were greatest at 94 and 102 hours of pregnancy when failure to recover blastocysts due to the initiation of implantation would be less likely than at 110 hours, when about equal numbers of ova were recovered from both halves.

When ova from the oviducal halves were considered there was a significant negative regression for the numbers recovered with advancing pregnancy (Text-fig.6, Table 21). While the equivalent positive regression for the cervical segments was nonsignificant, the results indicate that there was a prolonged and continual shift of blastocysts down the uterine horns which was not completed in all mice until between 102 and 110 hours of pregnancy. These results do not agree with statements by Snell (1956) and Wilson (1963) that ova were spaced shortly after their entry in to the uterine horns.

When attention is directed to the numbers of ova at each developmental stage at the time of recovery (Table 20), it is apparent that early or non-transformed (NT) blastocysts that had shed their zonae pellucidae were usually present only in the oviducal segments. This means most ova lose their zonae pellucidae whilst in the oviducal half and also that they are still being spaced after having lost this membrane.

It can be seen from Table 20 that about equal numbers of blastocysts within their zonae were recovered from each of the uterine horn halves. When

this is considered together with the conclusion that more blastocysts lost this membrane while in the oviducal segment it suggests that the uterus spaces zona-clad and zona-free blastocysts differently. For about equal numbers of blastocysts within their zonae pellucidae to have been present in either horn segment, it would follow that the uterus transports zona-clad more rapidly than zona-free blastocysts from the oviducal to the cervical half.

Conclusions drawn for the stages of pregnancy when ova begins to implant (page 46, Section I (a)) and when they are spaced apparently contradict one another as both these events take place early on day 4. This can be explained however, by taking into account the variation in developmental status of blastocysts from different mice at the time of recovery. It was assumed previously (page 44, Section I(a)) that ova were fixed to the uterus when they showed signs of giant cell trophoblastic transformation and at 94, 102 and 110 hours 16.5, 58 and 100 per cent, respectively, showed evidence of such transformation. Of the partially or completely transformed blastocysts 69 and 55 were recovered from the oviducal and cervical halves, respectively (Table 20). While their distribution favours the oviducal halves the difference was not significant. However, of the untransformed or moveable blastocysts 59 and 25 were found in the oviducal and cervical halves at 94 and 102 hours. This difference was significant ($P < 0.01$, Table 20). Thus blastocysts showing evidence of transformation were about evenly distributed in each horn while those at an earlier stage of development were not. It was the late positioning of the latter class, in mice where pregnancy was relatively less advanced, that gives the impression that blastocysts are being spaced at the time when implantation is beginning.

The conclusion that ova move slowly down the uterine horns has two important implications. First, spacing was not completed until a few hours

prior to the initiation of implantation, and secondly, that muscular contractions responsible for the movement of ova need not be vigorous. No precise estimate of the time interval between spacing and implantation can be made from the present data, though ova distribution favoured the oviducal half at 102 but not at 110 hours of pregnancy. With regard to the force of the uterine contractions necessary for ova distribution conflicting ideas are present in the literature.

Hollander and Strong (1950) doubted the existence of any agency for the spacing of ova 'other than the churning of the uterus and chance distribution' and McLaren and Michie (1959) suggested the only mechanism for the spacing of ova was a 'simple stirring brought about by uterine movement'. The former workers reached their conclusion from general observations made on 1080 pregnancies and because of the occurrence of placental fusions between adjacent embryos due to close spacing. McLaren and Michie (1959) observed that the injection of 0.01 to 0.02 ml of saline in the lumen of uterine horns on day $2\frac{1}{2}$ of pregnancy increased the incidence of placental fusion (two placentae) and reasoned that the saline partially inhibited the normal uterine contractions, 'leading to inadequate stirring of the uterine contents and therefore uneven distribution of the eggs'. In support of this explanation they reported work where donor eggs collected at $3\frac{1}{2}$ days post coitum (p.c.) and introduced into the uterine horns of pregnant recipients at $2\frac{1}{2}$ days p.c. usually implanted near the cervix. The recipient's own eggs entered the uterus after the time of transfer and implanted near to the oviduct and usually not inbetween the transferred ova. This 'zonation' was again attributed to the retardation of the normal mixing processes brought about by the action of injecting the eggs in saline. The interference is that zonation is abnormal and that normally eggs are free to and do, pass one another during spacing. However, in the rabbit at least, blastocysts probably do not move

to and fro past one another during spacing.

Böving (1956) found that rabbit ova enter the uterus shortly after 72 hours p.c. and at 4 days p.c. the mean location of the blastocysts was 17 per cent of the distance from the utero-tubal junction to the cervical internal os. At 5 days the mean location was 39 per cent of the horn length and after this time spacing became more even. The greatest daily progression of blastocysts down the uterine horn occurred between day 4 and day 5 p.c. when the dominant myometrial contraction pattern is a high frequency local type. There is a marked reduction in the strength of the uterine contractions coincident with the development of functional corpora lutea (Reynolds, 1965). Forceful peristaltic and antiperistaltic myometrial contractions are seen in the rabbit only during and for time after oestrus (Markee, 1944), so that when ova enter the uterus the myometrium is relatively inactive.

Oestrogens are effective as antifertility agents when administered to mice with intrauterine ova (Martin, Emmens and Cox, 1960; Emmens and Finn, 1962; Martin, 1963; Stone and Emmens, 1964), and one way in which ostrogens act is by causing strong uterine contractions. In the rat Pincus (1965d) and rabbit Chang and Harper (1966) the administration of oestrogen can cause the expulsion of the eggs from the uterus. Further, when Adams (1965) progressively eliminated the number of corpora lutea from pregnant rabbits, in which eggs had been transferred into the uterus, there was a progressive decline in the number of eggs recoverable from this organ. With the decrease in the numbers of corpora lutea eggs were expelled into the vagina. This effect was thought by Adams, to be due to a shift from a progesterone dominated to an oestrogen dominated myometrium accompanied by more forcible uterine contractions. However, oestrogen can also upset spacing without expelling ova into the vagina (Greenwald 1957). Three consecutive days of oestradiol treatment, starting between days 3 and 5 p.c., often led

to a crowding of ova at either end of the horns where implantation does not ordinarily occur. These data infer that in the mouse strong uterine contractions would be neither needed nor helpful for spacing.

Despite the fact that oestrogen can be responsible for the expulsion of uterine eggs, there is evidence that it is required for ova spacing in mice. When Smith (1966) transferred untreated or blastocysts cultured in vitro in the presence of oestradiol, to the oviducal half of the uterine horns of ovariectomised-progesterone treated mice 'implantation sites were clustered near the top of the horn'. However, if the mice received systemic oestrogen at the time of transfer they had implantation sites spaced along the horn. To account for the difference Smith suggested that oestrogen acted to increase the contractility of myometrium necessary for blastocyst spacing. Further it seems likely that the delayed oestrogen release thought to be required for implantation in lactating mice, could be responsible for ova distribution. Figure 8 (Bloch, 1966) shows unspaced blastocysts on day 5 of pregnancy in a lactating mouse and the delay of around 20 hours between an injection of oestrogen and the initiation of implantation (Whitten, 1958) would allow sufficient time for oestrogen to influence spacing.

It is doubtful that the nervous system plays much part in the spacing of ova. Bőving (1956) reported that the propulsive activity of uterus in the rabbit was not 'neurogenic' but probably 'myogenic' in nature. Further, reproduction is apparently normal in rats after 'extensive abdominal sympathectomy' or removal of the pelvic parasympathetic nerves (Carlson and De Feo, 1965).

Although no information concerning the type of uterine contractions occurring in early pregnancy in the mouse was found, it seems probable that the myometrium is susceptible to and would respond in a similar way to oestrogen and progesterone as in the rabbit. And as indicated by the work of

Adams (1965) ova transport and spacing in the uterine horns may depend not so much on the levels of oestrogen and progesterone alone but on the relative levels of both hormones.

Böving (1956) postulated that spacing in the rabbit was accomplished by a hormone-regulated stimulus-effector system. The expanding blastocyst which distended the uterus acted as the stimulus and the progesterone-conditioned uterine muscle as the effector. Blastocyst expansion in the rabbit prior to implantation is relatively much greater than that which occurs in mice and rats. Ova have been accorded a passive role during spacing in mice and the first sign that the uterus has recognised their presence is the Pentamine Blue reaction (McLaren, 1966; Finn and McLaren, 1967) at a time when the blastocysts are thought to have been spaced. However, there are reasons on morphological grounds to believe that the blastocyst is recognized before this time. Bloch (1966) observed that as an early step in implantation, the blastocyst induced the formation of a spherical crypt in the endometrium. Reference to photographs in Bloch's work and to Pl. II, fig. 6, (Orsini, 1963), shows that unspaced ova in pregnant lactating mice also induce crypt formation. Although the formation of crypts in uterine mucosa by blastocysts is no guarantee that the uterus will react to this stimulus, it does present anatomical evidence of recognition and it also highlights two other points. First, it demonstrates that the uterine lumen in the mouse has a small cross sectional diameter which may restrict blastocyst movement and secondly, that the blastocyst may not implant in a pre-existing antimesometrial crypt but actively form the crypt in which it does implant.

Moreover, if it is accepted that nontransformed blastocysts are able to contract and expand as earlier reasoned from their shape on recovery and as seen <u>in vitro</u> by Borghese and Cassini (1963) and Cole and Paul (1965),

there is added reason to believe that the uterus would be aware of their presence prior to spacing. Thus regardless of whether the blastocyst pressed on the uterus or vice versa, it is conceivable that the myometrium may be stimulated to respond in a similar manner as Böving (1956) postulated to occur in the rabbit.

2. The Spacing of Implantation Sites

(a) The order of implantation in the uterine horns

The failure to demonstrate that there were significantly more partially or completely transformed blastocysts in the oviducal than in the cervical uterine horn halves (Table 20) means the present results do not support Mossman's (1937) hypothesis that implantation is serial, i.e., beginning at the oviducal end of the uterus.

Also, the failure to demonstrate significant within variation for the developmental ages (Table 29) or the weights of foetuses (Table 32) according to position, again suggests that there is no ordered progression in the time of implantation and that chance alone determines the positions occupied by the blastocysts that implant earliest.

The inability to find a positional influence on foetal weights disagrees with the results of Hashima (1956) and McLaren and Michie (1959) who found that the foetus closest to the oviduct was lighter than its neighbour. The live foetus in this position in horns with two or more sites was not significantly lighter or younger than foetuses from any other position in the experiments described. McLaren and Michie also found that foetuses were lighter in the central regions than at either end of the horns. While in the present work, although not statistically significant, the heaviest foetuses were found in the central areas of the horns. However, McLaren and Michie weighed foetuses at $18\frac{1}{2}$ days p.c. from superovulated mice and Hashima reported that the pregnant mice in his experiments were killed 'at the termination of pregnancy'.

Foetal weights are at best only an indirect estimate of the time of ova-implantation, and reference to the standard errors (Table 34) shows that foetuses with similar external features (and therefore reckoned to be the same age Gruneburg (1943)), often varied considerably in weight. Furthermore, it is an unproved assumption that the variation in foetal weight near term reflects only, or accurately, that variation relating to the time of implantation. On theoretical grounds it is possible that the growth rate of a foetus, especially near term, may depend on its position in the uterus. And for this reason it is considered that data collected at day 12½ should be more suitable for this type of analysis than those collected later on in pregnancy. Further, the estimates of the foetal age made, although approximate, did correlate quite well with the foetal weights and do provide a direct measure of the time of implantation.

It can be seen from Table 34 that the majority of foetuses killed had their ages set at 13 days, whereas most of these were removed at day 12½ of pregnancy (or 12½ days post coitum). Gruneberg (1943) killed pregnant mice in his experiments at 0900 hours and assumed that on average the litters would be about ⅓ of a day older than their nominal age. This correction of ⅓ of a day was not added to the age estimates in the present experiments, so in fact, at autopsy most foetuses had a developmental age of about 13 and ⅓ days, that is, 5/6 of a day in advance of their post coital estimates and would appear to have been about 5/6 of a day ahead in development when compared with Gruneburg's data. However, in the present work, relative and not absolute age determinations were important.

During Experiment A ovariectomy at 3½ days of pregnancy and daily progesterone administration depressed both the weight and the ages of the foetuses relative to those found in intact mice ($P < 0.001$). However, ovariectomy and hormonal substitution therapy had no such effect during

Experiment B. These treatment effects observed during Experiment A are consistent with what would have been expected had implantation been delayed. Also mice sham ovariectomised at $1\frac{1}{2}$ days, probably implanted ova later on in pregnancy than those sham operated on at $3\frac{1}{2}$ days. This interpretation is consistent with other findings, viz., that the uteri were shorter, the implantation sites were smaller and the body weights at autopsy were less for mice sham ovariectomised at $1\frac{1}{2}$ than at $3\frac{1}{2}$ days of pregnancy.

Reasons for the delayed implantation are not known and those for its occurrence only in Experiment A, are discussed in Section I (page 70).

(b) The effect of treatment on the size of the spacing interval between implantation sites

Analyses of variance showed that ovariectomised progesterone treated mice had implantation sites that were spaced more closely, smaller in size and that occupied a relatively smaller amount of a shorter uterine horn than in intact pregnant mice (Tables 7 and 22). However, analysis of covariance techniques for data from Experiment B demonstrated that these differences were largely dependent on site size differences, and did not reflect basic differences in the way in which foetuses were spaced.

The reason for wanting to consider volume and interval parameters simultaneously in an analysis of covariance was that the interval measurements each included the length of one implantation site (intervals were measured from the mid points of the sites). It is apparent then that the interval size depended largely on the size of the sites and the analysis of covariance test, which in effect removed variation due to site size, allowed concentration on the parameter of interest, viz., the distance between sites that did not include part of the sites themselves.

For the proportion of occupied horn length to be greater in intact than in ovariectomised pregnant mice, it means that the larger implantation sites

occupied relatively larger amount of the greater horn length. However, when the size variation of the sites was controlled by including the size as the covariate X in an analysis of covariance on data from Experiment B, only in group 5, which had significantly fewer implantation sites per mouse than in other groups ovariectomised on day 3, was the variation in occupied horn length not all explainable by variation in site size.

The administration of oestrogen to ovariectomised progesterone-treated mice (group 7, Experiment B) was associated with increases in site size and uterine horn length and nonsignificant increases in the spacing interval and in the proportion of the uteri occupied with sites. That is, mice that received oestrogen presented a picture midway between the ovariectomised mice that were given progesterone alone and the intact mice.

The failure to demonstrate that spacing was basically different after ovariectomy and hormonal substitution therapy implied compliance with the null hypothesis. However, there are reasons to question the failure to invalidate the null hypothesis.

When note is taken of the deviations in the longitudinal axis orientation of foetuses (Table 23) it is apparent that the only foetuses that are directed at right angles to the direction of the uterine horns are from the ovariectomised hormone-treated groups. Figure 1, Plate VIII (cf. fig. 3) shows a uterus prior to the removal of foetuses orientated in this unusual way. Uteri or segments of uterine horns that contained foetuses orientated at right angles were unusual in that foetuses were packed so tightly that the horns were rigid and not folded, in situ.

Other evidence of abnormally close spacing in ovariectomised hormone treated mice is illustrated on Plate IX, foetuses from two uteri (figs. 1 and 2) and from a third not shown (group 5, Experiment B), occupied more than one implantation chamber. Foetuses involved were deformed, most probably due

to pressure exerted by the annular constrictions present between implantation chambers. For example, the foetus shown in Figure 1 has a depression in its back, while two of those shown in Figure 2 had annular grooves impressed around their heads and in all three cases the constrictions between implantation chambers are positioned so as to exert pressure on the foetuses where the deformations occurred.

Further examples of excessive intrauterine pressure were observed in two mice during Experiment B (one each from groups 5 and 6) where the horns had ruptured, liberating foetuses into the peritoneal cavity. In the mouse from group 5 the anterior tip of the left horn had ruptured, expelling the most anterior foetus and its maternal placenta. In the second mouse two foetuses, one with and the other without placentae, were extruded from an opening between two implantation sites that contained live foetuses at autopsy. All the extruded foetuses were dead.

Thus distances between the midpoints of the sites when foetuses were orientated at right angles to the direction of the horn were not a suitable measure of spacing, as the distances between the edges of the sites were zero. Nor do the midpoint distances reflect an accurate picture of the spacing in the other examples of extreme uterine confinement. The question arises as to whether or not these extreme cases of close spacing can be considered as unique, while spacing was 'normal' in other ovariectomised pregnant mice. Evidence that contradicted an all or none explanation was that although foetuses from the ovariectomised pregnant mice in Experiment B were neither lighter nor younger, they were contained in smaller, more closely spaced sites than those found in intact mice. A likely explanation is that the size of the implantation sites was over estimated for intact mice.

The measured length of an implantation site included the length of the distended horn on either side of the foetus and in most cases was the

distance between the annular uterine constrictions. However, there is reason to believe that the sites from intact mice were more often elliptical in shape than were those from ovariectomised mice. This was the result of a problem in technique. Immediately on opening the peritoneal cavity of intact mice at autopsy, the uterine horns were seen to be folded on themselves and around other viscera. The horns owed their flexibility to narrow intervals between sites. However, after excision, the uterine horns rapidly shortened and the narrow intervals between the sites were obliterated. Moderate tension to straighten the horns did not bring about the return of the narrow intervals but instead caused distension of the horn immediately adjacent to the sites causing these to assume an elliptical shape. While the pregnant uterine horns in ovariectomised mice were relatively more rigid and the implantation sites were not usually separated by attenuated intervals. Further, a higher proportion of sites were dead in ovariectomised than in intact mice and intervals between dead sites did not contract in an irreversible way after excision. Thus the length and volume estimates, from the assumption that all sites were spheroid in shape, were probably biased in favour of being larger in intact mice.

The reduction of the interval size after uterine excision was more pronounced in intact than in ovariectomised mice. This means that the spacing distances between sites in intact mice were probably underestimated.

The size of spacing intervals also varied according to the viability of the sites on either side of the interval (Tables 27 and 28). This was not unexpected as the mean size of intervals in a horn was dependent on and positively related to the size of the sites. The intervals between two dead sites were shorter than those bordered by live, or one live and one dead site. However, the size of the intervals between live and dead sites were not shorter than those between two live sites. This suggests that the live site

extended further towards the dead site than it would have had that site been alive. As this indicates that the positive site-size interval-length relationship was inconsistent, spacing between live and dead sites is not strictly comparable with that between two live sites. Further, because the spacing intervals between dead sites were reversibly, and those between live sites irreversibly shortened after uterine excision, comparisons of this sort should not be made. This raises objections to the type of analysis carried out in the present work.

Whereas in both experiments about 92 per cent of the sites in intact mice were alive, 8.5 and 62 per cent were alive in ovariectomised hormone-treated mice from Experiments A and B, respectively. Thus, in Experiment A and to a lesser extent in Experiment B the spacing of dead sites has been compared directly with that of live sites.

It is probable then that implantation sites were more closely spaced in ovariectomised hormone treated mice than in intact mice. Evidence for this conclusion is based on; 1) the unmistakable fact that the implantation sites in some mice were very close together, resulting in the right angle orientation of the foetal longitudinal axis, foetal occupation of more than one implantation chamber and the rupture of uteri. 2) Contraction of the spacing interval at autopsy occurred more frequently in intact mice and probably led to a disproportionate reduction in interval size when compared with ovariectomised animals. Also spacing between sites of differing viability was not strictly comparable, and the proportion of dead sites in ovariectomised mice was higher than in intact mice. 3) The distortion in the shape of the implantation sites, produced by straightening the uterine horns, more often encountered in intact than in ovariectomised mice artificially increased the length and volume measurements. This led to the questionable conclusion that larger sites occupy a disproportionately

larger amount of horn and allowed practically all variation in interval length to be explained in terms of volume variation.

The uterus in ovariectomised rats exerts considerable pressure on foetuses present (Selye et al., 1935; Zeiner, 1943; Carpent, 1962). Carpent considered that the increased pressure was due to 'uterine hypertonicity' and noted that this often caused gross deformations in surviving foetuses consistent with changes expected from compression. This author also observed that some foetuses were expelled from the uterus into the peritoneal cavity after ovariectomy on day 12 of pregnancy. Uterine hypertonicity appeared to have been caused either by the absence of, or an imbalance of supplied oestrogen and progesterone. Hypohysectomy and pituitary transplantation during pregnancy in the rat, whether accompanied by exogenous hormonal substitution therapy or not, did not lead to uterine hypertonicity (Carpent and Desclin, 1967; Carpent, 1963).

Abnormally close embryonic spacing or crowding occurred in ovariectomised progesterone-treated mice that received blastocysts cultured in oestradiol (Smith, 1966) and in ovariectomised rats that underwent delayed implantation in response to progesterone and oestrogen therapy (Carpent, 1962).

The crowding of foetuses demonstrated in the present experiments could be due either to the failure of ova to be spread out along the uterine horns, or to the failure of the intervals between the sites to grow during pregnancy, or to a combination of both effects. As exogenous oestrogen given at the time of ova transfer in Smith's (1966) work counteracted the crowding effect and embryos were spaced throughout the horns, failure of adequate ova dispersal is implicated.

In the present experiments there was definite evidence of failure of the uterus to grow and distend adequately throughout gestation in ovariectomised hormone-treated mice. This condition appears very similar to the 'uterine

hypertonicity' described by Carpent. Treatment with both oestrogen and progesterone (group 7, Experiment B) was associated with larger sites and longer uteri than that which was limited to progesterone alone (group 6, Experiment B). Thus oestrogen probably stimulated uterine growth.

Preliminary experiments demonstrated that mice ovariectomised on day 3½ and given 2 mg of progesterone daily often showed irregular close spacing on days 7 or 8 of pregnancy. This suggests that the close spacing observed in the current experiments may have been due to inadequate dispersal as well as to the failure of the uterus to grow and expand with the enlarging foetuses.

Böving (1956) argued that the even or nearly even spacing of ova prior to implantation found in the rabbit, implied a stimulus-effector system and that a random distribution suggested that the blastocysts played a passive role and went where they were driven. However, it becomes difficult to know in the event of uneven spacing how uneven it should be before it is random. And on theoretical grounds at least it would be possible to have ordered uneven spacing in which blastocysts usually implant at a compatible distance from one another to make good use of the available length of uterus. The likelihood of ordered uneven spacing in the mouse would appear to be higher than in the rabbit for at the time of implantation the sum total length of the blastocysts in a uterine horn is less than 2 per cent of its length while it is about 15 per cent in the rabbit (Böving, 1956).

However, no detailed information is available as to how regular spacing is at the time of implantation in the mouse. And when attempts are made to estimate the eveness or otherwise of spacing at 12½ days of pregnancy factors other than the position at which the embryos first occupied confound

estimations.

Contractions of the excised horn, as recognized by Bloch (1966) predispose to even spacing and place emphasis on the size of the sites rather than on the intervals between them, as occurred in the present experiments. Further, it has been suggested that a variable growth rate of different parts of the uterine horn during gestation (Frazer, 1955), and especially accelerated growth in the region of the embryo, would make it inevitable that foetuses would become spaced about equidistant from their neighbours (Reynolds, 1965).

Another important influence is the number of sites that a horn contains. Hollander and Strong (1950) and McLaren and Michie (1959) observed that as the number of sites in a horn increased so did the incidence of placental fusion. This, these workers considered, was evidence of irregular spacing. The corollary to this was also true, that is, spacing became more irregular as the number of sites in a horn increased. The average rate of placental fusion in 1080 pregnancies was 1 in 125 embryos and for horns containing 9 or more sites it was 1 in 40 embryos (Hollander and Strong, 1950).

Spacing measurements from intact mice showed that there was an inverse relationship between the number of sites in a horn and the interval lengths between them (Tables 27 and 28). If the assumption that the contraction of the horns after excision was uniform throughout their lengths is allowed, then the conclusion that the size of the spacing intervals in the horns with the same numbers of implantation sites are not significantly different from one another (and are therefore even) can be sustained.

While differential uterine growth during pregnancy could 'space' implantation sites (Reynolds, 1965) it is considered unlikely that this alone would account for the inverse relationship between the size of the spacing distances and the number of implantation sites in the horns. Nor is it likely

that the even spacing of sites, especially in horns with four or less sites, is due soley to differential uterine growth. Close spacing is unusual when there are few sites in a horn; so there is no apparent need to space sites equidistant from one another. The inference is that the fewer the blastocysts in the horns the further apart they are spaced. It is difficult to see how this could happen in the absence of a purposeful blastocyst-uterine interaction during spacing.

Frazer (1955) found in the rat, that if there were more than five sites in a horn they were evenly spaced along the horns at 9 days p.c. However, if there were five or less sites then the mean centre point was closer to the cervix. Thus the number of embryos may determine both the distance at which they are spaced apart and the region of the horn they occupy.

It is advantageous for ova to be spaced at a reasonable distance from one another. The survival rate is low and there is a reduction in the weight of foetuses whose placentae are fused in the crowded horns of superovulated mice (McLaren and Michie, 1959). As reasoned by Mayer (1960) the fact that ova can and do implant very close together in superovulated mice make it unlikely that spaced predetermined sites for blastocyst implantation exist. However, Orsini (1963) found that increases in vascularity, and implantation, in parous mice occurred between the placental scars of the preceding pregnancy.

This suggests that some areas of the uterus may be more favourable, rather than predetermined, for implantation than others. Shelesnyak (1966) has argued that in the rat, there are specific regions of the uterus set aside for decidua formation and that the blastocysts are attracted to these. Nonlocalized decidualizing stimuli in pseudopregnant rats and mice (Finn and Hinchliffe, 1964) can result in the formation, initially, of discrete deciduomata that are spaced along the horns. Further, Coppola, Ball and

Brown (1966) claimed to have observed spontaneous deciduomata formation in pseudopregnant rats that also first appeared as localized swellings. However, other workers (Psychoyos, 1966a; Finn, 1966b) considered that the uterus was uniformly sensitive to decidualizing stimuli throughout its length.

Thus while there is reasonable evidence that blastocyst spacing is influenced by their number present there may also be favourable and less favourable regions of the uterus for implantation.

SUMMARY AND CONCLUSIONS

Prior to implantation ova were found to move slowly and progressively down the uterine horns. Spacing was not completed in the present experiment in all mice until sometime between 102 and 110 hours of pregnancy. The number of ova implanting in the oviducal and cervical horn halves were not statistically different.

More ova lost their zonae pellucidae while in the oviducal half than in the cervical half of the uterine horns. Nontransformed blastocysts were still being spaced after the loss of their zonae.

Mild uterine contractions would have been adequate to distribute ova throughout the uterine horns in the present experiment. Reviewed work suggested that the strength of the uterine contractions depended on the balance of circulating oestrogens and progesterone (Reynolds, 1965; Adams, 1965; Smith, 1966).

There are two reasons why pre-spaced blastocysts may play an active part in an inter-relationship with the uterus to effect ordered spacing. First, blastocysts may undergo alternate contraction and expansion during spacing (Section I (a), page 48) and secondly, prior to spacing blastocysts were able to induce uterine crypt formation (Bloch, 1966). Either process could provide the blastocyst with a means of communicating its presence and position to the uterus.

Evidence was not found, either in intact or in ovariectomised hormone-treated mice, to suggest that ova implant in a definite positional order in the uterine horns.

The number of implantation sites in the uterine horns of intact mice was inversely related to the size of the spacing distances between them. The size of the spacing distances within uterine horns with the same number of implantation sites were not significantly different from one another.

Implantation sites were more closely spaced in ovariectomised hormone-treated than in intact mice. Failure to demonstrate that this difference was statistically significant was probably due to technical problems. The net result was that variation in the size of the implantation sites masked variation in the size of spacing distances between them. Pregnant uteri from ovariectomised mice that received both oestrogen and progesterone resembled more closely those from intact mice than those of ovariectomised progesterone-treated mice.

EXPLANATION OF PLATES

PLATE I

1. An 86 hour blastocyst within its zona pellucida (ZP). The zona is in contact with periphery of the blastocyst (size, outside diameter of ZP 120 u). x300.*

2. A 94 hour blastocyst within its zona pellucida. There is a gap (arrow) between the blastocyst and its zona (cf. fig. 1). x300.

3. An 86 hour blastocyst free of its zona pellucida. This is an example of a 'contracted' blastocyst. Note that the blastocoele is not distinguishable and that it is smaller than blastocysts within their zonae. (size, diameter 70 and length 80 u). x 300.

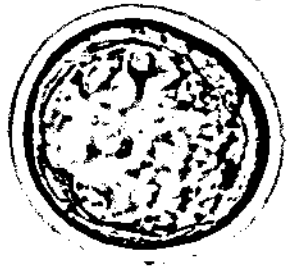
4. A 94 hour 'contracted' blastocyst.

5. A 102 hour blastocyst less than one half transformed. There is a distinct projection at the abembryonic pole (arrow). Giant cell trophoblast transformation has just begun at this pole. (ICM = inner cell mass). x300.

6. A 110 hour blastocyst less than one half transformed. Giant cell trophoblastic transformation having begun at the abembryonic pole (arrow). This blastocyst shows an expanded form. (size, diameter 130 and length 170 u). x300.

* Ova were photographed with a different microscope than the one with which they were measured and the magnification given is approximate only as the ova may have moved in the saline drop during transfer.

PLATE I



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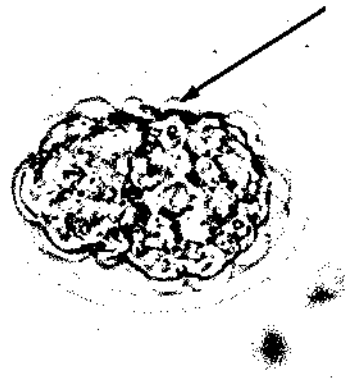

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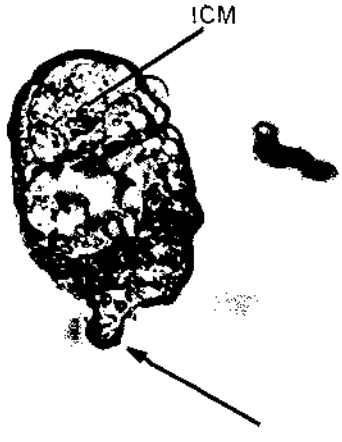

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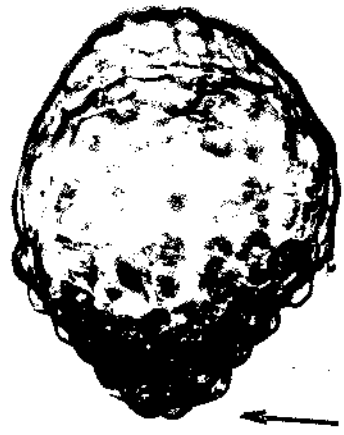

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PLATE II

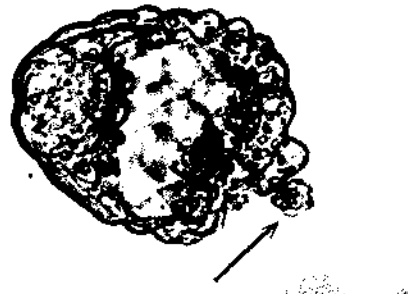
1. A 102 hour blastocyst less than one half transformed from the same mouse as figure 5, Pl. I. Note again how the cells at the abembryonic pole are organised to form a projection (arrow). x300.

2. A 102 hour blastocyst less than one half transformed. Note the debris (arrow) adhering to the abembryonic pole. x300.

3. A 118 hour egg cylinder. EC = the region of the ectoplacental cone; PC = the prospective region of the proamniotic cavity; Tr. = trophoectoderm; UE = uterine epithelium. x300.

4. A damaged 118 hour egg cylinder. The trophoectoderm has been lost during recovery. x300.

PLATE II



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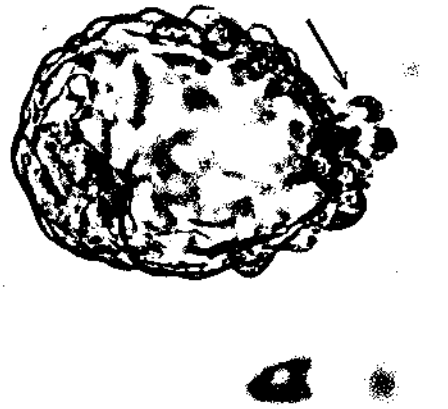

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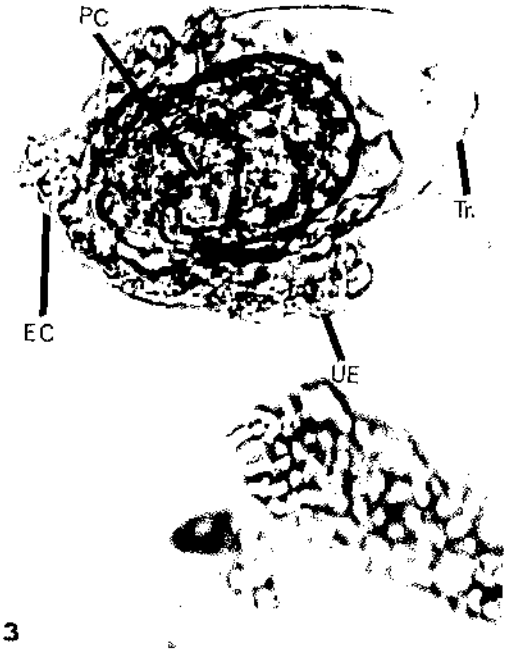

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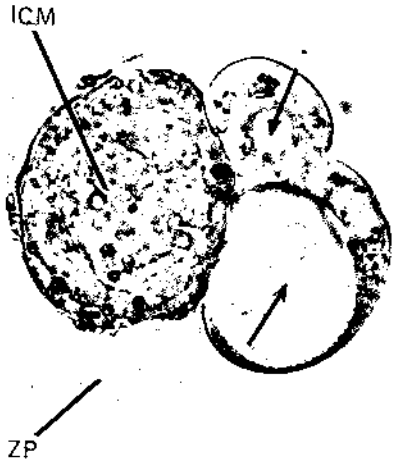
PLATE III

1. A 94 hour blastocyst during blastocoele formation. Two blastomeres have cavities (arrows) which probably coalesce during the final stages of cavitation. ICM = inner cell mass; ZP = zona pellucida. x550.

2. A 94 hour blastocyst showing no signs of giant cell transformation (size, diameter 70 and length 90 u). x777.

3. A 110 hour blastocyst fully transformed. The trophoblastic giant cells have all but obscured the blastocoele (Bl). (size, diameter 80 and length 150 u). x550.

4. A 118 hour egg cylinder with adherent uterine epithelium (UE) on the abembryonic pole Em. Ect. = embryonic ectoderm; Ex. Ect. = extra-embryonic ectoderm; PC = proamniotic cavity; Prox. Ent. = proximal entoderm. (size, diameter 100 and length 180 u). x550.



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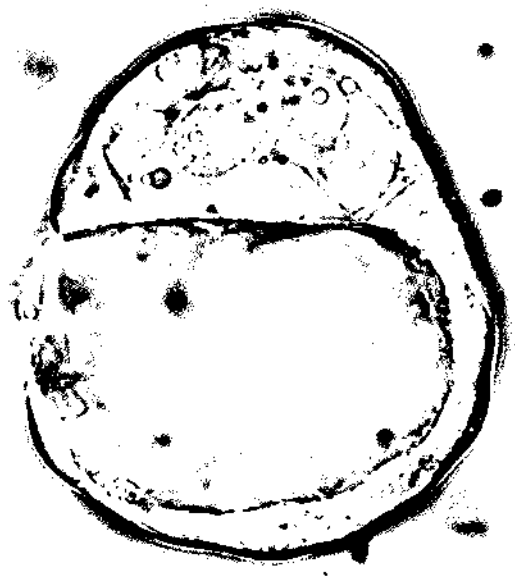

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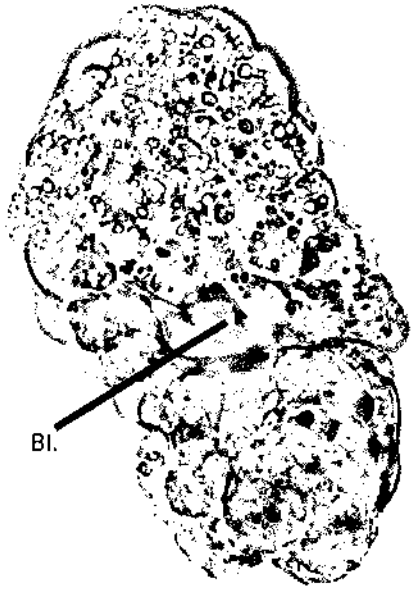

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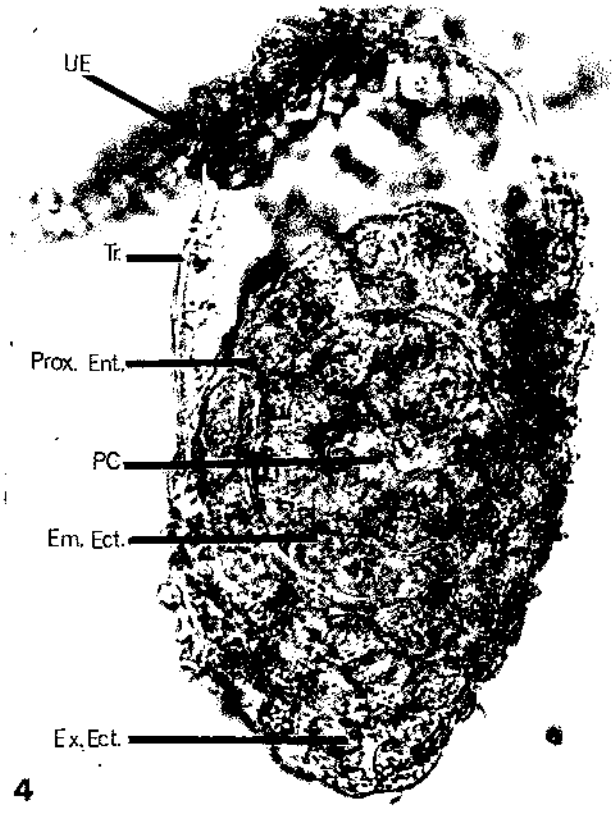

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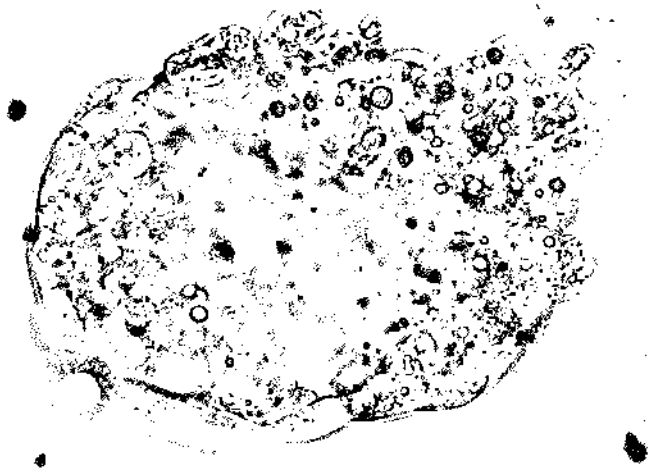
PLATE IV

1. A 110 hour blastocyst less than one half transformed. ICM = inner cell mass; AP = abembryonic pole. x427.

2. A 110 hour blastocyst, from the same mouse as figure 1, more than half though less than fully transformed. Note the irregular outline of the abembryonic pole. x427.

3. A blastocyst recovered at $12\frac{1}{2}$ days from a mouse ovariectomised on day $3\frac{1}{2}$ and given daily injections of vehicle during Experiment A. While there are no typical signs of trophoblastic transformation, the individual trophoblast cells are outlined more clearly than in blastocysts undergoing continuous development (cf. Pl. III, fig. 2). This blastocyst is unusually large for its state of development, i.e. diameter 120 and length 180 u . x427.

1



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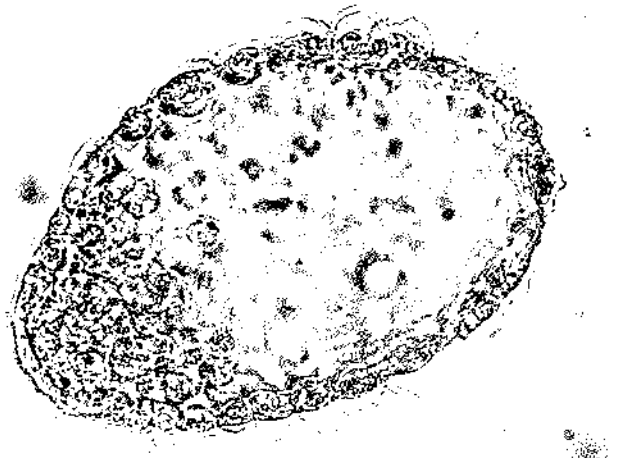

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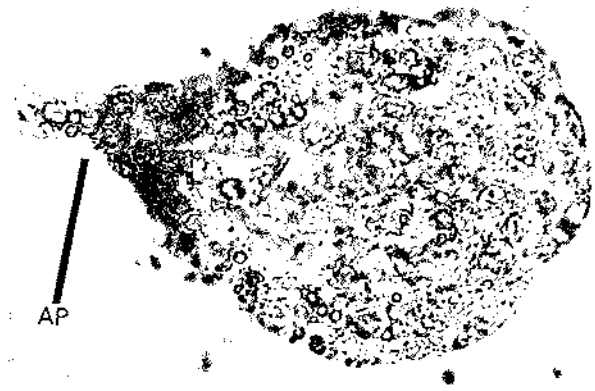
PLATE V

1. A 118 hour 'top' shaped fully transformed blastocyst. The trophoblast cells at the abembryonic pole (AP) are organised to form a point. The size of the blastocyst is within the expected range (diameter 120 and length 160 u). x427.

2. A 102 hour 'abnormal blastocyst'. Blastocoele (Bl) formation would appear to be unusual. The cellular knob on the left of the figure has a small cavity (arrow) and is connected to the rest of the 'blastocyst' by a stalk (ST). The blastocyst is smaller than usual (diameter 40 and length 120 u). x733.

3. An unidentified object recovered from a mouse 118 hours pregnant. It is distinctly cellular in make up and is 50 u wide and 140 u long. x733.



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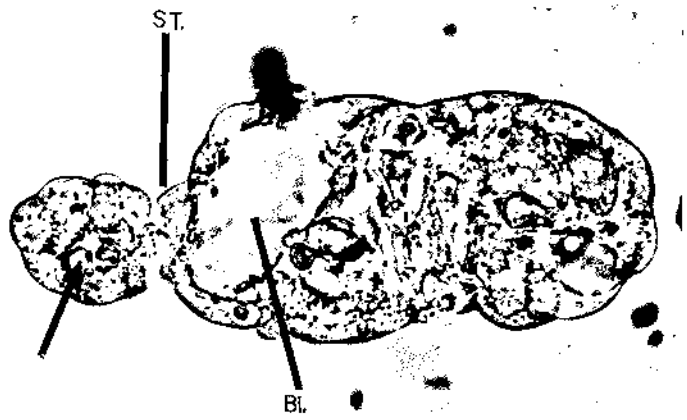

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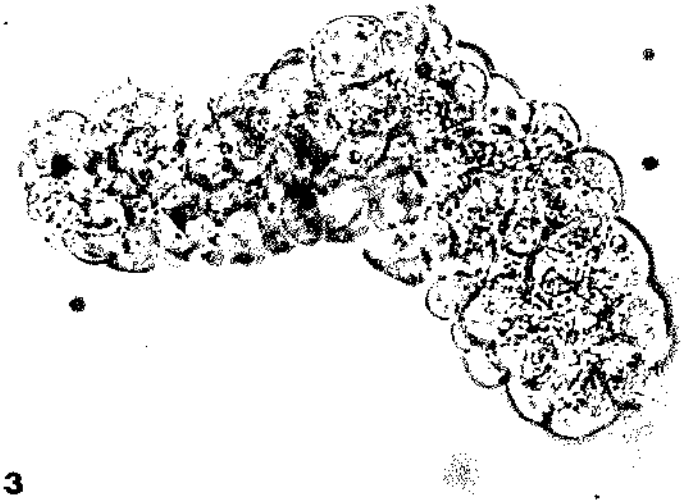

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PLATE VI

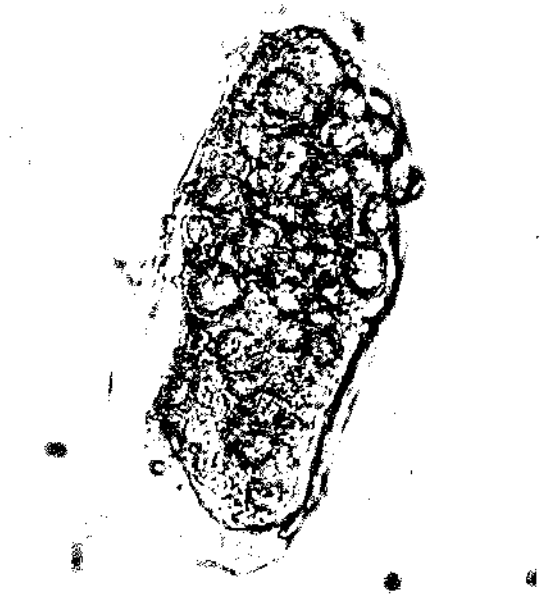
1. An unidentified object from a mouse pregnant 70 hours. This object bears little resemblance to other unidentified objects and to a late morula present in the same uterus (size, 60 u wide and 130 u long). x 592.

2. Material flushed from the uterus of same mouse as the object in figure 1. The round 'cells' may be secretion droplets adhering to uterine debris. x 592.

3. A blastocyst recovered at 12½ days from the uterus of a mouse ovariectomised at, and given no further treatment from 3½ days. This blastocyst does not show typical changes associated with giant cell transformation. The trophoblast cells are clearly delineated and swollen in their central regions. This blastocyst was recovered from the same mouse as the 'blastocyst' shown on Plate VII, figure 2. x650.

4. A 110 hour blastocyst, less than one half transformed. The blastocoele has collapsed, giving the wrong impression that transformation has proceeded to the inner cell mass (ICM) along the lower border. x427.

PLATE VI



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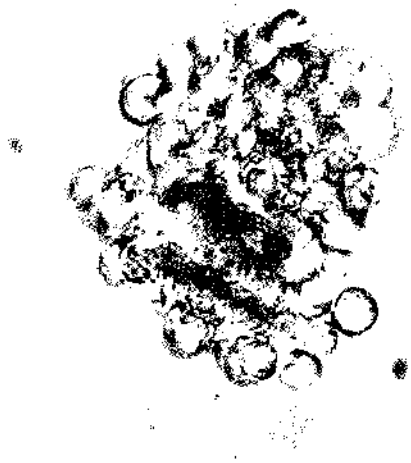

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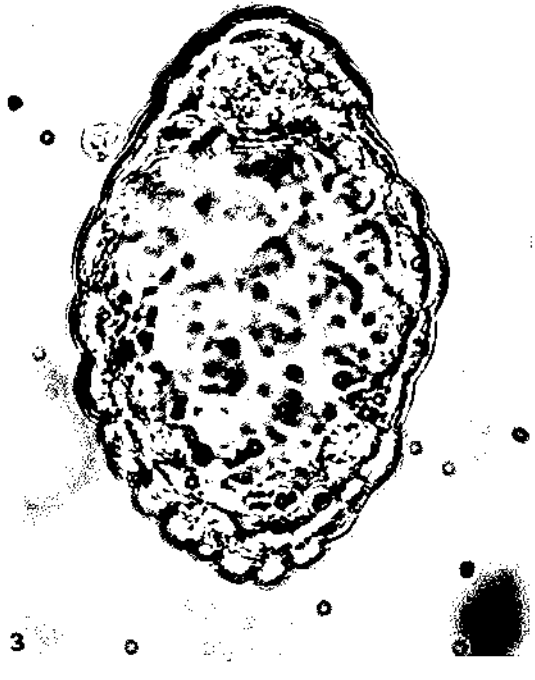

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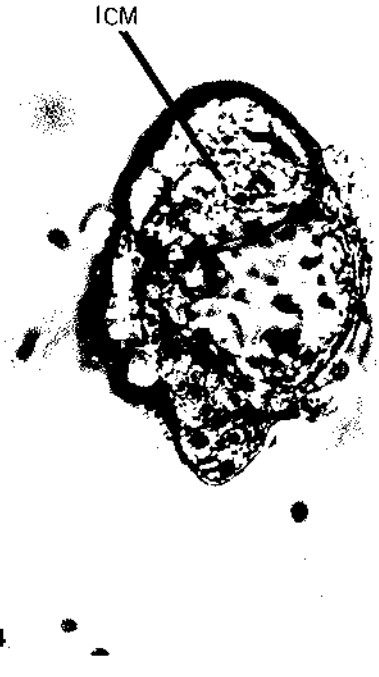

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PLATE VII

1. An unidentified object recovered from a mouse 110 hours pregnant. It is cellular in make up, smaller (diameter and length both 70 u) than normal blastocysts and has a distinct cavity (C). x787.

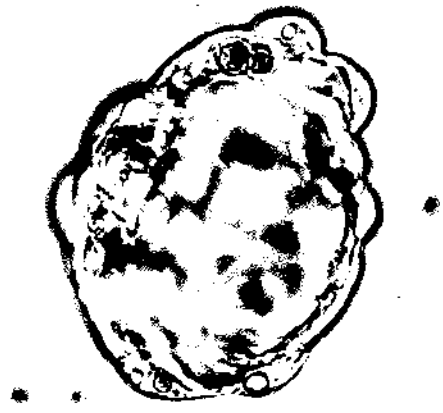
2. An abnormal 'blastocyst' recovered at 12½ days from a mouse ovariectomised at, and given no further treatment from 3½ days. It is small (diameter 50 and length 60 u) and has no obvious inner cell mass, but is distinctly vesicular (C = cavity). x787.

3. An unidentified object recovered from a mouse 102 hours pregnant. The object is cellular and has two distinct cavities (C) and is small (diameter 45, length 90 u). x787.

4. An unidentified object recovered from the same mouse as the object shown in figure 3. The object is cellular with no evidence of cavitation (diameter 40 u, length 130 u). x787.

PLATE VII

1



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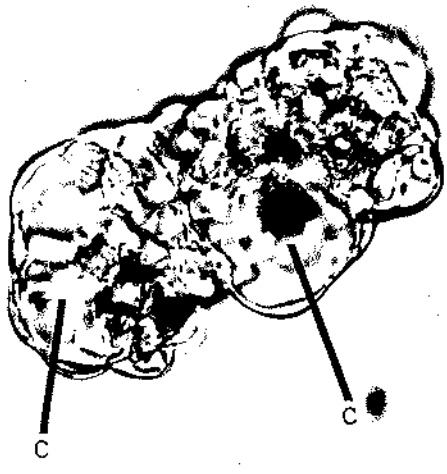

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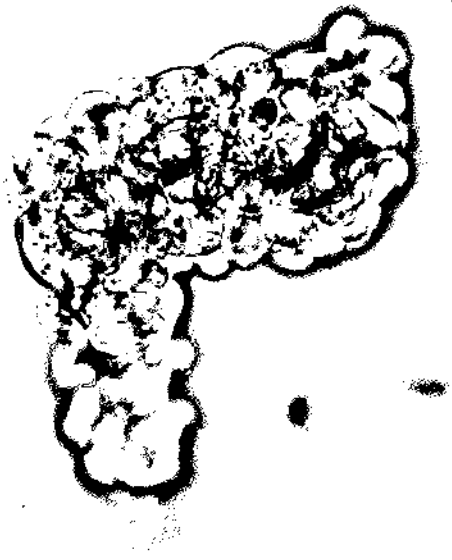


4

PLATE VIII

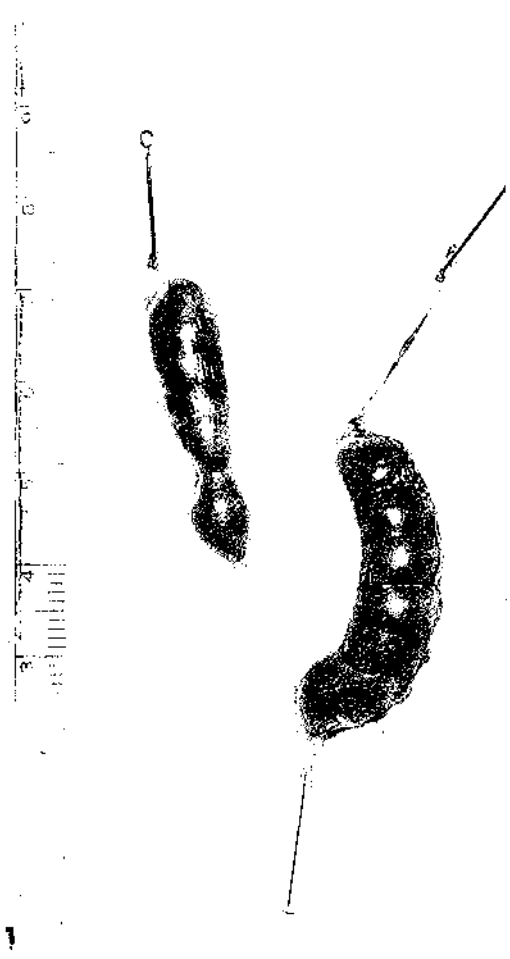
1. A 12½ day uterus from a mouse ovariectomised at 3½ days and given 2 mg of progesterone in vehicle daily. Note the crowded regions of both uterine horns that contain the foetuses and the thin unoccupied regions. Further, the occupied segments were rigid in shape and did not contract on excision and moderate tension failed to straighten the right horn (RH). Of the five foetuses in the left horn (LH) numbers 3 and 4 (numbered from the cervix) were dead and of the seven in the right horn number 2 was dead. The fifth foetus in the left horn and numbers 1 to 6 in the right were orientated so that their long axes were at right angles to, and not as is usual parallel to, the direction of the uterine horns. Partial placental fusion was present and septa between implantation chambers were not well formed.

2. A 12½ day uterus from an intact untreated control mouse. The implantation sites are relatively evenly spaced along the uterine horns. In situ the spaces between the sites are longer and thinner than shown in the figure. Although moderate tension was applied to straighten the horns, the very rapid contraction of the unoccupied regions after excision is largely irreversible.

3(a) A formalin-fixed 12½ day uterus from a mouse ovariectomised on day 3½ and given ½ mg of progesterone in vehicle daily. There were no living embryos in either horn. It is difficult to accurately count the number of implantation sites in such horns as the between site uterine constrictions were often absent. Both horns contained confluent coagulated remains of either partially resorbed and recognizable or unrecognizable conceptual remains. Although this mouse had a closed cervical canal the form of the uterus is similar to those seen in aborting mice.

(b & c) Formalin-fixed $12\frac{1}{2}$ day uteri from mice ovariectomised on day $3\frac{1}{2}$ of pregnancy and given $\frac{1}{4}$ mg of progesterone in vehicle daily. Note that the size of the implantation sites are smaller than in figure 3(a), and also that the spacing is irregular. All embryos in both mice were dead and being resorbed.

The scale shown is in cms.



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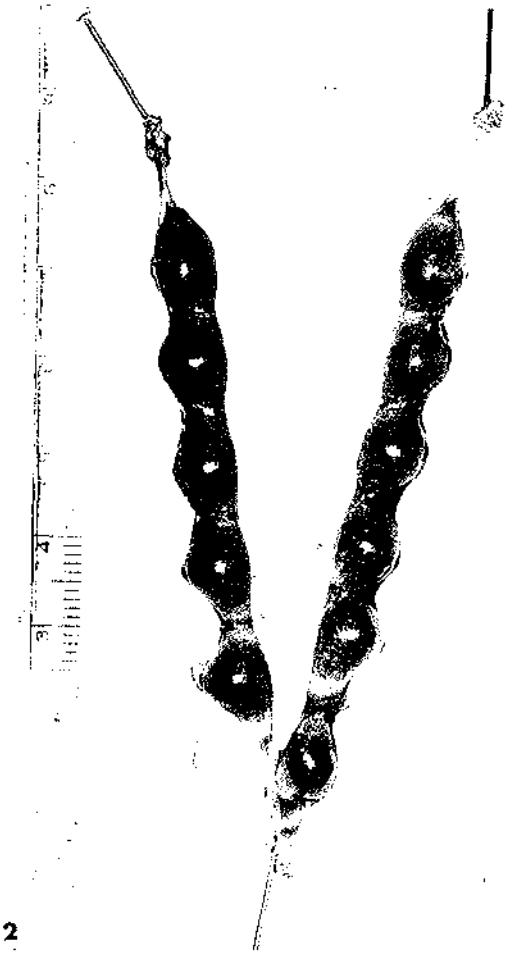

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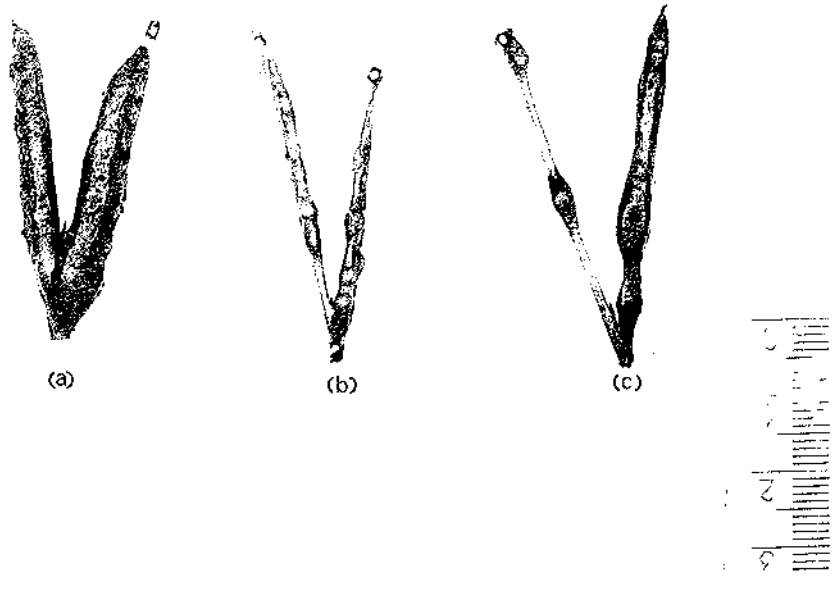

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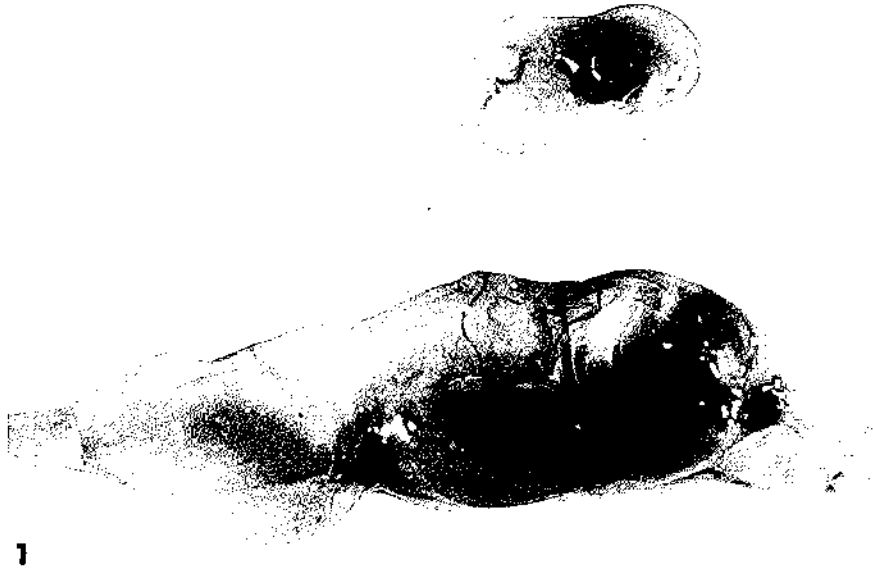
PLATE IX

1. The anterior portion of the left uterine horn and live foetus removed at 12½ days from a mouse ovariectomised at 3¼ days of pregnancy and given about 2 mg of progesterone daily. The foetus occupied the two implantation chambers shown directly beneath it. The placenta of the live foetus is based on the chamber on the extreme right of the figure implicating this as the site of implantation. Note the depression of the foetuses back that resulted from the pressure exerted by the uterine constriction between the two occupied chambers. The foetus weighed 97 mg and had a developmental age of about 12 days.

2. Three foetuses arranged in series recovered from the same uterine horn at 12½ days from a mouse ovariectomised at day 3½ of pregnancy and given about 2 mg of progesterone daily. The smallest foetus occupied the position closest to the cervix and in order from the left to right, weighed 17, 59 and 60 mg and had developmental ages of about 9, 11 and 11 days, respectively. Of note are the mishapen heads of (a) and (b) cf. with (c) and Pl. X. The heads were found to extend from their native chambers into the adjacent chambers and the uterine constrictions normally aligned between foetuses exerted pressure on the foetal heads to cause formation of the annular grooves (arrows) shown.

3. Scale in cms to correspond with figures 1 and 2.



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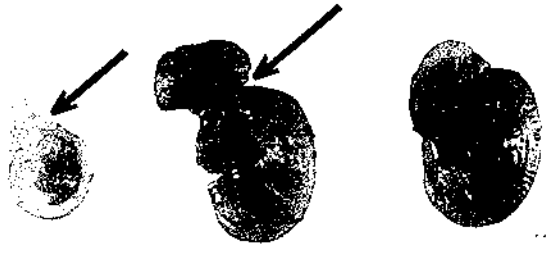

(a) (b) (c)

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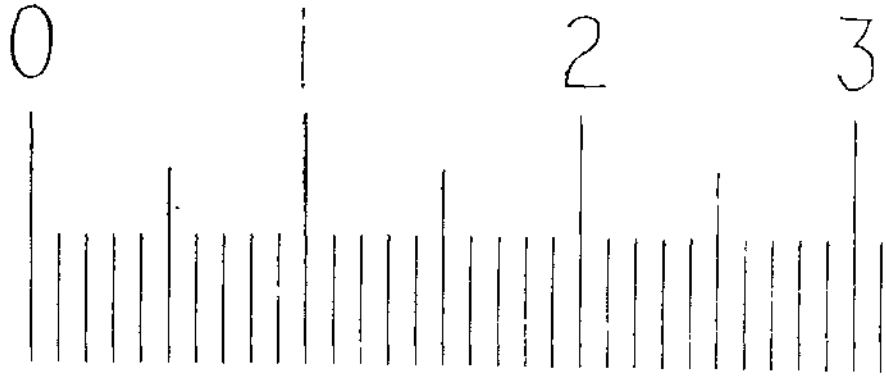
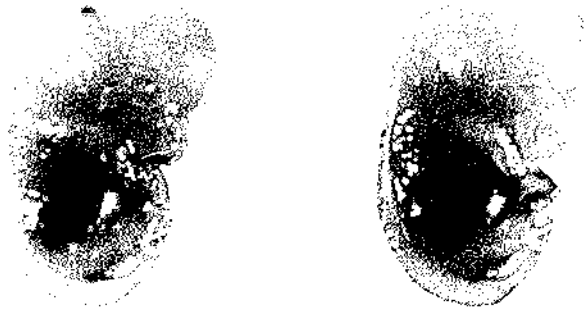


PLATE X

1. Two foetuses recovered from the left uterine horn of a mouse (No. 77) sham-ovariectomised on day $1\frac{1}{2}$ of pregnancy. These foetuses have the external features and crown-rump length measurements characteristic of the day 14 of pregnancy; foetus (a), weighed 171 and (b), 181 mg. These foetuses are about one day older than would be expected if autopsy was at day $12\frac{1}{2}$ of pregnancy (cf. fig 2). This discrepancy resulted from the time of mating in the absence of a vaginal plug being dated one day too late.

2. Adjacent foetuses recovered from a mouse (No. 44) at day $12\frac{1}{2}$ of pregnancy after sham ovariectomy on day $1\frac{1}{2}$. Foetus (a), weighed 89 and (b), 94 mg and both had external features and crown and rump lengths characteristic of about 13 days as did the majority of foetuses recovered from both experiments A and B at $12\frac{1}{2}$ days of pregnancy.

TABLE X



1 (a) (b)

(a) (b)

2

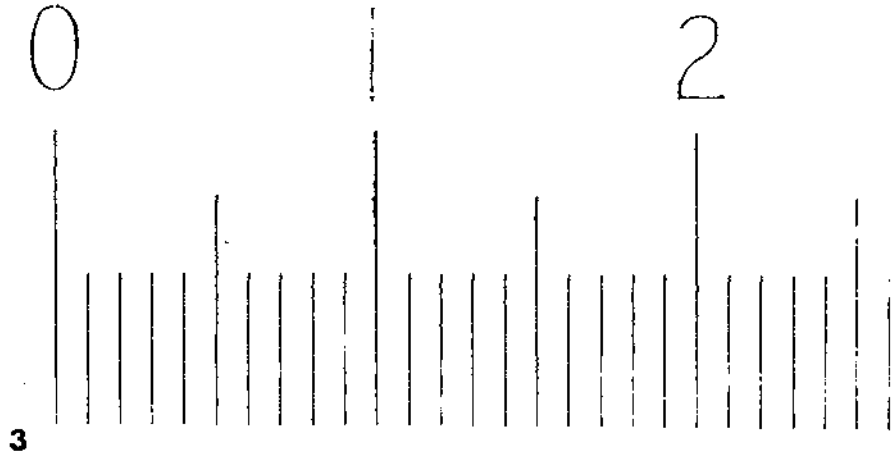


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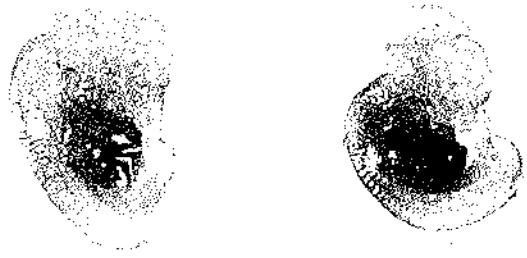
PLATE XI

1. Two foetuses recovered from the left uterine horn at $12\frac{1}{2}$ days from a mouse (No. 22) sham-ovariectomised on day $1\frac{1}{2}$ of pregnancy. There is a difference between the developmental ages of the foetuses of about one day. The body, weights and ages of foetuses (a) and (b) were 77 and 57 mgs and about $12\frac{1}{2}$ and about $11\frac{1}{2}$ days, respectively. As other foetuses in the uterus were aged about $12\frac{1}{2}$ days it is evident that foetus (b) is delayed and not foetus (a) advanced in development.

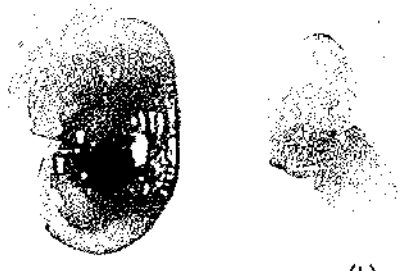
2. Adjacent foetuses recovered from the left uterine horn at $12\frac{1}{2}$ days from a mouse (No. 33) sham ovariectomised on day $1\frac{1}{2}$ of pregnancy. Foetus (a), weighed 95 mgs and had a developmental age of about 13 days, while the retarded foetus (b), weighed 32 mg and was about 11 days old. The six other foetuses in the litter weighed between 76 and 93 mg and were about $12\frac{1}{2}$-13 days old.

3. Scale for figures 1 and 2 in cms.



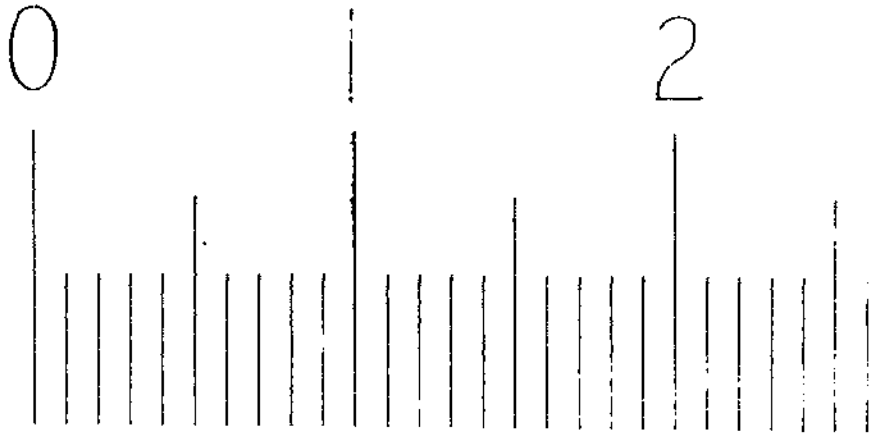
(a) (b)

1



(a) (b)

2



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