EFFECTS OF MELENGESTROL ACETATE ON RABBIT UTERINE PROTEIN SECRETION

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EILEEN FRANCES BOSTWICK

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ABSTRACT

EFFECTS OF MELENGESTROL ACETATE ON RABBIT UTERINE PROTEIN SECRETION

By

Eileen Frances Bostwick

Blood was collected daily via ear vein from 12 mature female New Zealand White rabbits before and during treatment with 10 μg MGA/kg body weight/day dissolved in corn oil or vehicle alone. Serum samples were also taken during subsequent pseudopregnancy induced by HCG injection. Does were sacrificed on Days 6 or 7 of pseudopregnancy and the uterine horns flushed with saline to collect uterine fluid. Steroids were quantified by radioimmunoassay. No differences in serum progesterone or estradiol concentrations were detected between treated and control animals. Concentrations of pregesterone in uterine flushings were similar in each group as were number and types of follicles present on the ovaries at sacrifice.

Measurement of blastokinin concentrations in uterine flushings of 54 does on Days 1-9 of pseudopregnancy revealed a delayed secretion pattern in MGA-treated animals versus controls. In does receiving corn oil alone blastokinin increased from 0.004 mg/ml on Day 1 to 1.5 mg/ml on Day 3, plateaued at 1.94-2.39 mg/ml from Days 4 to 8, and decreased on Day 9 to 0.29 mg/ml. The concentration of blastokinin in MGA-injected does was 0.1 mg/ml on Day 3, increased to

values comparable to controls on Days 4-7, but began to decline on Day 8. These data suggested a possible refractory period to progestin administration.

Blastokinin in uterine fluid was also assayed during MGA treatment. Control does maintained concentrations of 0.0004 mg/ml throughout the corn oil injection period. However, does receiving MGA exhibited a blastokinin secretion pattern of lesser magnitude, but highly correlated ($r = 0.89$) with the pattern of pseudopregnancy. No differences were detected in the progesterone concentrations of uterine flushings from MGA-treated rabbits during post-injection or pseudopregnancy.

Seventy-two hour embryos transferred to pseudopregnant recipient does pretreated with MGA failed to implant when the transfer media was supplemented with rabbit albumin; addition of 1.5 mg blastokinin to the media resulted in a 4.3% implantation rate on Day 11 of pseudopregnancy. The addition of blastokinin also enhanced implantation in corn oil injected does when compared to albumin (32.6% and 23.1%, respectively). The concentration of blastokinin in uterine fluid on Day 3 of pregnancy therefore appears to affect implantation rates in the rabbit.

Examination of frozen lyophilized uterine tissue from MGA and corn oil injected rabbits via scanning electron microscopy revealed greater secretory activity, as evidenced by the presence of glandular epithelium and secretory globules, in control Day 3 pseudopregnant does compared to does given MGA.

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INTRODUCTION

The maternal uterus provides a receptive environment for the developing embryo. Prior to implantation the fluids of the reproductive tract are responsible for both the protection and nurturing of the fertilized ovum. Studies have shown that this environment can be influenced by the maternal hormonal state. Specific proteins in this uterine fluid, formed by both selective transudation from serum and active secretion by the uterine epithelium, have been demonstrated in a number of species. In the rabbit, blastokinin (or uteroglobin) is present in detectable quantities only during early pregnancy, up to and including the period of implantation. However, to date there is no proof of a unique function for this protein despite its comprising an average of 50% of the total protein in uterine fluid immediately before nidation.

Melengestrol acetate (MGA) administration has been shown to cause changes in reproductive efficiency in a variety of species, possibly due to aberrations in the uterine environment. Development of a radioimmunoassay for measurement of blastokinin provided a means for detecting smaller changes of this protein in uterine fluid compared to previous efforts with electrophoresis and immunofluorescence. In addition use of scanning electron microscopy allows alterations in secretion patterns to be correlated with changes in uterine epithelial surface ultrastructure.

In particular, the objectives of this work were: (1) to quantify serum estradiol and progesterone concentrations in rabbits during treatment with MGA and during subsequent pseudopregnancy, (2) to describe the blastokinin secretion pattern in rabbits injected with MGA or corn oil, both during treatment and pseudopregnancy, (3) to determine if administration of exogenous blastokinin could enhance embryo implantation under conditions which are known to cause poor fertility, and (4) to correlate possible differences in serum hormones and uterine blastokinin concentrations with changes observed at the ultrastructure level.

REVIEW OF THE LITERATURE

Estrus Synchronization in the Bovine

Melengestrol acetate (MGA), a synthetic progestin, offers a potentially simple and effective method for controlling the bovine estrous cycle. However conception rates at the synchronized estrus in MGA-treated cows are lower than in control cows (Henricks et al., 1973; Roche, 1974). Aberrations in serum hormone concentrations (Randel et al., 1972; Wetteman and Hafs, 1973), abnormalities in uterine histology (Smallwood and Sorenson, 1969; Wordinger et al., 1971), and alterations in ovarian and oviducal function (Hill et al., 1971) are factors implicated in the lowered conception rates.

Henricks et al. (1973) observed low blood serum progesterone concentrations in MGA-treated beef heifers for 11 to 13 days prior to estrus compared to only a two day duration of low progesterone in untreated controls. However, in dairy heifers, both Britt and Ulberg (1972) and Randal et al. (1972) found progesterone concentrations almost double those observed in control animals for several days preceding onset of estrus. Dobson and coworkers (1973) reported that MGA-treated animals had circulating estrogen concentrations similar to controls during the first estrous cycle following treatment, but progesterone concentrations were lower. Wetteman and Hafs (1973) observed similar progesterone values, but 3-fold greater estradiol concentrations at proestrus and estrus in MGA-treated animals.

Conflicting reports on blood luteinizing hormone (LH) values are also reported (Randel et al., 1972; Wetteman and Hafs, 1973).

Differences in serum hormone concentrations may be due to length of treatment, mode of administration, and dosage. Roche (1974) found that heifers treated with progesterone implants for 9-12 days did not have different fertility rates from controls, but progesterone implants for 12-18 days caused lowered fertility and altered estrogen concentrations.

Measurements of ovarian and uterine activity indicate that individual animal responses to the administration of exogenous progestins may be responsible for the differences reported in circulating hormone values. Zimbelman and Smith (1966) administered 0.4 mg of MGA daily for 18-32 days in heifers and noted an increase in the size and incidence of palpable ovarian follicles which tended to persist for some time. Hill et al. (1971) achieved more precise synchronization of estrus in beef heifers by starting treatment on Day 4 of an estrous cycle. They found fewer Graffian follicles and more very large follicles in MGA-treated animals, and also observed that many heifers receiving the progestin had prolonged proestrus.

Although ovulation was completed inhibited, Johnson and Ulberg (1965) found that progesterone (50 mg, IM, daily for 14 days), acetoxyprogesterone administered orally, or progesterone plus estrogen treatment did not alter various parameters used as indicators of hormonal status. These included normal fluctuations in mucus viscosity or abundance, cervical mucus pattern formations, rectal temperatures, or follicle formation during treatment or at the post-treatment estrus when compared to control estrous cycles. Hill and

coworkers measured chlorine to protein ratio in cervical mucus of heifers beginning MGA treatment on Day 4 of an estrous cycle and compared these findings with those made in non-treated animals. Peak ratios coincided with estrus in control heifers but occurred a day or two before estrus in MGA-treated animals.

The effects of MGA treatment on the endometrium have also been studied. Wordinger et al. (1970, 1971) reported that glycogen content in surface epithelial cells of MGA-treated animals was lower than that in controls. The lamina propria and connective tissue stroma appeared less dense and less edematous after MGA, but endometrial and glandular epithelial cell heights were greater in treated animals. This confirmed work by Smallwood and Sorenson (1969) who found that uterine gland epithelium height increased to Day 4 and then decreased on Day 5 following treatment with medroxyprogesterone acetate (MAP). Hill et al. (1971) reported that more uncleaved ova were recovered from MGA-treated animals than from controls suggesting that a possible cause for reduced fertility was either more rapid transport of eggs through the reproductive tract or unfavorable conditions for fertilization.

Treatment of animals with progestins has continued to receive attention for its beneficial effects. Britt et al. (1974) reported that MGA treatment in early postpartum cows resulted in a shorter interval to first recorded estrus, fewer inseminations per conception and fewer days open for those cows conceiving.

Some Effects of Progestins in the Rabbit

Nutting and Mares (1970) reported two distinct periods of lowered fertility in progesterone treated rabbits. The first occurred

about the third day of daily progesterone injections and was of short duration, the other lasted from the seventh to the eleventh days of treatment. Inhibition of fertilization was dose dependent between 0.05 and 4 mg/day (ED_{50} was 0.4 mg). They implicated accelerated movement of ova through the oviduct, inhibition of sperm transport, and altered contractility patterns of the myometrium as probable causes of infertility. Chang (1966, 1967) and others also correlated rapid egg transport with reduced fertility. Administration of orally active medroxyprogesterone acetate or subcutaneous injections of progesterone (2 mg/day) for 3 days before ovulation induced complete degeneration of the eggs (Chang, 1966). In another experiment Chang (1967) found that the transportation of eggs from the fallopian tube to the uterus was significantly hastened by treatment of rabbits with 1-4 mg of progesterone or its derivatives (medroxyprogesterone acetate, chlormadinone acetate). He concluded that ova which reached the uterus too quickly either degenerated or were expelled from the uterus before they could be fertilized. When Pauerstein et al. (1974) injected rabbits with 2.5 mg of progesterone (IM) on the day of ovulation and on each of the 2 preceding days, they reported that progesterone appeared to act at the proximal isthmus as well as at the ampullary-isthmic junction to hasten ovum transport.

It is also possible that sperm transport and capacitation are altered in progestin treated rabbits. Chang and Hunt (1970) found that rabbits injected with progesterone (2 mg/day for 3 days) had fewer sperm in the uterus 1 day after insemination (does were inseminated 2 hours after the last injection) and fewer sperm were recovered from the fallopian tubes than in untreated controls. They concluded

that the inhibitory effect of such treatment is therefore on the entire female tract. Subcontraceptive doses of progesterone ($\leq$1.0 mg/day for 3 days) did not affect follicular development, number of ovulations, or fertilization rate (Allen and Foote, 1973). Implantation rate was decreased, apparently due to suppression of the zygote's development to blastocysts, but implanted embryos developed normally.

It would appear that exogenous progestins have their main effect on the rabbit uterus. Hamner et al. (1968) could detect no change in the specific gravity or viscosity of rabbit oviducal fluids when animals were treated with progesterone (1 mg/kg) or estrogen (5 μg/kg) alone or in combination. Sperm capacitation occurred in the progesterone dominated oviduct, but capacitation in the uterus required some estrogen in addition to progesterone (Bedford, 1969).

In the rabbit, progesterone stimulates endometrial proliferation. Lee and Dukelow (1972) reported that short preovulatory release of 20α-hydroxypregn-en-4-one immediately after mating may be important in the stimulation of endometrial proliferation observed during early pregnancy. Uterine responsiveness to progesterone, however, can apparently be altered by the introduction of progestins when endometrial growth is most responsive to estrogen (the estrogen priming phase). Progestin treatment during the proliferative phase caused an early regression in the ability of the rabbit uterus to respond to additional progesterone.

Melengestrol acetate (MGA), the synthetic progestin which has been used for synchronization of estrus in cattle, also causes reduced fertility in rabbits. Pritchard et al. (1970) injected does with 70 μg/kg of MGA dissolved in corn oil for 12 days and then

inseminated the rabbits vaginally or in the upper uterus. They also inseminated rabbits 1 or 3 days after treatment with 10 or 50 μg/kg/day of MGA. Their results suggested an inhibition of sperm transport, but little effect on capacitation as uterine inseminations were not significantly affected by the treatment. MGA did not inhibit follicular maturation as indicated by similar numbers of ovulation points on the ovaries of treated and control animals, but ova recovery was reduced with MGA injections. To examine the possibility that an altered uterine environment was responsible for the reduction in fertility in MGA-treated rabbits, Britt and co-workers (1973) transferred ova into rabbits given 10 μg/kg MGA for 10 days. Does were made pseudopregnant with an injection of human chorionic gonadotropin (HCG) 3 days after the last MGA injection and embryos from donor rabbits were transferred at 48 or 72 hours after HCG. Most MGA-treated animals were not able to maintain pregnancy. Altering the chronological relationship between donors and recipients resulted in pregnancy rates similar to controls when 4 day blastocysts were placed in Day 5 recipients pre-treated with MGA. The authors suggested that MGA delayed the onset of the time when the uterus was most receptive to transferred ova.

Ovum Transfer and the Study of Uterine Environment in Rabbits

Preparation of the uterus for pregnancy requires an intricate and accurate interplay of ovarian hormones in terms of sequence, ratio, and absolute amounts (Black, 1974). The influence of progesterone is apparently critical around the time of implantation in rabbits. While estrogen is not essential at this time, estrogen

priming does enhance the progestational response of the uterus (Hisaw et al., 1937). Alteration of the endometrium could therefore be a factor in the infertility action of exogenous progestins. Pseudopregnant rabbits are capable of maintaining pregnancy when ova are transferred to the uterus from a donor rabbit by the sixth day of pseudopregnancy. Pseudopregnancy can be induced in does by administration of exogenous gonadotropins and allows study of implantation rates under various conditions using a known number of control or pre-treated embryos. This is an important advantage as conception rates and litter size vary from animal to animal with time of insemination, natural versus artificial insemination, and pre- and post-implantation losses. Seasonal differences in pregnancy rates in rabbits have been reported by O'Ferrall, 1973. Rabbits artificially inseminated in March had higher conception rates than those inseminated in either January or October, and animals giving birth in January had greater prenatal losses than those which gave birth in June.

Adams (1960) investigated the effects of transferring varying numbers of ova to recipient rabbits. He transferred 5, 10, 15, 20, 25, or 30+ eggs to one or both horns of 72 hour pseudopregnant recipients. With increasing numbers, there were more implantations, but the proportion of embryos surviving decreased. Adams concluded that the cause of mortality was primarily related to local environmental factors (e.g., inadequate uterine secretion, lack of placental development).

Noyes and Dickman (1960a,b) studied the relationship between age of the ova and the stage of endometrial development in rats. They found that when ova first contact the uterine environment, chance

for survival depends on the absolute stage of maturation of the uterus and ova, and the particular relationship between their stages of development. Ova one day younger than the uterus underwent morphological changes and did not stimulate deciduomata or implant. One day "older" eggs, on the other hand, waited for the uterus to reach the proper stage so that implantation could occur at the normal time.

In the rabbit, egg development fails even earlier as the degree of asynchrony between ovum and uterus increases. Adams (1971) transferred 60 hour morulae to recipients up to 8 1/2 days of pseudopregnancy in advance of the donor. Ovum development was arrested and degenerative morphological changes took place within 2 days of transfer. Adams suggested that degenerative changes were due to an alteration in the uterine environment. This hostile reaction of the advanced progestational uterus was most fully developed by Days 9-12.

Estrogen injections on Days 1 and 2 of pregnancy cause a 2 to 5 day delay in the protein secretion pattern normally observed in the pregnant rabbit (Beier et al., 1972). Eggs transferred to uteri of estrogen-treated does of the same age (i.e., Day 4 ova to a Day 4 uterus) were retarded. Although early blastocyst stages were observed, implantation did not occur. Normal implantation occurred only when blastocysts of corresponding physiological rather than similar chronological ages were used for the transfer. The best results were achieved when Day 4 ova were transferred to Day 8 uteri. This suggested that the 2-5 day delay in protein secretion was responsible for differences in the ability of developing embryos to implant.

Steroid Hormones in the Rabbit

Although the rabbit is a reflex ovulator, there is some evidence of cyclic reproductive phenomena (Hamilton, 1951). Changes in vaginal cell type determined by microscopic examination of vaginal smears recurred at 4-6 day intervals and increased blood estrogen concentrations were correlated with an abundance of cornified cells in the smear.

Younglai (1972) reported a rapid increase in progesterone and estradiol concentrations in follicular fluid at the time of mating in rabbits. However, both estradiol and progesterone levels had decreased below premating levels by 6 hours post coitus. Likewise, rabbits administered human chorionic gonadotropin (HCG) to cause ovulation had elevated progesterone in ovarian venous blood by 1 hour post injection. Peak concentrations in mated animals were observed 1.5-4 hours post coitus (Shaikh, 1972).

It is well established that castration of pregnant rabbits causes abortion, but that pregnancy can be maintained in castrate does with exogenous progesterone. This evidence would suggest that progesterone is an adequate progestational agent in the rabbit (Mikhal, 1961). The two days following implantation are very critical for embryonic development in the rabbit, so inadequate progesterone prior to this time may result in a delay in implantation until conditions are more ideal (Hafez and Pincus, 1956). Peripheral plasma concentrations of progesterone rise from about 1.5 ng/ml before breeding to a peak level of more than 13 ng/ml on Day 18 of pregnancy (Baldwin and Stabenfeldt, 1974). Hilliard and coworkers (1973) measured progesterone secretion into the ovarian venous blood of pregnant rabbits and

found that secretion rate increased dramatically after implantation and peaked on or near Day 18 of pregnancy.

In the hysterectomized rabbit, pre-mating concentrations of progesterone as measured by radioimmunoassay were less than 1 ng/ml; these values increased until Day 9, when maximum concentration was 12 ng/ml. Serum progesterone decreased during Days 12-15 after mating (Hilliard et al., 1974).

Estrone values remain constant throughout gestation in the rabbit (Challis, 1973), while estradiol concentrations increase slightly during early pregnancy. The ratio of progesterone to estradiol, however, nearly doubled from Day 3 to Day 6 of gestation. The follicle is apparently the sole source of ovarian estradiol in the rabbit since Eaton and Hilliard (1971) were unable to stimulate estradiol output with injections of 0.5 IU of adrenocorticotropic hormone (ACTH).

A significant amount of testosterone is also secreted by the ovary (Younglai, 1973). Concentrations of this steroid increased 5-fold by 1 1/2 hours after breeding, but decreased to premating levels by 3 hours.

The rapid changes in steroid concentrations following mating are under control of pituitary gonadotropins. The mating stimulus causes a rapid decline in pituitary luteinizing hormone (LH) concentration in does (Desjardins et al., 1967). This sudden release of gonadotropin is reflected by an increase in ovarian progestin output, until just prior to ovulation when there is a sudden decrease in circulating LH to below precoital levels (Hilliard et al., 1964). In the rabbit follicle stimulating hormone (FSH) does not respond to the

same releasing mechanism as LH and apparently does not have a role in ovulation (Dufy-Barbe et al., 1973; Younglai, 1974). In greater than physiological doses, however, LH is luteolytic in the rabbit (Hilliard, 1973). Pseudopregnancy in the rabbit, then, ends when progesterone output from the corpora lutea can no longer check pituitary LH discharge (Hilliard et al., 1968).

Oviducal and Uterine Fluid: Identification of Blastokinin

During the interval between fertilization and implantation the ovum's environment consists of the fluids of the maternal reproductive tract. Oviducal fluid is secreted continuously throughout gestation in the rabbit, but the rate of secretion is markedly reduced 3 days after mating (Riddick, 1975). It is similar to blood plasma in sodium, chloride, and bicarbonate concentration, but contains significantly greater amounts of potassium. Hamner (1970) reported that the sodium concentration of oviducal fluid decreased after the first 3 days of pregnancy and calcium content increased following ovulation. The pH of oviducal secretions decreased from 7.75 during estrus to 7.43 with pregnancy. The pH of both uterine secretions and blood increased slightly from ovulation to Day 6 of gestation. Day 6 blastocyst fluid pH of 7.62 differed significantly from uterine secretions (McLachlan, 1970).

Uterine fluid is a source of free amino acids for the developing embryo. Jaszczak (1972) established that a definite concentration gradient existed between the uterine fluid and blastocoelic fluid. In the rabbit uterine fluid concentrations of free amino acids

reach a maximum immediately before implantation possibly under the control of ovarian steroids.

Biochemical studies have also identified nine specific proteins in rabbit endometrial secretion (Beier, 1974). Before Day 6 of pregnancy, albumin and transferrin are the major uterine luminal proteins. During early pregnancy selective transport of serum proteins into the uterine lumen combined with the secretory activity of oviducal and endometrial tissues produce certain proteins peculiar to this reproductive state. The predominant fraction in the post-albumin region is the pregnancy-specific protein uteroglobin (Beier, 1968). In the uterus pre- and post-albumin proteins and uteroglobin are strictly controlled by progesterone, even estrogen priming is apparently not required for onset of secretion of these proteins (Beier, 1974). Oviducal and uterine enzymes such as β-glycoprotein, a uterine protease which reaches peak activity during attachment and invasion of the trophoblast, are also regulated largely by progesterone.

Protein secretion by the uterine epithelium begins within the first three days post coitus as the embryos are migrating through the fallopian tubes. Under the increasing influence of progesterone, the endometrium increases secretion until nidation of the blastocysts (Beier, 1974). Throughout pregnancy the absolute protein content of the uterine secretion climbs continuously.

In 1967, Krishnan and Daniel reported partial purification of a specific protein fraction, isolated from rabbit uterine flushings, which could apparently induce and regulate blastocyst development of rabbit embryos since it was effective in promoting cavitation and expansion of blastocysts <u>in vitro</u>. They christened this protein

"blastokinin." Analysis of its composition revealed a lack of sialic acid, a high number of sulphur-containing amino acids, and few aromatic amino acids, as is generally the case in glycoproteins. Approximately 74% of the total weight was amino acid and the remainder largely a composite of hexoses and hexosamines (Krishnan and Daniel, 1968).

In an independent report, Beier identified a globular protein having a molecular weight of 30,000 which he called "uteroglobin" (1968). Investigators now agree that these two proteins are one and the same. Murray and McGaughey (1972; and Yarus, 1972), reported the existence of nonidentical subunits when this protein was treated with 2-mercaptoethanol. The minimal polypeptide weight of those subunits was estimated at 14,166.

Early studies by Kirchner (1972) using immunofluorescence, indicated that blastokinin was first detected 16 hours post coitus; 4 hours after ovulation. Synthesis apparently began before mating and was correlated with the age of the epithelial cells. Most parts of the endometrium stopped secreting by 5 days after mating. In attempting to quantify blastokinin, Johnson and coworkers (1972a) achieved only poor immunogenicity in a heterologous assay system, probably caused by the protein's low molecular weight. Purification and storage of the protein may also have altered its antigenicity. However, by using immunofluorescence, Johnson (1972b) was able to study the distribution of blastokinin in the uterus of Day 5 pregnant or pseudopregnant rabbits. Although low concentrations were found in the vagina, the amount of protein throughout the uterine cornua was uniform. Johnson found evidence of surface secretion on the villous epithelia and in the crypts. Blastokinin was also detected in the

crypt cytoplasm during estrus, but this might represent the reaction of degraded subunits rather than synthesis.

In 1974, Mayol and Longenecker reported the development of a radioimmunoassay for blastokinin using a guinea pig antiserum. This assay was capable of detecting as little as 0.3 ng of protein. Using this system they reported maximum concentrations of blastokinin on Day 6 with gradually declining, but still detectable levels to Day 12. There were approximately 160 ng/ml of blastokinin present in the uterine fluid at the time of insemination.

Hamana et al. (1970) found that this protein was most highly concentrated in blastocoelic fluid between 6 1/2 and 7 days post coitus but was absent after 8 days. Because histotrophic nutrition of the rabbit blastocyst ends on the eighth day of gestation, they suggested that blastokinin's physiological action was restricted to embryonic development between the shedding of the zona pelucida and implantation. Mayol and Longenecker (1974) also found small amounts of blastokinin in Day 6 blastocysts which could not be accounted for on the basis of non-specific contamination.

Daniel (1972) examined the possibility that a single region began secretion of blastokinin followed by a progressive expansion of secretory areas. Instead, using gel electrophoresis, he found that before implantation the uterus produced blastokinin throughout its length. However, with implantation there was a progressive tendency for the production of all uterine proteins to become localized at the sites of nidation and placentation. This is likely due to local steroid stimulus caused by estrogen production by the embryo.

Murray and Whitson (1974) demonstrated that actinomycin D and puromycin blocked or repressed the production of blastokinin by the epithelial cells of the endometrium and uterine glands. This indicated that continued synthesis or transcription of RNA is required for blastokinin production, especially for the high secretion rate on Day 5 of pregnancy or pseudopregnancy. Puromycin also tended to depress total protein production by the rabbit uterus.

Using poly(A)-rich RNA as a marker for the isolation and characterization of mRNA, Levey and Daniel (1976) evaluated endometrial RNA as a potential source of specific blastokinin mRNA. The proportion of newly synthesized poly(A)-rich RNA in uterine polysomal RNA increased from estrus through progressive days of preimplantation pregnancy. Maximal synthesis was observed on days 3-4 after mating and it remained constant through Day 6. Moreover, polysomal and poly(A)-rich RNA extracted from Day 4 endometrium could evoke synthesis of a blastokinin-like protein when introduced into the uterus of long-term castrate rabbits. These results would be consistent with expression of a specific blastokinin gene.

It still remains to be proven that blastokinin is essential for growth and development of the rabbit blastocyst and that it performs some specific function. Blastocysts transferred to progesterone-treated (3.0 mg/kg body weight/day) castrate recipients in which blastokinin was the dominant feature of the uterine environment, were capable of growth and differentiation (Arthur and Daniel, 1972). Daniel (1970) and El-Banna and Daniel (1972a,b) investigated the effects of uterine protein fractions on embryonic growth and development. When the protein components of uterine fluid were separated

by gel filtration (Sephadex G-200), fraction V (blastokinin) stimulated uridine and amino acid uptake, and growth of morulae and blastocysts *in vitro*. Embryos grew best and incorporated greater amounts of amino acids and uridine when the medium contained both progesterone and uterine fluid components. They concluded that progesterone was probably essential to the early embryo when provided either in complex with uterine proteins or bound to proteins as carriers. In contrast, Beier and Maurer (1975) cultured rabbit embryos from the 2- and 4-cell stages to the expanded blastocyst without the addition of uterine specific proteins. However, by comparison, development *in vitro* was slower than similar *in vivo* stages. Their results also indicated that blastocysts, although capable of synthesizing rabbit albumin *in vitro*, could not synthesize blastokinin. The blastokinin previously reported (Beier, 1968; Hamana, 1970) in blastocyst fluid must therefore originate from maternal proteins synthesized in the uterus. Indeed, endometrial cells from mature estrous rabbits grown in cell culture are capable of synthesizing blastokinin following addition of progesterone (Whitson and Murray, 1974). Monolayer mixed cultures of uterine endometrium proliferated under the influence of insulin, but produced very little, if any, blastokinin (immunoelectrophoresis) after 5 days of culture. Addition of 10 μg/ml progesterone produced a distinct blastokinin band after 3 days in culture. Treatment with 0.1 μg/ml estradiol-17β had an inhibitory effect by 48 hours.

Blastokinin and Steroid Hormone Interactions

In 1974, Goswami and Feigelson reported a low molecular weight protein from uterine and oviducal fluid which gave a cone-shaped

profile upon polyacrylamide gel electrophoresis followed by a modified Amido Black stain. This protein, apparently identical to blastokinin, could be induced in ovariectomized rabbits by progesterone and estradiol. Progesterone administration caused the "cone-protein" to appear in uterine fluid, while estrogen controlled its appearance in the oviduct.

Uterine blastokinin is probably under dual hormonal control. Supression of its progesterone-induced synthesis by estradiol is consistent with both the pattern of steroid secretion, and the time course of blastokinin appearance during early pregnancy. Another possibility is that estrogen may increase the degradation of blastokinin as plasma estradiol begins to rise at about the time of the protein's disappearance (Bullock and Willen, 1974). Although Arthur and Daniel (1972) found the kinetics of blastokinin appearance and disappearance in uterine fluid to be similar for pregnant and progesterone-treated does, Barfield et al. (1976) reported no marked decrease in blastokinin concentration for ovariectomized progesterone-treated rabbits. Estrogen (1 μg/day) in addition to progesterone caused a marked reduction in protein secretion but not until days 16 to 17. They suggested a blastokinin-estrogen complex could act as a negative control on blastokinin concentrations.

Rahman et al. (1975) found that adrenocorticotropic hormone (ACTH) was also capable of inducing blastokinin secretion in the rabbit. They suggested that the rabbit adrenal may be an important source of progesterone secretion. It has been established that maintenance of pregnancy requires the ovaries; the adrenals alone cannot prevent abortion in the ovariectomized rabbit. However, the

role of adrenal progesterone during the preimplantation period in intact does is still unknown.

Quantities of steroids capable of inducing blastokinin also cause changes in uterine histology corresponding to a McPhail index (McPhail, 1934) of 4 (distention of the glands with a lacelike appearance of the emdometrium). Two hundred μg per day of progesterone was generally adequate to induce full secretory changes in the uterus, but large priming doses of estrogen reduced progesterone induced endometrial changes (Giannina and Meli, 1976). Studies with monolayer culture of rabbit endometrium also suggest that progressive differentiation is mediated by progesterone (Whitson and Murray, 1974).

Arthur and coworkers (1972) proposed a model where progesterone was transferred from a serum binding protein to a carrier in the uterus to explain the time-course relationship between blastokinin and progesterone. Evidence of a rabbit serum protein which bound progesterone and differed in size and composition from blastokinin had already been reported (Chader and Westphal, 1968). Urzua et al. (1970) had also demonstrated that Day 5 uterine fluid had a greater binding affinity for progesterone than blood serum and that when uterine fluid was incubated with radioactive progesterone prior to chromatography, progesterone was eluted from the separatory column with the blastokinin peak. In affinity binding studies of their own, Arthur et al. (1972) found evidence of binding of estradiol and progesterone in the region of blastokinin production in Day 5 pregnant animals. Binding by one steroid could be inhibited by increasing the concentration of the other. In particular, small amounts of estradiol

could result in major changes in bound progesterone. Blastokinin would not bind testosterone.

Rahman et al. (1975) hoped to develop an assay using progesterone binding to blastokinin to quantify blastokinin content in uterine fluids. However, using Sephadex G-25 separation and equilibrium dialysis, they reported negative results for binding. The authors concluded that progesterone binding to blastokinin was a low-affinity, high-capacity type similar to that of albumin and was probably not of any greater physiologic significance than progesterone binding to albumin. Beato and Baier (1975), however, were able to show that 1 molecule of progesterone binds with a blastokinin molecule and that blastokinin is composed of 2 subunits of similar size. Using the technique of equilibrium dialysis the authors observed a saturable binding of progesterone in uterine fluid which was dependent on the presence of millimolar concentrations of dithioerythritol in the incubation medium. In immunological experiments 100 µl of antisera prepared in guinea pigs against purified blastokinin almost completely inhibited the progesterone binding activity of both uterine fluid and a highly purified blastokinin preparation. The effect of dithioerythritol on progesterone binding suggested that the cysteine residues of the blastokinin molecule are somehow involved in the steroid binding, and might account for conflicting results in the literature.

In 1976, Muechler and coworkers isolated a heat labile progesterone receptor in the cytosol of the rabbit oviduct and uterus made up of 7-8s and 4-5s components. The 7s component was more prevalent in uterine cytosol while the 4s was probably nonspecific. They detected a decrease in binding in the oviduct 3-12 hours post

coitus which coincides with a fall in ovarian estradiol. A steady decline in uterine binding was observed, whereas the lowest binding in the reproductive tract tissues corresponds to the peak blastokinin concentration as determined by Mayol and Longenecker (1974).

Further work is needed to determine if the observed steroid binding by blastokinin is of physiologic significance. As the presence of steroid receptors has been shown in the serum and uterus, it would not be unreasonable for blastokinin to function as a high affinity carrier between the maternal organism and the embryo, or as a progesterone receptor once inside the blastocyst. Indeed these transport functions have already been suggested in the literature and are under further investigation. As Beato and Baier (1975) have pointed out, a most interesting situation in endocrine regulation may exist: a steroid hormone (progesterone) induces the synthesis and secretion of a protein (blastokinin) which in turn binds the steroid and mediates its action in the blastocyst.

MATERIALS AND METHODS

Experiment I

These studies were undertaken to describe changes in hormone concentrations in blood and uterine fluid and changes in uterine protein secretion in rabbits treated with melengestrol acetate. Twelve mature virgin New Zealand White does, isolated a minimum of 3 weeks, maintained on a 12:12 light: dark photoperiod, and given feed and water ad libitum were injected subcutaneously daily for 10 days with either 10 μg/kg melengestrol acetate (MGA)[1] dissolved in corn oil or vehicle alone. Half the does assigned to receive each treatment were allotted to one of two groups and rabbits in each group were bled via an ear vein on alternate days beginning twelve days prior to first MGA or corn oil injection. On the third day following the last injection, all animals received 100 I.U. human chorionic gonadotropin (HCG) (Follutein, E. R. Squibb and Sons, Inc.) intravenously to induce pseudopregnancy. Does were killed by cervical dislocation on the sixth or seventh day of pseudopregnancy. Uteri were excised, immediately trimmed of fat, rinsed with tap water and blotted to remove all traces of blood. Each uterine cornus was flushed with 3 ml of 0.33 M sodium chloride. Fluid from each pair of horns was pooled, centrifuged (700 x g) to remove cellular debris, and frozen at -20°C until

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[1] 6-dehydro-16-methylene-6-methyl-17α-acetoxyprogesterone furnished by the Upjohn Company, Kalamazoo, Michigan.

assayed for blastokinin and progesterone. Blood serum samples were also stored frozen until subjected to radioimmunoassay for progesterone (Louis et al., 1973) and double antibody radioimmunoassay for estradiol as described below.

Triplicate aliquots of serum (0.5 ml) were placed in 16 x 125 mm culture tubes. Five thousand cpm of ^{3}H-1,2,6,7-estradiol 100 μCi/mM, repurified by column chromatography) were added to a fourth aliquot from 10 representative unknowns to account for procedural losses. All samples were extracted with 5 ml redistilled nanograde benzene for 1 minute by mixing on a vortex-type shaker. Tubes were centrifuged for 5 minutes at 2500 x g and the aqueous phase was frozen by touching the tube bottom to a bath of solid CO_2 and ethanol at -79°C. The benzene layer was decanted into 12 x 75 culture tubes for assay or into scintillation vials for determining procedural loss. Radioactivity in those vials was averaged to compose a single correction factor for all unknowns.

Estradiol (New England Nuclear) standards (0,1,2,3,4,5,6,7,8, and 10 picograms) from a stock solution of 50 pg/ml in benzene and high and low estradiol standard sera samples were included with each assay. Standards and serum extracts were evaporated under vacuum at 50°C. Assay tube walls were rinsed once with 0.5 ml benzene.

Antiserum[2] (0.2 ml) diluted 1:5000 with 0.1% gelatin (Knox Gelatin, Inc., Johnstown, N.Y.) in 0.1 M phosphate buffered saline (PBS) containing 1:100 normal rabbit serum was added to each tube,

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[2]MSU anti-estradiol #74 was prepared in rabbits against estradiol-6-oxime human serum albumin.

mixed and incubated at room temperature for 2 hours. Two hundred µl of 0.1 percent gelatin in 0.1 M phosphate buffered saline, containing 10,000-15,000 cpm of ^{3}H-1,2,6,7-estradiol were added to each tube, mixed and incubated at 4°C overnight. Four hundred µl of castrated sheep anti-rabbit gamma globulin[3] diluted in PBS-gelatin were then added to the tubes, mixed and allowed to incubate for 48 hours at 4°C. Tubes were centrifuged at 2500 x g for 30 minutes and 0.5 ml of the supernatant fluid was diluted with scintillation fluid[4] for quantification of radioactivity in a liquid scintillation spectrometer.

Radioactivity in samples was counted for 10 minutes. The percent of labelled hormone bound was derived by plotting the average counts of the standards as a percent of the zero tubes. The amount of hormone in each sample was then calculated, after correction for recovery, by interpolation between standards.

Statistical differences in serum hormone or protein concentrations in these animals were determined by t-test (Sokal and Rohlf, 1969).

Experiment II

Fifty-four virgin female New Zealand White rabbits, isolated for 3 weeks and weighing a minimum of 3.0 $\pm$ 0.2 kg, were subjected to the same treatment regime as in the first experiment. Three does per treatment were sacrificed on each day of Days 1-9 of pseudopregnancy.

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[3] MSU second antibody was prepared in sheep against rabbit gamma globulin (Sigma Chemical Co., St. Louis, Missouri) and each bleeding titered for maximum binding.

[4] 3a70B preblend scintillation fluid; Research Products International Corp., Elk Grove Village, Ill.

Uterine fluid samples were collected to determine if differences in blastokinin secretion patterns existed between treatment groups during early pseudopregnancy. The uterine horns were flushed as described previously, and ovaries were examined for number of hemorrhagic follicles, large follicles (>2 mm), and old corpora lutea. Uterine fluid samples were assayed for blastokinin content by the double antibody radioimmunoassay method of Mayol and Longenecker (1974).[5] The iodination procedure was as follows.

Twenty-five µl of 0.5 M sodium phosphate buffer (pH 7.6), 4.6 g purified blastokinin, 2 mCi of $Na^{125}I$ (80-140 Ci/ml, Amersham Searle Corp., Arlington Heights, Ill.), and 10 µl of chloramine T (2.5 mg/ml in phosphate buffered saline) prepared immediately before use were agitated together for 60 seconds. To stop the reaction 25 µl of potassium metabisulfite (2.5 mg/ml in PBS) were added. Next a few drops of glycerol were added and the entire iodination mixture was layered on to 1 x 25 cm Sephadex-25 column (10 ml bed volume) previously equilibrated with phosphate buffered saline and 2% bovine serum albumin. Fractions (0.5 ml) were eluted from the column with PBS. The ^{125}I-blastokinin elution profile in 10 µl samples from each of the fractions was determined by quantifying radioactivity with an automatic gamma counter. Fraction 9 contained the iodinated hormone. Iodinated material remained stable for several weeks when stored at -20°C.

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[5]Guinea pig anti-rabbit blastokinin antisera, purified blastokinin for iodination and standards, and goat anti-guinea pig gamma globulin generously supplied by Dr. D. E. Longenecker, Mead Johnson Research Center, Evansville, Ind.

Mayol and Longenecker (1974) described the sensitivity, cross-reactivity, and accuracy of the blastokinin assay, and it was revalidated in our laboratory using our iodinated hormone. The useful range of assay sensitivity was between 0.127 and 2.280 ng (91 to 27% binding). Precision of the RIA was obtained by including a common uterine fluid sample in each assay. Specificity was examined using blood serum samples and fractions which contained greater than 50 μg/ml of non-blastokinin protein from blastokinin purification procedures described below. No significant interference (<1%) could be detected. Increasing amounts of aqueous extracts from Day 6 pseudopregnant rabbit uteri gave a dose response curve parallel to the standard curve. Increasing concentrations of blood serum gave no such response (Figure I).

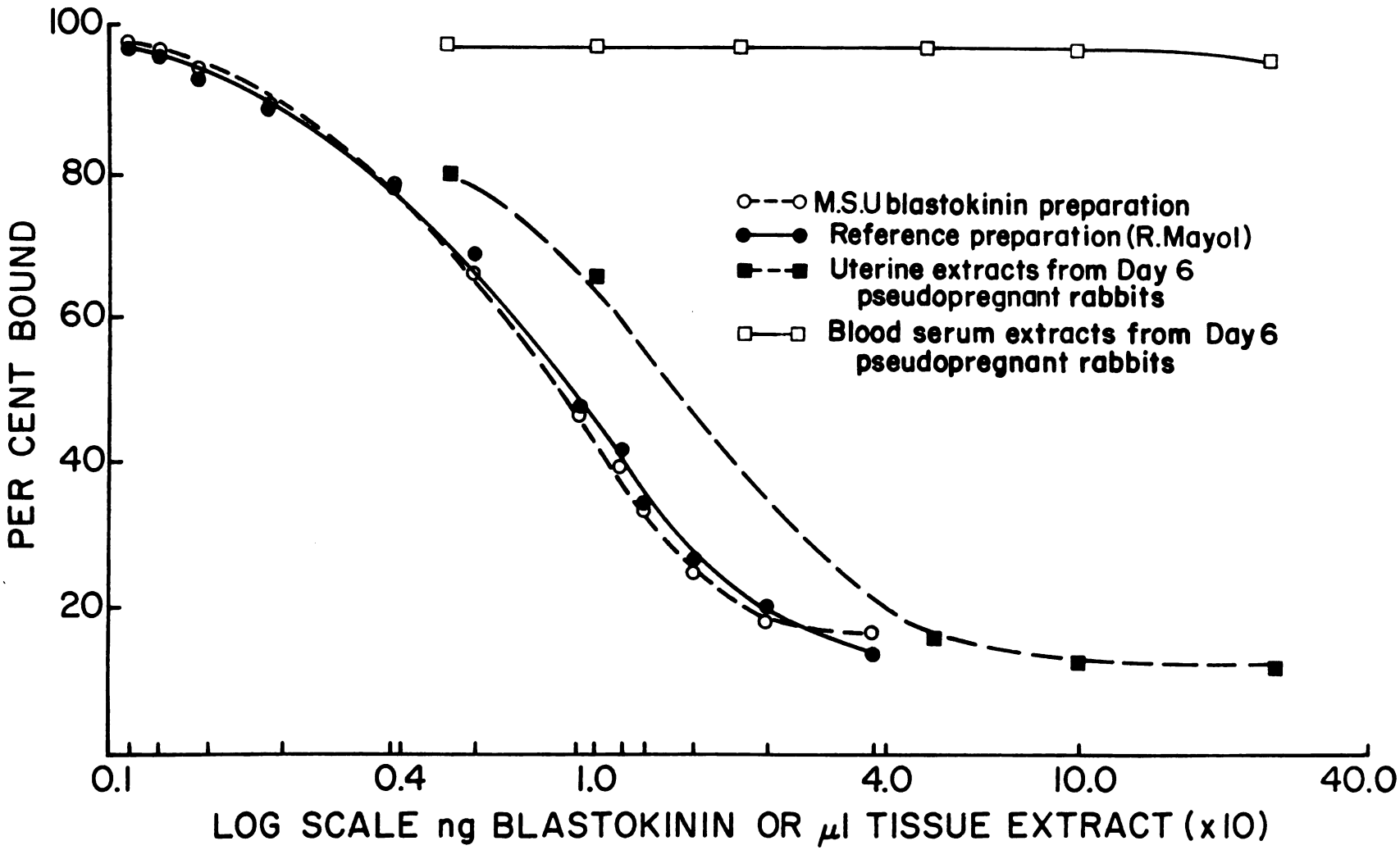
In a repeat of Experiment I, rabbits were sacrificed (3 MGA-treated and 3 controls each day) on Days 3, 6, or 9 of MGA or corn oil injections, on the second day following these injections, or on Day 2 of pseudopregnancy. Blastokinin concentration in these samples was determined.

Differences in uterine fluid blastokinin concentrations were determined by t-test. The product-moment correlation was used to determine the linear relationship between blastokinin secretion patterns in MGA-injected rabbits during MGA-treatment and early pseudopregnancy (Sokal and Rohlf, 1969).

Experiment III

The final experiment was conducted to determine if lower concentrations of blastokinin in uterine fluid of Day 3 pseudopregnant

FIGURE 1.


100
80
60
40
20
PER CENT BOUND
M.S.U blastokinin preparation
Reference preparation (R.Mayol)
Uterine extracts from Day 6 pseudopregnant rabbits
Blood serum extracts from Day 6 pseudopregnant rabbits
0.1
0.4
1.0
4.0
10.0
40.0
LOG SCALE ng BLASTOKININ OR μl TISSUE EXTRACT (x10)

MGA-treated rabbits had any physiological role in reduced fertility. If blastokinin is required, for example, to transport maternal steroids into the blastocyst or supply nutrients during implantation, then increasing its concentration should enhance implantation. Embryos (32-64 cell morulae) were transferred into does which received the same MGA or corn oil injections as described previously. Superovulation was induced in the donor animals with subcutaneous injections of follicle stimulating hormone (FSH) (0.4 mg twice daily Armour FSH-P)[6] for 3 days before insemination. Semen from four males was pooled and extended in 0.1 M phosphate buffer (pH 7.1) for each insemination. Human chorionic gonadotropin was injected at the time of insemination, 12 hours after the last FSH injection. Control and MGA-treated recipient does also received an HCG injection at this time.

Donor rabbits were killed by cervical dislocation 72 hours after insemination. Uteri and oviducts were excised and flushed with Krebs-Ringer bicarbonate buffer. Ova were collected in watch glasses and examined for evidence of cleavage. Normal-appearing ova were placed in a protein supplemented buffer before transfer to the recipient uterus. This buffer consisted of heat-treated bovine serum (Onuma et al., 1968), penicillin G (75 μg/ml), streptomycin sulfate (50 μg/ml), and 2 mg/400 μl of either purified blastokinin or rabbit serum albumin.[7] A minimum of 4 ova in either the albumin or blastokinin supplemented buffer was surgically transferred to the upper end of the uterine horns. (Staples, 1971). Each animal served as its

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[6]Armour-Baldwin Laboratories, Omaha, Nebraska.

[7]Sigma Chemical Co., St. Louis, Missouri

own control. Rabbits were sacrificed on the eleventh day of pregnancy and the uterus examined for evidence of implantation.

The blastokinin was purified in our laboratory as described below. Ten New Zealand White rabbits were made pseudopregnant by injecting 100 I.U. HCG (I.V.). On Day 6 of pseudopregnancy all animals were sacrificed and their uteri flushed with 0.33 M sodium chloride. Flushings were pooled and centrifuged at 700 x g for 20 minutes to remove cellular debris. The supernatant fluid was dialyzed against distilled water for 24 hours at 4°C. A Sephadex G-200 column (2.6 x 40 cm) was prepared by flushing for 48 hours with 0.05 M Tris-HCL buffer (pH 8.0). The dialyzed uterine fluid was frozen, lyophilized,[8] redissolved in 2.5 ml of the Tris buffer and layered onto the Sephadex G-200 column. Forty minute fractions were eluted with the same Tris buffer and protein concentration of fractions was measured by spectrophotometer (wavelength = 280 nm) and by the total protein method of Lowry et al. (1951). Peak fractions were identified, dialyzed, lyophilized, reconstituted with Tris buffer, and further purified on a DEAE cellulose column equilibrated with Tris-HCL (Mayol and Longenecker, 1974). Elution from the DEAE cellulose column was effected with a linear gradient of 0.05 M Tris-HCL to 0.5 M NaCl in 0.05 M Tris-HCL. Again the peak fractions were identified by absorbance at 280 nm. All column chromatography was performed in a cold room (4°C). The purity of the protein was tested using gel electrophoresis (Weber and Osborn, 1969). Purified blastokinin from Dr. Longenecker and normal rabbit serum were employed as standards and

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[8]Virtis Automatic Freeze Dryer, Model 10-010, Virtis Company, Gardiner, N.Y.

fractions from two minor peaks were also tested. Staining with Comassie Blue revealed a single band for the peak fraction identical with the known blastokinin sample. The peak fractions were again dialyzed against distilled water, lyophilized and then stored at 0°C until dissolved in the heat treated serum and used for the embryo transfers.

Analysis of the albumin versus blastokinin treatment required an arc-sine transformation of the data. Statistical comparisons of the means were then made using a t-test (Sokal and Rohlf, 1969).

Experiment IV

The technique of scanning electron microscopy allows a close examination of surface structure changes involved in the rabbit uterine epithelial cells change from estrus to highly secretory state of pregnancy. Mature virgin New Zealand White does were subjected to MGA or corn oil treatment as described above. Rabbits were sacrificed on Day 10 of treatment and Days 3, 4, 5, or 6 of pseudopregnancy. The ovaries of pseudopregnant animals were examined for ovulation sites. Uteri were excised immediately, trimmed of fat, and blotted to remove all traces of blood. The uterine cornua were then temporarily placed in 0°C-4°C physiological saline until sectioned and frozen.

Uterine sections were taken just beyond the cervix, at mid-uterus, and at the tubo-uterine junction. One section was taken from each location from each uterine horn; similar sections were pooled within treatments. Tissue was frozen by immersion in liquid nitrogen

and then lyophilized. Prepared samples were coated[9] with a minute quantity of gold ($\leq$300 A) for better conductivity under the electron beam.[10] A permanent record was made of sample observations via a polaroid-type camera attached to the scanning electron microscope.

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[9]Film-Vac, Inc., Mini-Coater Model EMS-41, Englewood, N.J.

[10]Super-mini scanning electron microscope, International Scientific Instruments, Inc., Mountain View, California.

RESULTS AND DISCUSSION

Experiment I

Since there appeared to be no report in the literature of serum estradiol and progesterone concentrations in progestin treated rabbits, daily blood samples were collected to describe these values in MGA-treated does.

Serum progesterone was less than 1 ng/ml throughout pretreatment and during both MGA and corn oil injections in all does. Following an intravenous injection of HCG, serum progesterone increased to about 1 ng/ml by 24 hours, and continued to rise until the rabbits were sacrificed on Days 6 or 7 of pseudopregnancy (Figure II). Peak progesterone for the two groups combined averaged 8.0 $\pm$ 2.6 ng/ml. These data support the findings of Hilliard et al. (1974) for untreated pseudopregnant rabbits in which serum progesterone reached a high of about 12 ng/ml on Day 9.

No differences were observed in serum estradiol concentrations between treatment groups. Early pretreatment values were high compared to later values. One possible explanation is that the commonality of environment had synchronized "cycles" in this group of does. Evidence of cyclic reproductive phenomena in the rabbit has been reported in the literature (Hamilton, 1951). Serum estradiol varied between 5 and 10 pg/ml during treatment, but there were no differences between treatment groups ($P>0.1$). There was approximately a 2-fold increase in estradiol following the onset of pseudopregnancy

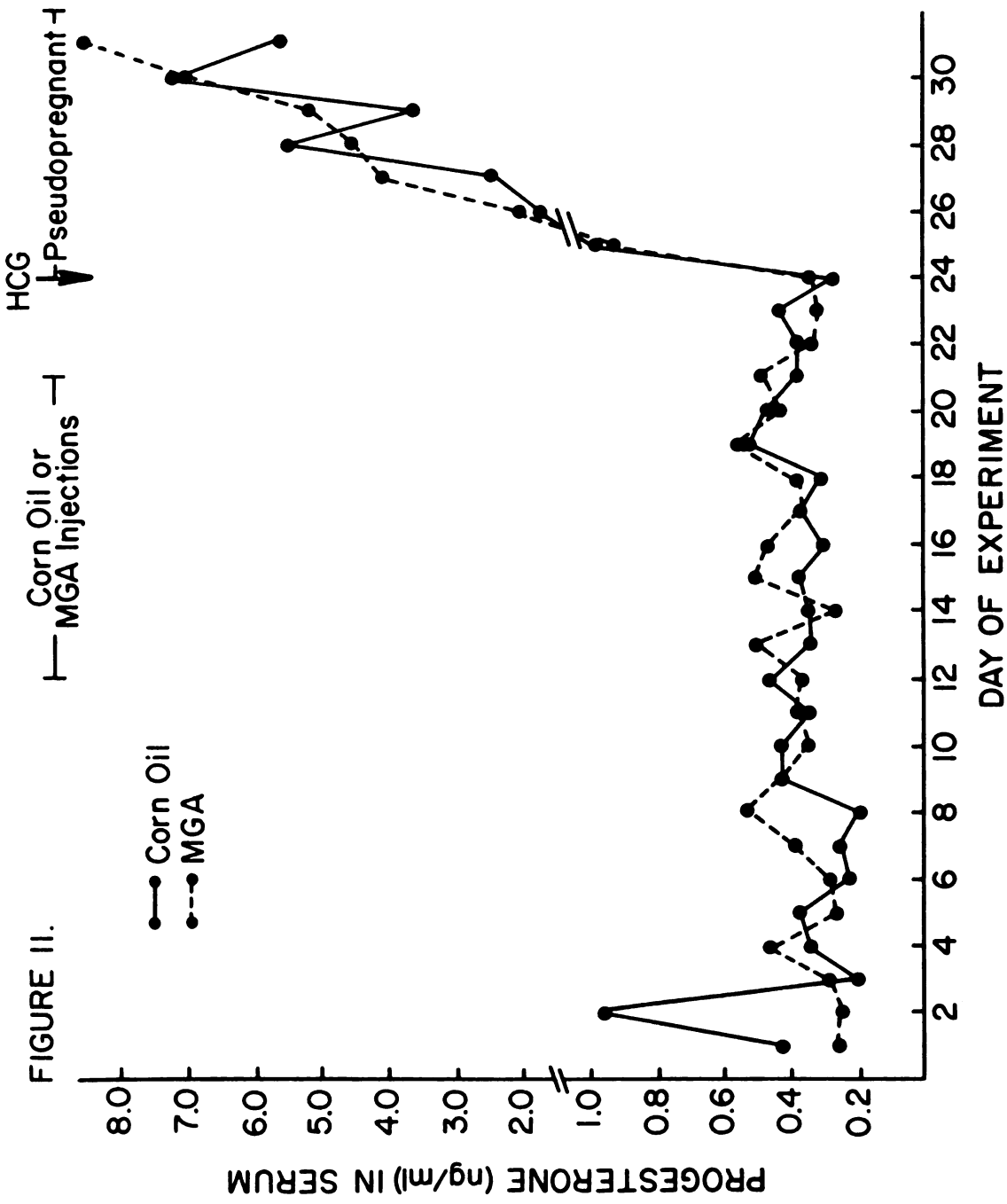


FIGURE II.
Corn Oil
MGA
Corn Oil or
MGA Injections
HCG
Pseudopregnant
PROGESTERONE (ng/ml) IN SERUM
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
DAY OF EXPERIMENT

induced by HCG (Figure III). Although estradiol concentrations observed in the present study followed the general trends reported in the literature (Younglai, 1972; Challis et al. 1973), actual values were lower than previously reported. This difference may be due to the highly specific double antibody radioimmunoassay utilized in this study. Challis and coworkers (1973) reported a peak (83 pg/ml) of estradiol-17β in the peripheral plasma of pregnant rabbits on Day 6 with a secondary increase which peaked on Day 15. In this experiment serum estradiol peaked on Day 5 of pseudopregnancy.

Rabbits were killed by cervical dislocation on Days 6 or 7 of pseudopregnancy and the ovaries were examined for the presence of corpora lutea, hemmorrhagic follicles, and large (>2 mm) follicles (Table I). Again corn oil injected rabbits did not differ significantly from those receiving MGA, although progestin treated does had higher means in each category. Apparently some other cause must be responsible for lowered fertility in progestin-treated rabbits as neither serum concentrations of progesterone or estradiol, nor ovarian development or maturation, were changed by MGA treatment.

Experiment II

With the development of radioimmunoassay for blastokinin (Mayol and Longenecker, 1974), a more definitive examination of uterine secretion was possible. Blastokinin averaged 0.004 mg/ml of uterine flushing for the first 2 days of pseudopregnancy in both MGA-treated and control rabbits. Blastokinin increased to 1.5 mg/ml by Day 3 in does given corn oil, continued to rise until Day 5, then plateaued (1.94-2.39 mg/ml) until Day 8. Blastokinin fell

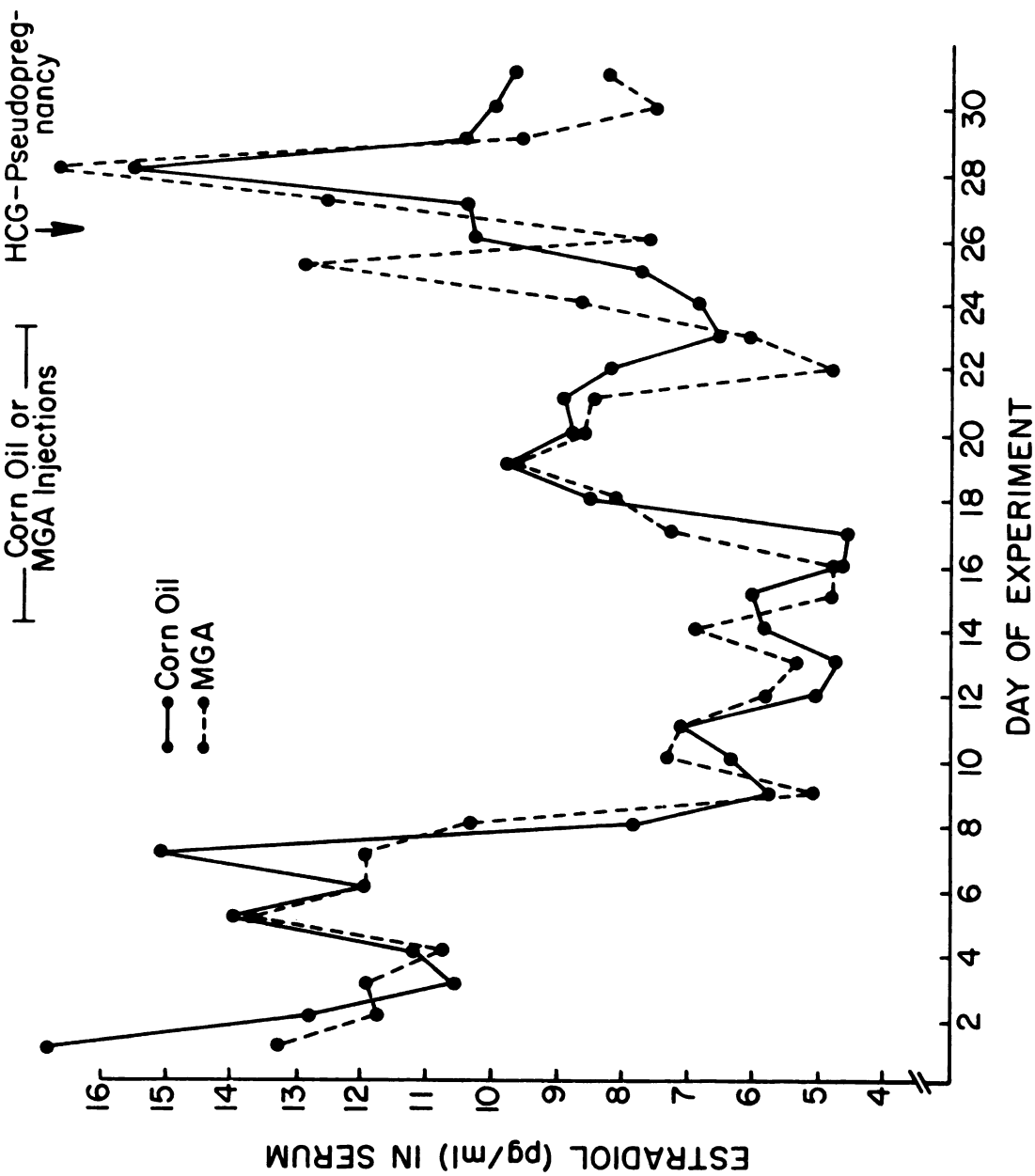

HCG-Pseudopregnancy
Corn Oil or MGA Injections
Corn Oil
MGA
ESTRADIOL (pg/ml) IN SERUM
DAY OF EXPERIMENT

FIGURE III.

TABLE I
OVARIAN MORPHOLOGY

Corpora lutea (total from both ovaries)

Control		MGA	
Rabbit #		Rabbit #	
1	10	2	9
6	7	12	17
9	8	7	10
3	9	5	6
11	11	8	13
4	17	10	12
	10.3 ± 1.4[a]		11.2 ± 1.5

Hemorrhagic Follicles

Control		MGA	
Rabbit #		Rabbit #	
1	3	2	3
6	2	12	5
9	3	7	3
3	3	5	4
11	1	8	3
4	3	10	2
	2.5 ± 0.3		3.3 ± 0.4

Large Follicles

Control		MGA	
Rabbit #		Rabbit #	
1	10	2	3
6	9	12	14
9	6	7	9
3	17	5	10
11	3	8	12
4	5	10	16
	8.3 ± 2.0		10.7 ± 1.9

[a] mean ± std. error

dramatically to 0.29 mg/ml on Day 9 (Figure IV). Rabbits treated with MGA had a different blastokinin secretion pattern. On Day 3 blastokinin values averaged 0.1 mg/ml, less than one-tenth the concentration in oil treated does. By Day 4 MGA-treated animals had blastokinin concentrations comparable to controls, but this plateau lasted only through Day 7. Blastokinin levels fell to an intermediate value of 0.75 mg/ml on Day 8 and were again similar (0.31 mg/ml) to rabbits receiving corn oil by Day 9.

Mayol and Longenecker (1974) reported blastokinin concentrations in uterine flushings of 0.02, 0.20, and 0.37 mg/ml on Days 3, 4, and 6, respectively, with a decrease to 0.01 mg/ml on Days 10 and 12. The difference in reported values may be due to breed of rabbit or differences between pseudopregnant and inseminated animals. Nevertheless, Mayol and Longenecker's work in Dutch-Belted rabbits still represents a 2000-fold increase in blastokinin concentration from Day 0 (day of insemination and gonadotropin administration) to Day 6, and the secretion pattern is similar.

Follicle numbers did not differ ($P>0.10$) between treatment groups, nor were there any macroscopically detectable differences in ovarian or uterine morphology (Table II).

Because blastokinin production is under progesterone control (Arthur and Daniel, 1972; Goswami and Feigelson, 1974), it could be assumed that the progestin treatment caused the difference in the blastokinin secretion pattern. However, despite previous authors' reports of blastokinin induction with progesterone alone (Arthur and Daniel, 1972), MGA appeared to be retarding the production of blastokinin during pseudopregnancy. One possible explanation was

FIGURE IV.

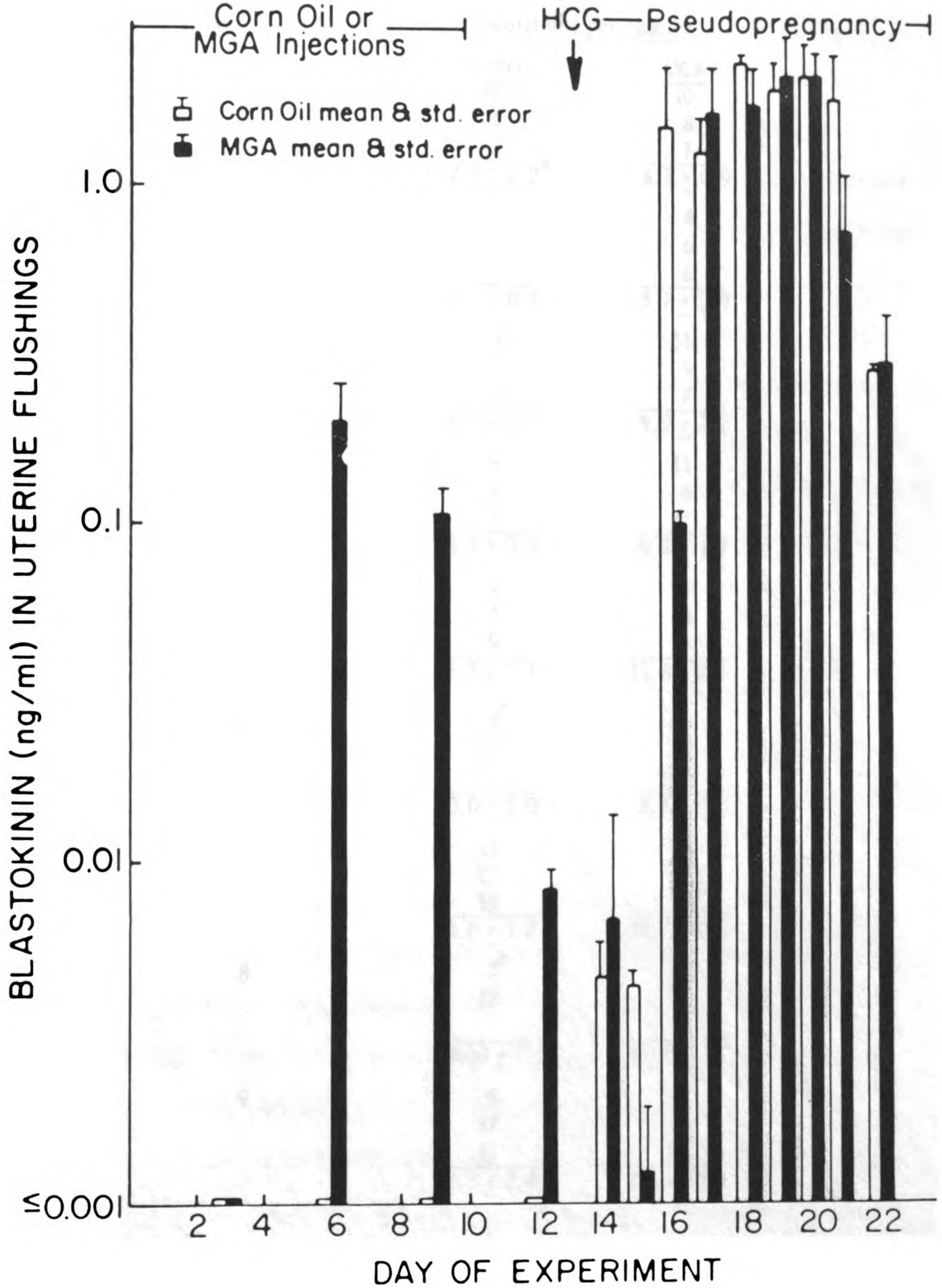

Corn Oil or
MGA Injections
HCG—Pseudopregnancy
Corn Oil mean & std. error
MGA mean & std. error
1.0
0.1
0.01
≤0.001
BLASTOKININ (ng/ml) IN UTERINE FLUSHINGS
2
4
6
8
10
12
14
16
18
20
22
DAY OF EXPERIMENT

TABLE II
OVARIAN MORPHOLOGY

Days Post HCG	Ovulations per rabbit	
	Control	MGA
1	10	10
	9	8
	3	7
	7.3 ± 2.2[a]	8.3 ± 0.9
2	4	4
	6	6
	6	6
	5.3 ± 0.7	5.7 ± 0.9
3	9	11
	7	9
	9	7
	8.3 ± 0.7	9.0 ± 1.2
4	9	11
	9	6
	8	11
	8.7 ± 0.3	9.3 ± 1.7
5	9	9
	7	8
	9	16
	8.3 ± 0.7	11.0 ± 2.5
6	8[b]	8
	12	9
		8
	10.0 ± 2.0	8.3 ± 0.3
7	11	10
	13	11
	15	10
	13.0 ± 1.2	10.3 ± 0.3
8	7[b]	11
	12	11
		16
	9.50 ± 2.5	12.7 ± 1.7
9	9	12
	17	10
	11	14
	12.3 ± 2.4	12.0 ± 1.1

[a] mean ± std. error
[b] two animals only

that the progestin treatment was inducing blastokinin during the injection period. The second part of the experiment was therefore designed to examine what effect MGA might have before induction of pseudopregnancy.

When uterine blastokinin secretion was measured during Days 3, 6, and 9 of MGA or corn oil injections, blastokinin averaged 0.0004 mg/ml for each day in controls. Rabbits injected with MGA had a concentration of 0.006 mg/ml on Day 3, 0.202 $\pm$ 0.06 mg/ml on Day 6, and 0.108 $\pm$ 0.07 mg/ml on Day 9. This secretion pattern was highly correlated ($r = 0.89$) with the blastokinin pattern of pseudopregnancy. On the second day following the last MGA injection the concentration of blastokinin had fallen to 0.08 $\pm$ 0.001 mg/ml, approximately 10-fold greater than in controls. By Day 2 of pseudopregnancy the amount of blastokinin in the uterine fluid was similar for both treatment groups and values were comparable to those found in the first part of this experiment (Figure IV).

It is conceivable that MGA-induced production of blastokinin temporarily exhausted secretion or caused a short refractory period to the rising progesterone concentrations of pregnancy or pseudopregnancy, thus delaying blastokinin secretion in the progestin-treated does. The lower concentrations of blastokinin observed during treatment compared to pseudopregnancy confirm work by Arthur and Daniel (1972) that less than 1.0 ng of progesterone per kg of body weight daily was not effective in eliciting a maximal secretory response. In an experiment with immature female rabbits, Giannina and Meli (1976) found that 200 µg/day of progesterone was the lowest amount of the hormone capable of inducing secretory change. The total

quantity of progestin administered daily in this experiment was well below that level. If progesterone induced endometrial changes and blastokinin production are closely related, the single day's delay in maximal protein concentrations could sufficiently alter the uterine environment so that ovum viability was drastically reduced.

Arthur and coworkers (1972) and Beato and Baier (1975) reported that blastokinin was capable of binding progesterone in uterine fluid of rabbits. In this study progesterone was measured in uterine fluid collected from rabbits on Days 3, 6, or 9 of treatment, the second day after the last MGA or corn oil injection, and Days 2, 6, and 7 of pseudopregnancy (Figure V). Although the average concentration of progesterone (0.180 $\pm$ 0.016 to 0.305 $\pm$ 0.020 ng/ml) appeared to increase as treatment progressed, the increase was non-significant ($P>0.10$) with the small number of samples. A peak concentration of progesterone was detected on Day 6 of pseudopregnancy (6.04 ng/ml $\pm$ 0.5 ng/ml). There was no difference between the mean progesterone concentration in the uterine fluid of MGA or corn oil injected does ($P>0.10$). This is particularly significant because by Day 3 of treatment there was a 10-fold difference in the blastokinin concentration in the uterine secretions of these animals. There is no way of determining with this assay procedure whether the progesterone was bound to blastokinin. The extraction procedure used may cause the blastokinin molecule to release the hormone or the concentrations may reflect only quantities of free progesterone in the uterine fluid.

Recently Cowan et al. (1976) reported less than 0.2 ng progesterone per µl uterine flushing for Days 0-3 of pregnancy using a competitive protein-binding assay. The concentration of progesterone

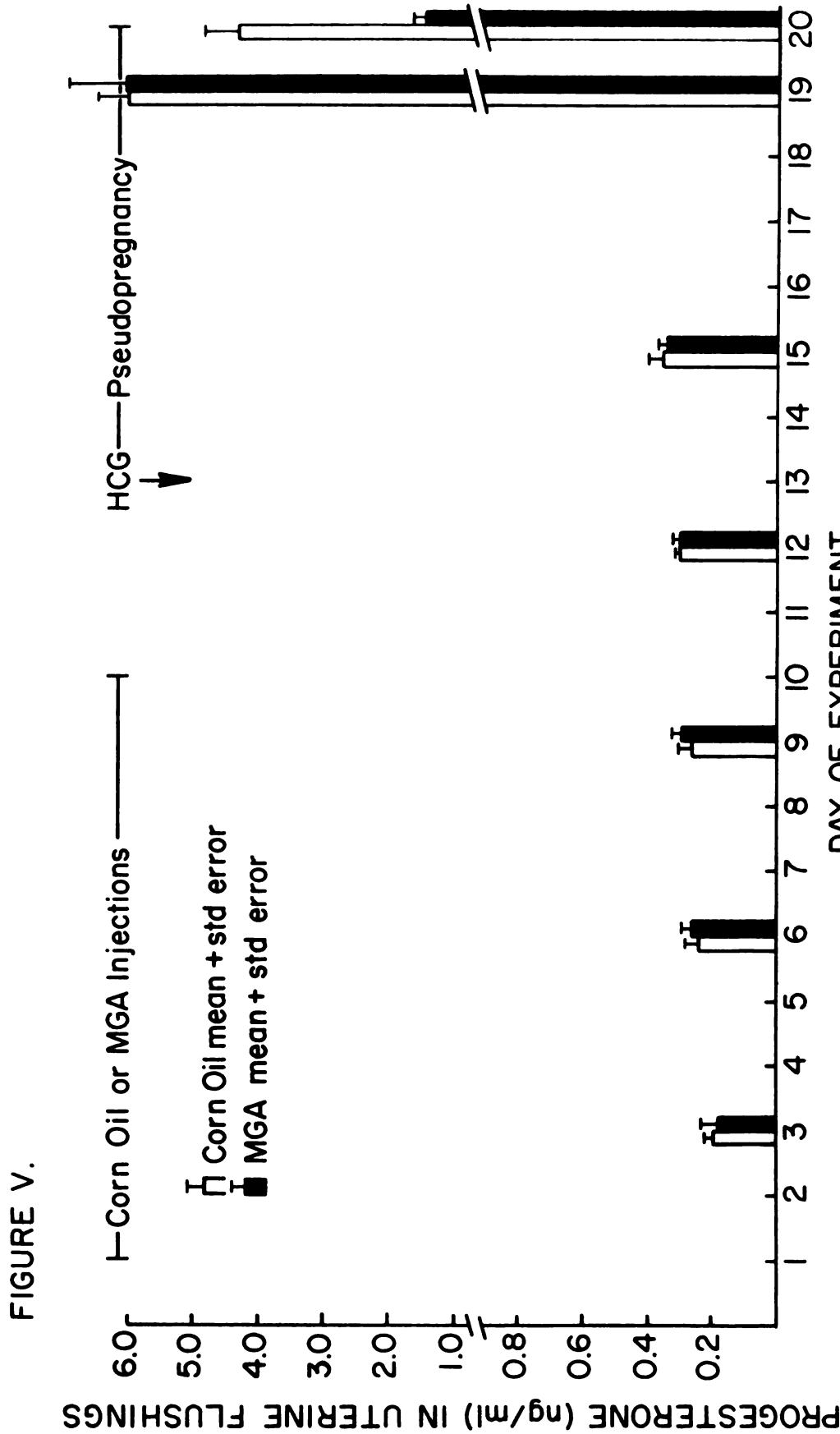

PROGESTERONE (ng/ml) IN UTERINE FLUSHINGS
6.0
5.0
4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
DAY OF EXPERIMENT
HCG
Pseudopregnancy
Corn Oil or MGA Injections
Corn Oil mean + std error
MGA mean + std error

FIGURE V.

increased to 1.1 ng/ml on Day 4, 5.4 ng/ml on Day 5, and 26.0 ng/ml on Day 6. Fowler and coworkers (1976) found even greater concentrations of progesterone in uterine flushings in pseudopregnant rabbits with a range of 41.2 to 104.8 ng progesterone recovered per uterus (2 ml flushing) on Day 5.

No increase in progesterone concentration of uterine flushings was observed in samples taken during MGA treatment despite an increase in blastokinin concentration. One possible explanation is that no more progesterone is available for binding to blastokinin at this time and that 0.2-0.3 ng/ml is the average quantity of progesterone always found in the rabbit uterus. Another possibility is that uterine fluid may contain MGA due to treatment and this may be bound instead by the blastokinin molecule. Our radioimmunoassay for progesterone does not crossreact with MGA (crossreactivity <0.1%) and therefore the progestin, if present, would remain undetected. More probably these progesterone concentrations reflect the absence of corpora lutea.

A composite sample of uterine fluid was eluted through a Sephadex G-200 column to separate the major protein fractions (Figure VI). Each uterine horn from Day 6 pseudopregnant rabbits was flushed with 3 ml of 0.33 M NaCl. Average fluid recovery was 83.44% (2.50 ml) with a range of 2.08-2.73 ml. Blastokinin comprised the major fraction; 47.28% of the protein detected by the Lowry assay method (1951). The elution profile was similar to that described by Mayol and Longenecker (1974) and by Krishnan and Daniel (1967).

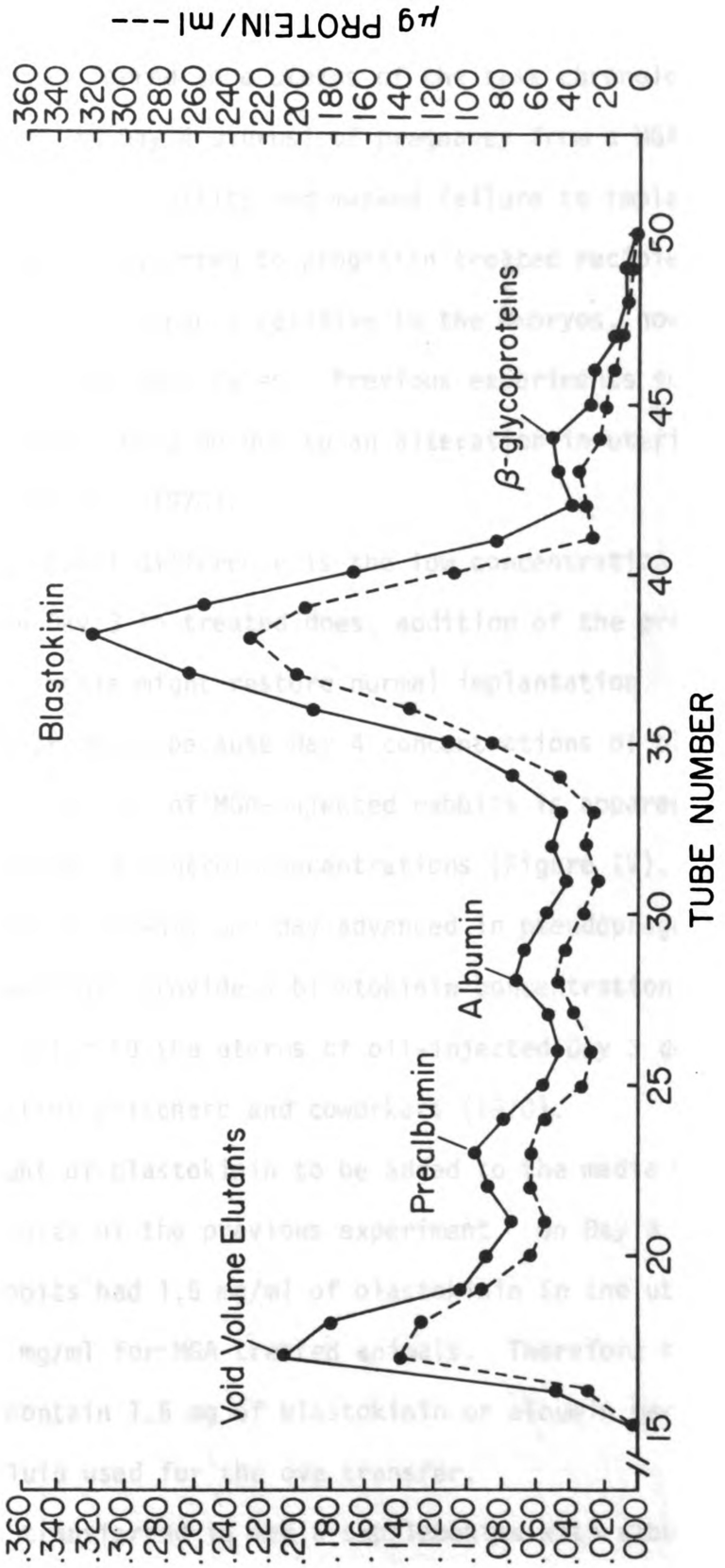


Figure VI. Sephadex G-200 Elution Profile

Experiment III

Embryos transferred to a uterus of the same chronological age (i.e., Day 4 embryo to Day 4 uterus) of pregnancy from a MGA-treated rabbit exhibit reduced viability and marked failure to implant (Britt et al., 1973). Ova transferred to progestin treated recipients one day advanced in pseudopregnancy relative to the embryos, however, resulted in normal pregnancy rates. Previous experiments suggest that this reduced survival could be due to an alteration in uterine secretions (Pritchard et al., 1970).

If the critical difference is the low concentration of blastokinin detected on Day 3 in treated does, addition of the protein to the ovum transfer media might restore normal implantation. Strength is added to this argument because Day 4 concentrations of blastokinin in the uterine secretions of MGA-injected rabbits is apparently equivalent to the Day 3 control concentrations (Figure IV). The uterus of treated recipients one day advanced in pseudopregnancy (Day 4) could therefore provide a blastokinin concentration to the Day 3 embryos similar to the uterus of oil-injected Day 3 does, as in the experiment of Pritchard and coworkers (1970).

The amount of blastokinin to be added to the media was determined by the results of the previous experiment. On Day 3 the corn oil injected rabbits had 1.5 mg/ml of blastokinin in the uterine fluid compared to 0.1 mg/ml for MGA-treated animals. Therefore the media was made up to contain 1.5 mg of blastokinin or albumin per 400 µl; the amount of fluid used for the ova transfer.

Embryos transferred in media supplemented with albumin to uterine horns of MGA-treated rabbits did not implant (Table III).

TABLE III
EMBRYO TRANSFER

Oil-injected	Albumin (%)	Blastokinin (%)
Group 1	1/8[a]	1/9
	0/4	1/4
	1/4	3/5
	1/5 (14.3%)[b]	0/6 (20.8%)
Group 2	1/4	2/5
	4/5	5/5
	0/5	0/5
	1/4 (33.3%)	2/4 (47.4%)
	9/39 (23.1%)	14/43 (32.6%)
MGA-injected		
Group 1	0/4	0/5
	0/4	0/5
	0/3	0/3
	0/6 (0%)	0/7 (0%)
Group 2	0/5	1/5
	0/5	1/5
	0/5	0/5
	0/4	0/4
	0/7 (0%)	0/8 (7.4%)
	0/43 (0%)	2/47 (4.3%)

[a] number implantation sites/number embryo transferred
[b] group total percentage of implantations

However when embryos in media containing blastokinin were transferred to the contralateral horns of these same does, 2 of 27 of the fertilized ova were capable of implanting. While these small numbers are not significant ($P>0.1$) they are indicative of a trend more clearly demonstrated in the corn oil injected does. The two embryos represent an overall implantation rate of 4.3%.

More than one-fourth of all embryos transferred to corn oil injected rabbits implanted. In these animals addition of blastokinin to the transfer media enhanced ($P<0.05$) the rate of implantation. When albumin was added to the transfer media, 23.1% of the embryos implanted compared to 32.6% for embryos transferred in blastokinin-containing media. These totals include one oil-treated doe that did not have any implantation sites on either side. If one accepts that she may have had an undetected fertility problem and excludes her from the calculations, the implantation rates become 26.5% and 36.8% for embryos transferred in albumin and blastokinin-containing media respectively.

In 15 of 17 transfers, the uterine horn receiving blastokinin-containing media had as good or better an implantation rate than the contralateral horn. The improved implantation rates from Group 1 to Group 2 probably represents improvement in the transfer technique. These transfers were peformed 2 days apart. Rabbits used as embryo donors were superovulated prior to insemination and in almost all cases, enough ova were recovered to permit transfer to at least one oil and one MGA-treated recipient. In no instance did the results indicate any infertility on the part of the donor rabbits.

Various authors have previously demonstrated blastokinin's ability to aid in the growth and development of blastocysts in vitro. In 1967, Krishnan and Daniel reported that addition of blastokinin to the culture media promoted expansion and cavitation of Day 3 rabbit morulae compared to the addition of maternal serum proteins. Later studies showed that rabbit blastocysts grew best in culture when placed in media containing both blastokinin and progesterone (El-Banna and Daniel, 1972), and that growth and differentiation could occur in rabbit embryos transferred to castrate recipients given sufficient quantities of progesterone to stimulate uterine blastokinin production (Arthur and Daniel, 1972).

There are several conclusions which could be made from the results of this experiment. First, as the in vitro studies indicated, addition of blastokinin to the culture media (in this instance the uterine environment) aided in the growth and development of the embryos transferred to oil-injected rabbits. Second, the additional blastokinin may have "rescued" more embryos from the trauma of transfer and allowed continued development. This trauma likewise falls into several categories. Embryos placed in albumin-containing media were washed free of any attached blastokinin and may have needed more time to recover once placed in the donor rabbit. Also, the effects of ether anesthesia on uterine protein production are unknown; the added blastokinin may have supplemented a temporary slowdown in uterine secretion.

Although the results of embryo transfers to the MGA-injected donors are statistically inconclusive, it is this author's opinion that further studies will show that addition of blastokinin to the

uterine fluid can overcome the low fertility caused by progestin administration. In part this optimism is caused by the marked improvement in implantation rate from Group 1 to Group 2. Familiarity with the procedure and more ideal working conditions should improve the chance for success even more.

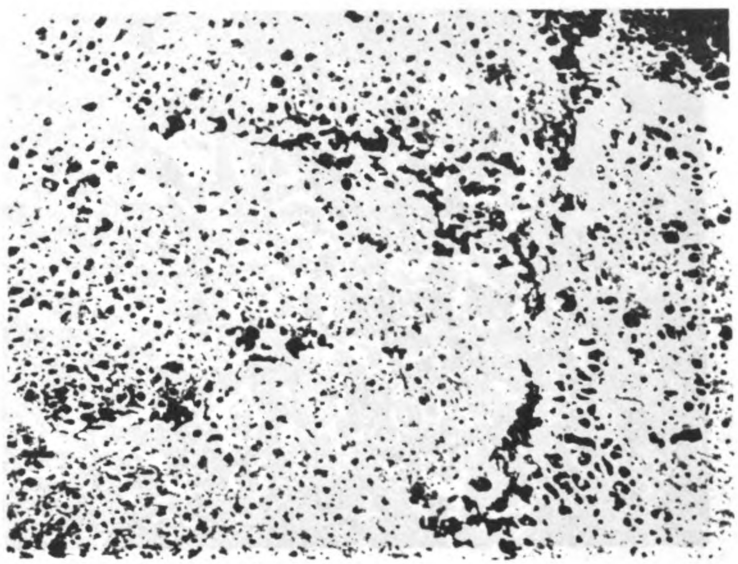
Experient IV

Kanagawa et al. (1972) described the ultrastructure of cell surface and tissue organization of the reproductive tracts of adult female estrous rabbits. Reports have also been published on reproductive tract epithelium at 3 and 12 hours after ovulatory stimulation (Hafez, 1972; Riches et al., 1975). It was hoped that study of MGA-treated and oil control rabbit uteri would help explain the differences observed in blastokinin concentration on Day 3 of pseudopregnancy.

Previous authors have described the arrangement of mucosal folds and crypts observed in the uterus. Few ciliated cells were observed in the majority of the samples examined. It is possible that the freeze-drying technique destroys these fragile structures. However Kanagawa and coworkers (1972) also reported a majority of non-ciliated secretory cells in the middle and upper sections of the uterus.

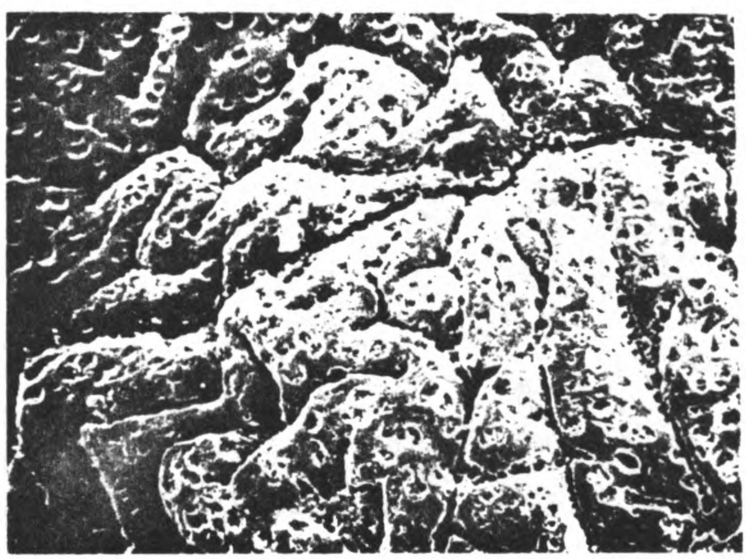
In oil-injected rabbits a highly complex system of cervical crypts was evident for all days of pseudopregnancy at low magnification (Figure VII). Secretory cells with many junctional complexes were clearly visible at higher magnification (Figure VIII). One series of photographs was taken at increasing magnification into the

Figure VII



(100x mag.)

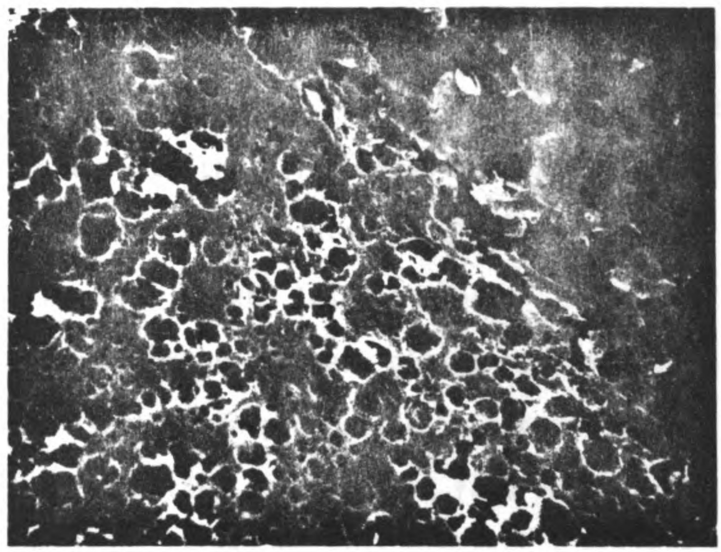
Day 3 pseudopregnant oil-injected rabbit uterus



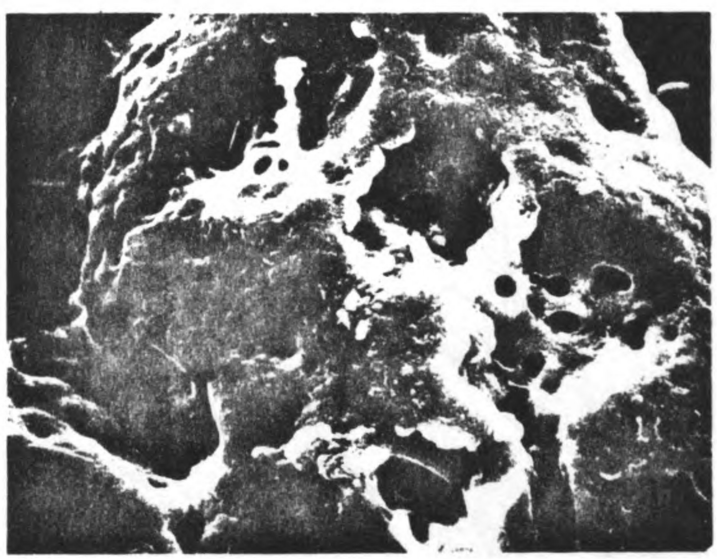
(100x mag.)

Day 6 pseudopregnant oil-injected rabbit uterus

Figure VIII



(200 x mag.)
Day 3 pseudopregnant oil-injected rabbit uterus



(1000x mag.)
Day 6 pseudopregnant oil-injected rabbit uterus

opening of a uterine gland (Figure IX). The presence of secretory granules is clearly demonstrated.

The appearance of epithelial tissue from progestin-treated does is similar in general, but does not show evidence of great secretory activity (Figure X). This is especially noticeable on Day 3 of pseudopregnancy when the differences in blastokinin concentration are greatest. There are fewer uterine gland openings, and secretory cells are smaller in size and less complex.

One interpretation of these photographs is that MGA administration affects blastokinin production by retarding secretory cell formation. Such an action could result from refractoriness to the animal's own rising progesterone concentrations or cell exhaustion from blastokinin secretion during the treatment phase. Further study and cataloging of photographs of this nature could result in establishment of a new McPhail-type index at the ultrastructure level.

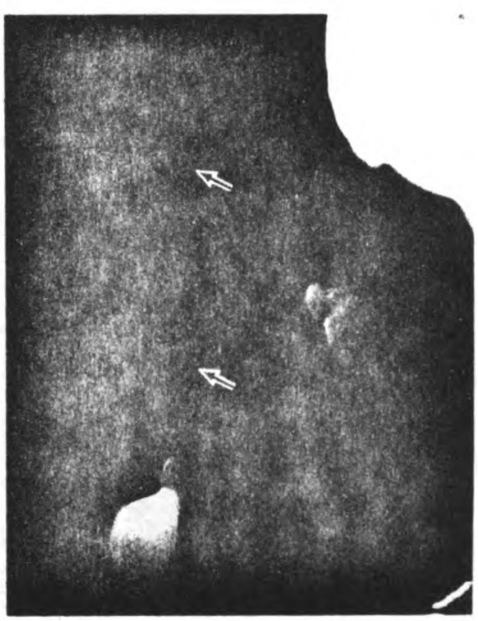
Figure IX

Day 3 pseudopregnant oil-injected rabbit uterus

(50x mag.)

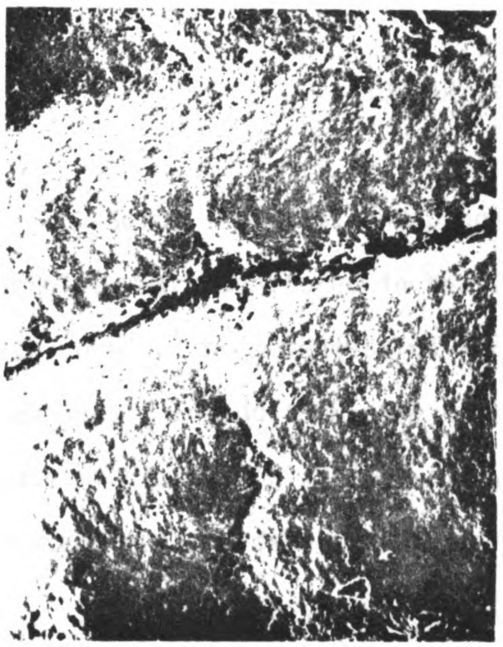
(200x mag.)

(700x mag.)

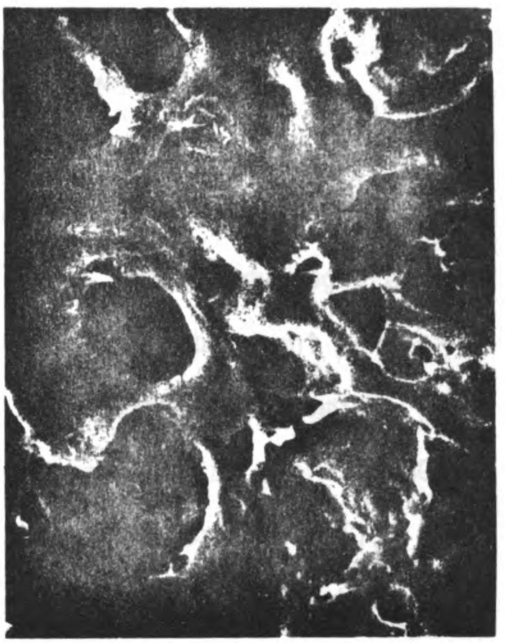


(3000x mag.)
arrows indicate
secretory granules

Figure X

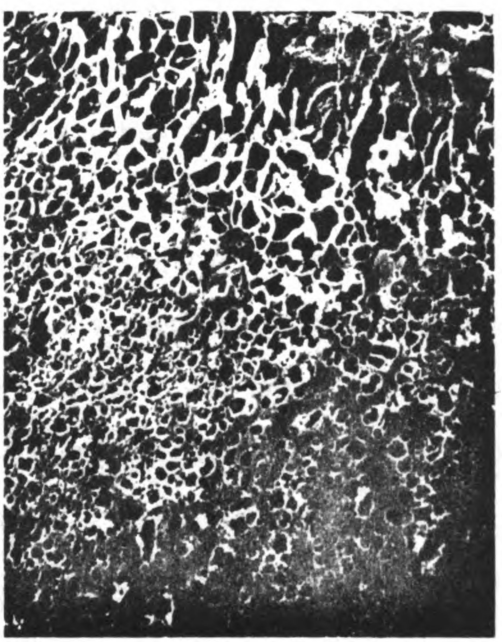


(50x mag.)



(700x mag.)

Day 3 pseudopregnant MGA-injected rabbit uterus



(100x mag.)

Day 3 pseudopregnant Oil-injected rabbit uterus

SUMMARY AND CONCLUSIONS

Serum estradiol and progesterone, and uterine blastokinin secretion were monitored in New Zealand White rabbits during treatment with corn oil or melengestrol acetate and during subsequent pseudopregnancy. In addition, the effects of blastokinin or albumin-containing media on 72-hour embryos transferred to the uteri of MGA-treated does was determined by the implantation rate on Day 11.

Serum progesterone averaged less than 1 ng/ml throughout pre-treatment period and during MGA and corn oil injections in all does. Both treatment groups responded to an intravenous injection of human chorionic gonadotropin with increasing concentrations of progesterone typical of the onset of pseudopregnancy. Peak progesterone values prior to sacrifice on Days 6 or 7 of pseudopregnancy of 7.3 ng/ml and 8.0 ng/ml for corn oil and MGA-treated rabbits, respectively, were not statistically different.

Serum estradiol concentrations of 5-10 pg/ml during treatment were lower than previously reported. Again there was no difference between the treatment groups. All animals responded to the induction of pseudopregnancy with an increase in serum estradiol which peaked on Day 5 at 15.6 pg/ml for controls and 16.8 pg/ml for MGA-treated rabbits. Similar numbers of large (>2 mm) follicles, hemorrhagic

follicles and corpora lutea were found on the ovaries of does receiving each treatment.

In oil-injected does blastokinin in uterine flushings averaged 0.004 mg/ml during the first 2 days of pseudopregnancy. On Day 3, the blastokinin concentration increased to 1.5 mg/ml and continued to rise until Day 5, plateaued at 1.94-2.39 mg/ml until Day 8, and then fell to 0.29 mg/ml on Day 9. In the MGA-treated rabbits Day 3 values were much lower (0.1 mg/ml), the blastokinin concentration increased on Day 4 to 1.6 mg/ml, plateaued at 1.75-2.23 mg/ml until Day 7 and then decreased to 0.75 and 0.31 mg/ml on Days 8 and 9 respectively.

Because progesterone administration was known to induce uterine blastokinin secretion, a possible explanation of the above results was that the MGA-injected does were exhibiting refractoriness due to the exogenous progestin. Indeed measurement of blastokinin concentrations in the uterine flushings of does during MGA treatment showed a secretion pattern of lower magnitude, but highly correlated ($r = 0.89$) with the blastokinin pattern of pseudopregnancy. The concentration of blastokinin in the oil-injected controls averaged 0.0004 mg/ml throughout the injection period.

Progesterone in the uterine flushings did not appear to differ between treatment groups, but this laboratory has no facilities to measure MGA and it is conceivable that the progestin may be present in the serum and uterine flushings.

When embryos were transferred to oil-treated does, blastokinin-containing media significantly enhanced the rate of implantation compared to albumin containing media (37.6% vs. 23.1%). The implantation rates in MGA-injected rabbits did not differ between uterine horns

receiving embryos in blastokinin or albumin supplemented media. However, I would suggest that the zero implantation for albumin versus 4.3% for blastokinin may be indicative of a beneficial effect of adding the specific uterine protein.

Scanning electron microscope photographs confirm that uteri of Day 3 pseudopregnant, MGA-treated rabbits exhibit less secretory activity than their oil-injected controls. Epithelial tissue is less convoluted and has fewer secretory crypts and glands in evidence.

Further experiments are needed to elucidate the physiological functions of blastokinin, but these data may be interpreted to suggest that blastokinin secretion is intricately involved with the processes of pre-implantation and implantation.

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