ACUTE EFFECTS OF REMOVING COMPONENTS, OR THE ENTIRE GRAVID UTERUS, ON SYSTEMIC PLASMA CONCENTRATIONS AND OVARIAN OUTPUT OF 20αOH-PROGESTERONE AND PROGESTERONE IN 21-DAY PREGNANT RABBITS

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CHAPTER I

INTRODUCTION

The Biological Subject of the Study

Man and many domesticated animals are viviparous. Successful reproduction incorporating this feature is characterized by the compatible existence and growth of the conceptus in the uterus and is accompanied in many species by alterations in ovarian endocrine function in the mother. Without these alterations in ovarian endocrine function gestation is not completed in a number of species. Fraenkel's reports of 1903 and 1910, on the effect of ovariectomy early in pregnancy were the first to demonstrate an essential role for the mother's ovaries associated with maintenance of pregnancy. His experiments, performed on rabbits, were followed by those of other investigators including Hammond, Allan, Corner, Robson, and Greep. From their studies it was learned that continuous ovarian luteal function during the entire period of gestation is essential for successful reproduction in the rabbit, since removal of the corpora lutea at any stage of gestation terminates pregnancy. Furthermore, the presence of the gravid uterus is required to sustain the functional integrity of corpora lutea beyond the time of midgestation, or duration of pseudopregnancy in the rabbit. The objective of this study was to investigate the regulatory role that the gravid uterus exerts on ovarian endocrine function at a time beyond the duration of pseudopregnancy in the rabbit.

The Experimental Scope of the Study

The advent of techniques such as mass spectrometric analysis and electron capture detection following gas-liquid chromatography make possible identification and measurement of specific hormonal steroids in systemic plasma and venous effluent from the gonads. Such physicochemical techniques have been successfully applied towards elucidating regulation of endocrine function of the male gonad by Ewing and of the female gonad by Eik-Nes, McCracken, McKerns, Dorrington, Kilpatrick, Hilliard, Keyes, and others. The objective of the thesis was pursued by performing experiments designed to use physicochemical techniques in conjunction with organ removal to answer the following questions: What are the acute effect(s) of removing the gravid uterus, at a time beyond the duration of pseudopregnancy, on ovarian endocrine function? From what anatomical part of the gravid uterus does the influence that sustains ovarian progestin secretions beyond the duration of pseudopregnancy originate?

The acute effects that removing the gravid uterus has on ovarian function were assessed by measuring 20α-OH-progesterone and progesterone concentrations in ovarian venous plasma in two groups of eight 21-day pregnant rabbits. Ovarian venous blood was collected from one group six hours after hysterectomy. Ovarian venous blood was collected from the other group 24 hours after hysterectomy. In these same rabbits the concentrations of the progestins in systemic plasma were measured before and either six or 24 hours after the operations. For comparative purposes, comparable ovarian venous and systemic plasma samples were collected from a group of eight 21-day pregnant rabbits before and 24 hours after laparotomy.

To resolve the question where in the gravid uterus the influence that sustains ovarian progestin secretion originates, all fetuses were removed by hysterotomy from eight 21-day pregnant rabbits, and all placentas and fetuses were similarly removed from another group of eight 21-day pregnant rabbits. To assess the effects of these treatments on ovarian endocrine function the concentrations of 20αOH-progesterone and progesterone were measured in the systemic plasma before the operations, as well as in systemic and ovarian venous plasma 24 hours after the operations.

In addition to experiments that addressed questions related to the biology of regulation of ovarian function, experiments were conducted to evaluate the specificities, precisions, and accuracies of the assays for measuring 20αOH-progesterone and progesterone that were used to monitor ovarian endocrine function.

CHAPTER II

REVIEW OF LITERATURE, DOCUMENTATION OF PREMISES, AND RATIONALE

Introduction

In eutherian mammals, viviparity involves attachment to the uterine wall, growth, and expulsion of the conceptus at a specific time (Page, 1967). The successful accomplishment of these events in most mammalian species (Conaway, 1971) depends upon the capacity of the adult female to react to the presence of the conceptus by altering uterine glandular and muscular function to be compatible with survival of the conceptus in the uterus. Past studies indicate that the adult female gonad produces steroids which are important in regulating uterine glandular and muscular function. Past studies also indicate that in some species the presence of the conceptuses in the uterus can influence steroid secretion by the mother's ovaries, and that this capacity of the conceptuses to influence steroid secretion by the mother's ovaries can be crucial to the viability of the conceptuses (Amoroso and Finn, 1962; Hisaw, 1963).

The principal objective of the first part of this chapter is to present some features that are known about the influences that the conceptuses have on endocrine functions of the maternal gonads in a number of species. The reason for the presentation of these features is that they provide precedences on which to base investigations of the

regulatory role that the conceptuses may have on ovarian endocrine function in other species. Information about the processes whereby the conceptus influences ovarian endocrine function has been compiled by many scholars including Amoroso (1960), Deanesly (1966), Denamur (1968), Everett (1961), Greenwald and Rothchild (1968), Hisaw (1963), Melampy and Anderson (1968), Moor (1968), Rothchild (1967), and Zarrow (1961).

The second part of the chapter forms a documentary basis for investigating the effects of the conceptus on endocrine functions of the female gonad in the rabbit.

Endocrine Aspects in the Evolution of Viviparity

The evolutionary development of viviparity constituted a lengthening of gestation. In 1959 and 1963, F. L. Hisaw drew together much information on the endocrine processes involved in the development of prolonged gestation in vertebrates. Several broad observations were made in his studies. A principal observation was that in mammals whose gestation period is longer than the luteal phase of their estrous cycles, a prolongation of the functional life of the corpus luteum occurs. In a number of species, prolonged corpus luteum function is associated with specialization of the chorion in the development of various methods of placentation (Amoroso, 1959). This specialization of the chorion can include endocrine functions among which is the capacity to secrete gonadotropins (Amoroso, 1960; Amoroso and Finn, 1962). While the chorionic gonadotropins are proteins or peptides and serve to prolong the functional life of corpora lutea in the species in which they are found, there are differences among them in both their chemical characteristics and physiological properties (Evans and

Simpson, 1950; Cole, 1964; Papkoff, 1969). Such differences suggest that the chorionic gonadotropins may be parallel adaptations that developed after the mammals in which they occur became differentiated from a common parent stock (Hisaw, 1963; Amoroso, 1952).

Hisaw (1959, 1963) noted that frequently adaptations found in the various viviparous animals hold in common an ability to evoke responses in the accessory organs of the female reproductive tract, which are integrated in an endocrine system where steroids play an important role. Therefore, it should not be unexpected to find that specialization of the chorion has involved elaboration of steroids with biological activities of gonadal steroids. As with elaboration of proteinaceous gonadotropins by the specialized chorion, there is considerable variation between species with regard to elaboration of steroids, particularly estrogens (Ainsworth and Ryan, 1966, Amoroso, 1960). Chorionic specialization in some species, notably primates, sheep, and guinea-pig has achieved mimicry of both pituitary and gonadal function (Amoroso and Finn, 1962).

The foregoing trends in endocrine processes with regard to the evolution of viviparity give rise to two points which merit consideration when investigating influences by the conceptus on endocrine processes of the mother. One is that the mechanisms of influences evolved by the conceptus may be different from species to species but can be expected to be functionally related to the endocrine framework of the adult female. Therefore, an appreciation of influences attributable to the conceptus entails some knowledge of endocrine processes in the non-pregnant female. Secondly, since different species appear to have arrived at viviparity by a variety of endocrine routes, premises for

investigating endocrine aspects of viviparity in any one species should be based as much as possible upon data available for that species. This does not preclude considering endocrine processes associated with viviparity in one species as precedences and their existence tested for in other species. As to whether or not the same endocrine processes do in fact exist in other species would have to be determined experimentally; nature has given no a priori assurances that they will. However, experiments that test for the presence of endocrine processes that are known to occur in some species would be justifiable approach for studying how the conceptus influences endocrine functions of the mother's gonads in other species.

Influences of the Conceptus on Endocrine Processes of the Female Reproductive Cycle[1]

Introduction

The full female reproductive cycle may be considered to consist of 1) follicle growth, 2) ovulation, 3) progravidity, 4) gravidity, 5) parturition, and 6) postpartum nurture (Everett, 1961). One of the most prominent and characteristic endocrine features of the female reproductive cycle is a period of relatively constant levels of progesterone in the female during the period of gravidity (Rothchild, 1967).

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[1]The effects of the uterus on gonadal endocrine function have been reviewed by Ginther (1966, 1967), Donovan (1967), Bland and Donovan (1966), Schomberg (1969), and Anderson et al. (1969). The present discussion is directed at influences of the conceptus on gonadal function in the mother and no attempt has been made to describe the effects of uterine influences except where the conceptus is involved.

It is generally accepted that progesterone acts at two sites where it exerts a dominant influence on female reproductive processes. The first site is the sex accessory organs associated with maintaining the conceptus during gestation and providing postpartum nutriment. The second site of progesterone influence is the hypothalamus. Here progesterone acts to inhibit the hypothalamic processes required to initiate ovulation and estrus (Rothchild, 1967). Differences in the female reproductive cycle between different species and alterations in endocrine processes between non-pregnant, pseudopregnant, and pregnant females of the same species may, in many instances, be attributed to variation of the sources and mechanisms of regulating progesterone secretion. A wealth of information on this subject has been presented in review articles (Amoroso, 1960; Anderson et al., 1969; Denamur, 1968; Greenwald and Rothchild, 1968; Melampy and Anderson, 1968; Moor, 1968; Schomberg, 1969). The source and regulation of progesterone secretion during gestation appear to be independent of the conceptus in some species, while in others, the source and regulation of progesterone secretion are affected by the conceptus.

Species in Which the Conceptus Does Not Affect Endocrine Processes During Gestation

Following ovulation, but in the absence of conceptuses, the corpora lutea in Mustelids and Marsupials undergo development indistinguishable from that of pregnancy (Moor, 1968; Sharman, 1970). These are functional corpora lutea since sex accessory gland development indicates that they secrete progesterone for a period corresponding to the length of gestation. Rothchild (1967) and Melampy and Anderson (1969) have

noted that the period during which the corpora lutea of the dog, cat, fox, and badger are functional during pregnancy is the same as their functional life span during pseudopregnancy. Thus, there exists a group of mammals in which following ovulation the reproductive cycle is very much the same in non-pregnant and pregnant females. The endocrine function of their gonads does not appear to be influenced by the presence of conceptuses in the uterus. This lack of regulatory function on the part of the conceptus appears to be restricted to Marsupials and large predaceous species in which the rates of reproduction are low (Conaway, 1971).

Effects of the Conceptus on Endocrine Processes During Gestation in Some Common Domestic and Laboratory Animals

Introduction. Perhaps because of their availability and economic value, the reproductive processes of domestic and laboratory animals were studied more thoroughly than those of undomesticated species. Therefore, the most detailed information on the mechanisms whereby endocrine functions in the female are influenced by the conceptus is available on domestic and laboratory animals. In keeping with the principal emphasis of the thesis, attention is given primarily to the effects of the conceptus on ovarian function of the maternal ovaries.

Ewe, Sow, and Cow. The presence of conceptuses in the uterus prolongs the life spans of corpora lutea in the ewe (Moor, 1967), sow (Masuda et al., 1967), and cow (Bowerman and Melampy, 1962). However, in these species, the conceptuses do not increase the functional activities of the maternal gonads. The level of progesterone secreted by

their corpora lutea of pregnancy is similar to the maximum level secreted during the estrous cycle (Moor, 1968).

Prolongation of the life spans of the corpora lutea in the ewe and sow appears to depend upon the conceptus being present in the uterus at days 12 to 13 and 7 to 14, respectively (Moor and Rowson, 1966; du Mesnil du Buisson and Rombauts, 1963). Some progress has been made towards determining if conceptuses exert their influence via mechanical, neural, or humoral mechanisms. There is some evidence that the mechanical distention of the uterus by the conceptus does not cause the prolongation of the life spans of corpora lutea. Plastic coils inserted into the uteri of ewes on day 12 do not provide the stimulus necessary over the critical 12 to 13 day period to prevent regression of corpora lutea on day 15 after ovulation (Moor, 1965). Similarly, uterine distention in the sow does not prevent regression of corpora lutea (Anderson, 1962).

Anderson et al. (1963) investigated the possible role of afferent innervation of the uterus on luteal function in gilts. Luteal regression and follicular development occurred normally after uterine denervation.

There is some evidence that an active principle in early conceptuses prolongs the life span and sustains progesterone secretion by the ewe's ovary. Daily intra-uterine infusion of homogenates of frozen and thawed 14-day and 15-day sheep embryos maintained the corpora lutea of nine test ewes until day 23 or 24 (Rowson and Moor, 1967). In contrast, similar infusions in the sow (Anderson, 1966) did not sustain corpora lutea. However, infusions of irritant or corrosive material did produce persistent corpora lutea in the sow (Anderson et al., 1961). Pyometria

in cows is frequently associated with persistent corpora lutea (Ginther, 1966). Hysterectomy in the ewe, sow, and cow has approximately the same effects on gonadal function as do the conceptuses (Anderson et al., 1963; Malven and Hansel, 1964; Collins et al., 1966). These observations indicate that early in gestation the conceptuses in the ewe, sow, and cow probably influence the endocrine function of the dam's gonad by protecting the corpora lutea from a lytic effect of the uterus or by preventing the uterus from acquiring its lytic properties (Moor, 1968). The conceptus's capacity to block luteolytic influence from the uterus appears to be a function of the placenta in sheep (Moor, 1968). This is indicated from the observation that in the ewe luteal function was maintained for an extended period of time if the placenta was left in situ when the fetuses were removed or destroyed by cautery during the first 50 days of gestation (Moor, 1968). In contrast, when placentas as well as fetuses were removed rapid regression of the corpora lutea of pregnancy occurred.

Teratogenic agents, a number of fetal diseases, and several experiments involving ablation of fetal pituitaries have demonstrated that an intact and healthy fetus is required to induce routine endocrine changes associated with parturition in cows and sheep (Kennedy, 1971). Prolonged gestation is frequently associated with dysfunctional pituitaries and adrenal cortices in sheep. The prolonged gestation in sheep resulting from experimental ablation of the fetal pituitary can be prevented by infusing cortisol into the fetus (Liggins, 1968). The mechanism whereby cortisol exerts its effects is not known. Adrenal hypertrophy of the type seen at normal parturition is a common response of fetuses aborted alive due to several infectious diseases in cows and

sheep (Liggins, 1969). Thus, the fetus's endocrine system may have some influence on the dam's endocrine processes at the time of parturition.

The foregoing observations indicate that in some species, exemplified by the ewe, sow, and cow, the main effect of the conceptuses in the uterus is principally a prevention of luteal regression at a time corresponding to the end of a non-fertile cycle. The embryos, placentas, and fetuses, have all been implicated as regulators of endocrine function of the dam's ovaries during gestation. Distention of the uterus does not appear to be an important factor in establishing an endocrine status of pregnancy in the ewe and sow.

Small Laboratory Animals. The effects of the conceptuses on ovarian endocrine function in the dam can be considered more pronounced in some of the small laboratory animals than in the ewe, sow, and cow discussed in the previous section.

In both pregnant and pseudopregnant mice luteal growth is similar until about the eighth day post coitum by which time the diameter of the corpora lutea is about 0.6 mm. Thereafter, the corpora lutea of pregnancy enlarge rapidly and reach a maximum diameter of 0.9 to 1.0 mm by the 16^{th} day of pregnancy (Deanesly, 1930). In pseudopregnant mice on days four to five fatty granulations are visible in the cytoplasm of the lutein cells. This lipid enables the corpora lutea of pseudopregnancy to be distinguished from those of pregnancy at the same stage after mating (Deanesly, 1930).

The course of pregnancy continues unaltered following removal of the fetuses if the placentas are left *in situ* in the mice (Selye *et al.*, 1935; Newton and Beck, 1939; Deanesly and Newton, 1940). The retained placentas continue to grow, corpora lutea are maintained and parturition

occurs at the normal time. Extracts of mid-term mouse placentas can maintain luteal function in virgin mice when injected into the peritoneal cavity (Cerruti and Lyons, 1960). However, extra-uterine trophoblasts established by transplanting blastocysts (Kirby, 1965) to various sites in host mice is without effect on the corpora lutea. This led Kirby (1965) to propose that secretion of placental luteotropin in the mouse is dependent upon an interaction between the maternal and fetal components of the placenta. Mammary tissue taken from midpregnant nulliparous mice and co-cultured with placentas, exhibit responses similar to those obtained with prolactin. Furthermore, placental fragments transplanted to mammary fat pads caused local lobuloalveolar development (Kohmoto and Bern, 1970). Transplants of five-day placentas stimulated the mammary gland only if the entire placenta with fetus was used. Whether the mammogenic and luteotropic substance from mouse placentas are the same is not known.

Corpora lutea in both pregnant and pseudopregnant rats reach a diameter of about 1.4 mm by the tenth day after mating. The corpora lutea of pregnancy then enlarge to attain a diameter of 1.9 mm on about the 16^{th} day gestation. Luteal regression occurs in pseudopregnant rats after day 12 (Long and Evans, 1922; Weichert and Schurgast, 1942). By the fifth day after mating, the corpora lutea of rats with conceptuses in the uterus secrete twice as much progesterone as those of pseudopregnant females at the same stage following mating (Eto et al., 1962). The mechanisms whereby the conceptuses elicit these responses are not known. It has been suggested that during the pre-implantation stages the embryo might exert both an anti-luteolytic and luteotropic effect on the corpora lutea (Ray et al., 1955).

In rats pregnancy is maintained when the fetuses are removed, although pregnancy is usually prolonged (Selye et al., 1933). The placentas must remain attached to the uterus or pregnancy is terminated in the rat (Klein, 1935). Placentas from 12-day pregnant rats when homogenized and injected for ten days into rats with normal estrous cycles produced vaginal mucification and development of the mammary glands (Ray et al., 1955). Saline extracts of rat placentas injected into pseudopregnant rats after hypophysectomy on the fourth day after mating sustained enough luteal activity to produce deciduoma, although fresh placental extracts injected from the 19^{th} day of pregnancy onward did not delay parturition (Astwood and Greep, 1938).

In the guinea-pig, the endocrine function of the maternal ovaries is noticeably affected by the conceptus. The volume of corpora lutea in pregnant and non-pregnant guinea-pigs grows to about 2 mm^3 by the ninth to eleventh day after ovulation. Luteal regression then occurs in non-pregnant animals, whereas in pregnant females the corpora lutea continue to grow until they reach a maximum volume of 3 mm^3 on about the 20^{th} day of gestation (Rowlands, 1956). The concentration of progesterone in systemic plasma remains low until about the end of the second week after mating. Between the 15^{th} and 20^{th} day of pregnancy the progesterone concentration rises (Heap and Deanesly, 1966; Heap et al., 1967). Surgical removal of the entire conceptus at any time during gestation in the guinea-pig results in regression of the corpora lutea within two to three days (Bland and Donovan, 1966). Fetectomy does not cause regression of corpora lutea, although specific changes in ovarian progestin secretions are not known (Klein, 1939; Nelson, 1934). Hypophysectomy as early as day three after mating in guinea-pigs does not

interrupt pregnancy or alter progesterone levels provided viable conceptuses are present in the uterus (Heap et al., 1967). Evidently, a relationship between the conceptuses and corpora lutea that is independent of the pituitary, is established at an early stage of gestation in guinea-pigs. The conceptuses secrete enough progesterone by the fourth week after mating to maintain pregnancy in the absence of ovaries (Deanesly, 1963).

Corpora lutea of non-pregnant hamsters regress at about nine days after ovulation (Greenwald and Rothchild, 1968). The corpora lutea in pregnant hamsters continue to grow and reach a maximal diameter of about 1.05 mm by days 14-16 (Greenwald et al., 1967). Beginning on about day 12 of pregnancy there is an increase in the number of large antral follicles (Greenwald, 1964). In the hamster, removing the pregnant uterus between the eighth and thirteenth days causes a premature and rapid regression of the corpora lutea of pregnancy and the ovarian cycle is reinitiated (Klein, 1938). After fetectomy in the hamster the corpora lutea of pregnancy are maintained and inhibition of the ovarian cycle continues provided the placentas remain attached (Klein, 1938).

In summary it can be said that in the mouse, rat, guinea-pig, and hamsters the conceptuses cause substantially more growth of luteal tissue and secretion of progestins than occurs in nonfertile cycles. In all these species the placentas appear to be the components of the conceptuses which manifest the most significant effects on the dam's ovaries. Removing the fetuses does not appear to greatly influence ovarian function during gestation in these small laboratory animals.

The cells responsible for the secretion of placental gonadotropic factors in small laboratory animals have not been identified but the

evidence favors the giant trophoblast cells. Mayer and Canivenc (1965) have found that rat placental autographs with prominent large trophoblasts were responsible for luteotropic, mammotropic, and lactogenic effects. In mice, Kohmoto and Bern (1970) found in organ transplants and cultures that the blastocyst and giant cells have lactogenic activity on days six and seven and that the placental labyrinth and giant cells can cause lactogenic effects after eight days of pregnancy.

It should be noted that while there is ample evidence indicating that gonadotropins are elaborated by the placentas in some of the small laboratory animals, no purified gonadotropins from these sources have been reported to date.

Species in Which the Occurrence of Specific Gonadotropins and Some Other Hormones Can Be Related to the Presence of the Conceptus

Women. The conceptus in women gives rise to several hormones that have effects on the mother's ovaries and accessory sex structures. These include human chorionic gonadotrophin (HCG), human placental lactogen (HPL), estrogens, and progesterone (Hobson, 1971). There is very likely an interplay in hormone production by the fetus and placenta (Diczfalusy, 1964).

Midgley and Pierce (1962) and Thiede and Choate (1963) obtained histochemical evidence of localization of HCG in the placental syncytium. The precise role of HCG during pregnancy is not clear. Suggestions have been made that it might stimulate placental steroid secretion (Hobson, 1971). Since HCG appears in serum of pregnant women before commencement of the menses that normally follows infertile

ovulations (Lyon et al., 1955), it is possible that HCG acts to block ovarian cycles during early gestation (Page, 1967). HCG is capable of prolonging the life of the corpus luteum of the normal cycle in women (Brown and Bradbury, 1947), monkeys (Hisaw, 1944), and rats (Yochim and De Feo, 1962). HCG has been indicated as the cause of prolonged progesterone secretion by the ovaries following cases of abdominal pregnancies in women in which the placenta was not wholly extirpated (Kim et al., 1971).

HPL is a protein hormone immunologically related to human pituitary growth hormone. It is secreted in steadily increasing amounts during pregnancy and has been found to be localized in the syncytiotrophoblast (Sciarra, Kaplan, and Grumbach, 1963). Although its physiological role remains to be established, it has somatotrophic, lactogenic, and luteotropic effects in test animals, and can potentiate the biological effect of HCG and human growth hormone (Joseimovich, 1967).

Progesterone and estrogens are produced by the human syncyteotrophoblast. The syncytium becomes the main site of steroid synthesis during later pregnancy (Ryan, 1962) and can maintain pregnancy in the absence of the ovaries after day 41 of the last menstrual period (Melinkoff, 1950; Tulsky and Koff, 1957).

Thus, women are an example of a species in which the conceptus is known to effect changes on endocrine processes of the mother. Furthermore, in women a compound has been isolated that is elaborated by the conceptus and that has pronounced effects on progesterone secretion by the mother's ovaries.

Non-Human Primates. Hobson (1971) has indicated in a recent review article that very little is known about the endocrinology of pregnancy

in non-human primates. However, it is known that chorionic gonadotropin (CG) is produced in several species of non-human primates including the gorilla, the baboon, the rhesus monkey, and the marmoset (Hobson, 1971). The urinary excretion patterns of CG in these species resembles that of human chorionic gonadotropin (HCG). It is assumed that CG in non-human primates acts on the endocrine processes of the mother in a fashion similar to that of HCG in women. Thus, elaboration of gonadotropins by the conceptus is not a phenomenon peculiar to women.

Mare. The mare serves as another example of an animal in which the conceptus elicits the presence of a particular gonadotropin in the mother. The particular compound found in the mare is called pregnant mare's serum gonadotropin (PMSG). PMSG appears in the serum of mares between days 35 to 41 of gestation (Cole and Hart, 1930). Maximum levels occur at about day 60. The compound disappears from the serum by days 130-150. Earlier studies indicated that the decidual cells of the endometrial cups were probably the source of PMSG (Cole and Goss, 1943). Later studies have implicated the glandular epithelium of the uterus (Clegg et al., 1954).

In the mare, super-ovulation and luteinizing of ovarian follicles has been correlated with peak titers of PMSG in serum and indicates a gonadotropic function for PMSG in the mare related to the provision and maintenance of a new crop of corpora lutea (Cole and Hart, 1930; Cole et al., 1931).

The nature of the stimulus that initiates elaboration of PMSG has not been reported in the literature. Attempts to induce production of endometrial cups by mechanical distention of the uterus with a balloon were not successful (King, 1966). A jenny donkey mated to a stallion to

give a hinny fetus attained high levels of PMSG (203 I.U./ml) in plasma compared to a donkey with donkey fetuses (32 - 53 I.U./ml) or mares carrying mule fetuses (17 - 22 I.U./ml) (Allen, 1969; Clegg et al., 1962), indicating that the conceptus's genotype may be important in inducing the phenomenon of PMSG production.

Conclusions

The foregoing material on some of the effects the conceptus has been observed to have on maternal endocrine processes during gestation illustrates that in some species the presence of the conceptus not only prolongs luteal function as in the ewe, sow, and cow, but can also cause an alteration or increase in ovarian endocrine function, as indicated in the mouse, rat, guinea-pig, hamster, women, and mare. In these species up to the time of parturition, the conceptus's capacity to influence the endocrine function of the mother's ovaries appears to stem principally from the placenta. No specific factor(s) resembling what has been found in women or the mare has been isolated from the mouse, rat, guinea-pig, or hamster. However, preparations including saline extracts, homogenates, and transplants of conceptus tissue from rats and mice have been shown to have gonadotropic activity.

The significance of these observations is twofold. First, they document that the conceptus can have a regulatory influence on the endocrine function of the adult female gonad. Secondly, in some species, in which growth of luteal tissue and ovarian output of progestins is enhanced by the conceptus--notably in the mouse, rat, guinea-pig, hamster, women, and mare--the presence of the conceptus appears to result in the elaboration of gonadotropins from the uterus.

Further understanding of the processes that regulate the adult female gonad would be enhanced by determining the particular tissues of the ovary that are subject to influence by gonadotropins emanating from the gravid uterus and determining the tissues from which the gonadotropins originate.

During the course of the present review of literature no reports were found in which ovarian endocrine function was directly assessed by measuring specific progestins in ovarian venous plasma following fetectomy, conceptectomy, or hysterectomy. The effects of such treatments on the endocrine function of the female gonad were indirectly assessed by interpreting gross changes apparent on the ovary or changes in secondary sex organs. The observations on changes in endocrine function of the ovaries based on biological endpoints need to be confirmed by monitoring specific species of hormonal steroids in ovarian venous blood.

Endocrine Functions of the Maternal Ovary During Gestation in the Rabbit

Introduction

The first part of this chapter established that the gonads in adult females are subject to regulatory influences from the conceptus in women and some laboratory and domestic animals. However, the mechanisms whereby the conceptuses can influence the endocrine functions of the gonads in the mother remain incompletely described in most species. Ideally a complete description of the mechanisms whereby the conceptuses regulate ovarian endocrine function would include the following points:

1) unequivocal evidence that a gonadotropic principle existed, 2) localization of its site of origin, and 3) localization of its site of action. These are general points which need to be established in a number of species to provide a broad base for understanding the regulatory influences acting upon the gonads of adult females. Particular questions related to these points are: 1) how long acting are the regulatory influences from the gravid uterus, 2) from what anatomical part of the gravid uterus do the influences that regulate ovarian endocrine function originate, and 3) is luteal tissue the principal ovarian tissue subject to influences from the gravid uterus? The purpose of the last portion of this chapter is to establish the premises on which experiments can be based that pursue answers to these questions in the rabbit.

There are well defined ovarian compartments which are known to produce specific species of steroids in the rabbit. Furthermore, in the rabbit maintenance of luteal tissue during the last half of gestation appears to be dependent upon the presence of factors emanating from the gravid uterus. These features suggest that the rabbit is particularly suited for experiments to determine which of the ovarian tissues are particularly subject to influences from the gravid uterus, from which part of the gravid uterus they originate, and how long acting the regulatory influences are that come from the gravid uterus. Particular premises that need to be documented with regard to rabbits are the types of hormones that are elaborated by the rabbit ovary, which ovarian tissues produce them, their function during pregnancy in the rabbit, and the regulation of their production. Special emphasis is given to estrogens and progestins since these are the hormones

produced by the ovary which appear to be subject to influence from the gravid uterus and about which the most information is available.

Steroids Secreted by the Rabbit Ovary

20αOH-Progesterone. Simmer et al. (1963) isolated material from ovarian venous blood of estrous rabbits which they identified as 20αOH-progesterone. They based their identification on several criteria that included migration on paper chromatograms, color reactions, oxidation products, formation of the acetate derivative, ultraviolet absorption, and infrared spectroscopy. Several other investigators have subsequently confirmed the presence of 20αOH-progesterone in rabbit ovarian venous blood (Dorrington and Kilpatrick, 1966; Exley et al., 1968; Hafez et al., 1965; Okano et al., 1966). In these studies ovarian venous blood was found to contain several fold more 20αOH-progesterone than systemic blood indicating that 20αOH-progesterone was secreted by the ovary.

Progesterone. Progesterone in ovarian venous and peripheral blood of pregnant rabbits was measured by physicochemical methods for the first time by Mikhail et al. (1961). They based their identification of progesterone on ultraviolet absorption and infrared spectroscopy following isolation procedures that included partitions with organic solvents and paper and alumina column separations. Simmer et al. (1963) confirmed the identity of progesterone in ovarian venous blood of rabbits treated with gonadotropins using essentially the same criteria. The maximum level of progesterone Mikhail et al. (1961) found in peripheral blood of pregnant rabbits was 0.14 μg/ml whereas ovarian venous blood at the same stage of gestation, day 16, contained 2.36 μg/ml.

These results support the contention that progesterone is a hormone secreted by the ovary during pregnancy.

Estrogens. Estrogens have been measured in rabbit systemic and ovarian plasma by Eaton and Hilliard (1971). In three rabbits bled by heart puncture two minutes after mating, plasma concentrations of estradiol were six, 22, and five pg/ml and estrone concentrations were two, three, and 22 pg/ml. At 125 minutes after mating estradiol concentrations in systemic plasma of the same animals were nine, 24, and six pg/ml and estrone concentrations were 21, 25, and 22 pg/ml, respectively. In contrast, estradiol measured in ovarian venous plasma of estrous rabbits averaged 323 pg/ml plasma indicating estradiol secretion by the ovary. While the authors could not provide classical physicochemical evidence to establish the structure of the compound they assayed, there is indirect evidence that it was 17β-estradiol. The radio-ligand assay procedure (Korenman et al., 1970) used in these experiments makes use of estrogen binding properties of rabbit uterine cytosol which is quite specific for compounds with estrogenic biological activities (Korenman, 1969). The different estrogens are separated from each other on a celite column before the sample is assayed. Other studies suggest that 17β-estradiol could be the principal estrogen in rabbits (Saldarini et al., 1970). It is 100 times more effective in sustaining luteal function in hypophysectomized rabbits and ten times more effective in promoting uterine growth in immature ovariectomized rabbits than the α-isomer.

Ovarian Tissues Associated With Steroid Secretion

Interstitial Tissue and Corpora Lutea. Recently two studies have been reported on steroid secretion by interstitial tissue and corpora lutea in ovaries of pregnant rabbits. Endo et al. (1969) collected ovarian venous blood from eight rabbits at mid-pregnancy for 30 minutes before and after surgical removal of corpora lutea by cauterization. Evidence that cauterization destroyed only corpora lutea was obtained histologically. Progesterone concentrations dropped from 25 ± 2.6 μg/100 ml blood before cauterization to less than 2 μg/100 ml blood after cauterization. In contrast, 20αOH-progesterone concentrations decreased to a lesser extent, from 59 ± 16.2 μg/100 ml blood before to 44 ± 12.2 μg/100 ml blood after cauterization. Eaton and Hilliard (1971) also demonstrated that in pregnant rabbits progesterone secreted by the ovary comes principally from the corpora lutea and 20αOH-progesterone principally from the interstitial tissue. They anesthetized rabbits within two to four hours after mating and removed surface follicles 1 mm or more in diameter from one ovary by cauterization, thereby preventing subsequent formation of corpora lutea in that ovary. Sixteen to 26 days later blood was collected simultaneously from both ovaries and the steroid outputs compared. Progesterone output by the ovaries with corpora lutea present averaged 30.5 ± 4.1 μg/ovary/hr compared to 6.5 ± 2.0 μg/ovary/hr from the ovaries in which corpus luteum formation was prevented by cauterization. Average 20αOH-progesterone output by ovaries without corpora lutea was 67.0 ± 14.8 μg/ovary/hr which was not different from the 63.5 ± 9.6 μg/ovary/hr average output by ovaries with corpora lutea.

Follicles. When surface vesicular follicles 1 mm or more in diameter were destroyed in one ovary by cauterization immediately before simultaneous cannulation of the ovarian veins of both ovaries of an estrus rabbit, the output of estradiol from the cauterized ovary was less than that of the control ovary, 12.5 and 1.0 ng/ovary/hr, respectively (Eaton and Hilliard, 1971). Subsequent intravenous injection of luteinizing hormone increased estradiol output by the control ovary to 20.1 ng/ovary/hr, whereas the ovary that had cauterized follicles produced only 2.5 ng estradiol/ovary/hr, indicating that in estrus rabbits the follicles are probably the main source of estrogens. In pregnant rabbits, destruction of follicles by x-irradiation causes a subsequent regression of corpora lutea which can be overcome by giving exogenous estrogens (Keyes and Nalbandov, 1967), indicating that follicles may be a source of estrogen during pregnancy.

The foregoing discussion indicates that 20αOH-progesterone from interstitial gland tissue, progesterone from luteal tissue, and probably 17β-estradiol from follicles can be considered as compounds secreted by ovaries in rabbits. The next section documents the output of these steroids from the ovaries at different times during the reproductive cycle of the rabbit and indicates that ovarian endocrine function is closely related to the reproductive status of the rabbit. Since it is known that specific ovarian tissues produce specific hormonal steroids, secretion of a particular steroid would be indicative of the site of action of regulatory influences on the female gonad during gestation in the rabbit.

Ovarian Morphology and Steroid Secretion During Pregnancy and Pseudopregnancy in the Rabbit

Ovaries from pregnant and pseudopregnant rabbits differ from each other with respect to the average weights and size of their corpora lutea. In pregnant rabbits corpora lutea reach a maximum average weight of about 20 mg between days 15-20 after mating and show only a slight decrease in weight, to about 18 mg, at days 26-30. In contrast, the average weight of corpora lutea in pseudopregnant rabbits reaches a maximum of approximately 18 mg at days eight through 14 after mating and by 21 days after mating weigh only 7-8 mg (Hilliard et al., 1968). Hammond (1917) measured the diameters of corpora lutea in ovaries taken from pregnant and pseudopregnant rabbits and rabbits with decidual cells produced by trauma. Unfortunately the system of units of his measurements were not given. By day 15 after coitus corpora lutea obtained from pregnant rabbits attained diameters of about 14 units which remained approximately the same until day 30, the last date recorded. Corpora lutea in pseudopregnant rabbits or rabbits with deciduoma attained diameters of 13.2 units at days 13-14, but thereafter declined to 6-7 units by day 27-29. Hughes and Myers (1966) observed that as early as day 12, corpora lutea of pseudopregnancy show irregularities in the outline of the nuclei and an abundant influx of leucocytes and incipient fatty degeneration of some luteal cells. Changes occurring in follicular tissue during pregnancy and pseudopregnancy in the rabbit are not as thoroughly documented as the changes in corpora lutea. Hughes and Myers (1966) indicate follicles are prominent structures on ovaries of pseudopregnant rabbits. Adams (1968) noted that there is an increase in the number of Graafian follicles before day 17 of

pregnancy and that the number of follicles remains high until near term.

The data from Hilliard et al. (1968), Okano et al. (1969), and Mikhail et al. (1961) are in close agreement with each other with regard to the amounts of progestins secreted by the rabbit ovary during pregnancy and pseudopregnancy. Ovaries in pregnant rabbits secrete progesterone at less than 10 μg/g ovary/hr at days one to four after mating, secretion increases to a maximum of 70-80 μg/g ovary/hr at days 15-20, after which progesterone secretion declines to 10-20 μg/g ovary/hr at days 26-30. 20αOH-progesterone secretion by ovaries in pregnant rabbits rises from less than 10 μg/g ovary/hr at days one to four after mating to a maximum of 150 μg/g ovary/hr at days eight to 14, thereafter 20αOH-progesterone declines to about 70-80 μg/g ovary/hr until after parturition. Estradiol secretion (Hilliard and Eaton, 1971) rises from less than 5 ng/ovary/hr at days one through three after mating to 10-30 ng/ovary/hr at days four through ten, the last dates at which estradiol was reported measured.

Patterns of progesterone and 20αOH-progesterone secretion during pseudopregnancy differ from those of pregnancy (Hilliard et al., 1968). Progesterone secretion from ovaries in pseudopregnant rabbits reaches a maximum of 40-50 μg/g ovary/hr at days eight through 14 and then declines to less than 10 μg/g ovary/hr by day 21; 20αOH-progesterone secretion increases from less than 10 μg/g ovary/hr at days one through four after mating to 160-170 μg/g ovary/hr at days 15-20 and remains elevated through at least day 21.

Thus, the presence or absence of the conceptuses in the uterus following mating can be associated with definite increases in

progesterone and decreases in 20αOH-progesterone output by the rabbit ovary.

Thus, endocrine function of specific tissues, most notably interstitial and luteal, of the ovaries appear to undergo characteristic changes during gestation in the rabbit; the presence of the conceptus can be said to result in lesser output of 20αOH-progesterone by interstitial tissue and greater output of progesterone by corpora lutea at days 15 through 25 following ovulation, than occurs during pseudopregnancy.

Extra-Ovarian Sources of Progestins and Estrogens During Pregnancy in the Rabbit

Preceding sections have indicated that interstitial, luteal, and follicular tissues in the rabbit ovary are sources of specific steroids, the secretion of which appears to be correlated with the reproductive status of the female. Investigations were conducted determining if the gravid uterus and placentas are a source of steroids resembling steroids secreted by the ovary. Progestins were measured during pregnancy in the peripheral serum of rabbits by the Hooker-Forbes bioassay based on stromal cell hypertrophy of the uterus in overiectomized mice (Zarrow and Neher, 1955). When rabbits were castrated on the 24th day of gestation progestins measured in peripheral serum dropped 40 to 60 percent within the first 12 hours and decreased to 75 percent of their original value by 24 hours after castration. An almost identical drop in serum progestins was observed when castration was combined with hysterectomy. Since the Hooker-Forbes bioassay gives a response to both 20αOH-progesterone and progesterone (Zander et al., 1958) it is

generally considered that the placenta in the rabbit is not an important source of 20αOH-progesterone or progesterone. Implantation of placentas into vaginas of immature mice did not produce cornification (Astwood and Greep, 1938) indicating the absence of estrogens. These findings are consistent with the observation by Ainsworth and Ryan (1966) that aromatizing and 17β-ol-dehydrogenase systems appear to be lacking in rabbit placentas.

Thus, the ovaries appear to be the principal source of progestins and estrogens during pregnancy in the rabbit. Indications of fluctuations in progestin and estrogen production based on sex accessory gland function or structure during gestation suggest ovarian production of these compounds.

Significance and Function of Ovarian Steroids During Gestation in the Rabbit

Up to this point the review has been concerned with documenting the particular species of steroids secreted by the ovary, ovarian compartments from which they originate, and temporal sequence in which they are secreted in the rabbit. The following section documents the function of ovarian steroids during gestation in the rabbit.

When both ovaries were removed from rabbits at anytime during the first six days of pregnancy, the uterus was always found empty when examined ten or more days later (Fraenkel, 1910). Corner (1928) found only degenerate embryos when he examined on days four through seven after mating seven does that had been ovariectomized 14-18 hours after mating. Courrier and Kehl (1938) castrated does on days four, ten, thirteen, and eighteen and examined them five through seven days later.

They observed that pregnancy had been terminated in all rabbits due to lack of implantation, reabsorption of conceptuses, or abortion. Bilateral oophorectomy on the 16th and 21st days of pregnancy caused abortion four days later (McIlroy, 1912). Robson (1936) performed castration on several dates between the 20th and 29th days of gestation. All does aborted 24-50 hours later. If the corpus luteum alone is removed from the ovaries of pregnant does gestation is terminated (Corner, 1928). Destruction of follicles by x-irradiation terminates pregnancy (Keyes and Nalbandov, 1967). Thus, it is apparent that the ovaries are required at all stages of pregnancy in the rabbit and that both the corpora lutea and follicles, serve vital functions in maintaining gestation.

Allen and Corner (1930) succeeded in maintaining pregnancy to term in rabbits ovariectomized 18 hours after mating by injecting extracts of swine corpora lutea. Later it was found that the crude extracts contained two ovarian hormones, estrogen and progesterone (Allen, 1932), and that the more purified extracts (Allen and Meyer, 1933; Wintersteiner and Allen, 1934) containing low titers of estrogen, while capable of sustaining pregnancy in castrates (Allen, 1937), did not produce the same effects that the crude extract produced. The difference in effects produced by crude and purified extracts with regard to uterine and mammary gland function and development was quite apparent in castrate pseudopregnant rabbits. The uteri of castrated pseudopregnant rabbits injected with progesterone alone developed progestational proliferation identical to intact pseudopregnant or pregnant rabbits up to day eleven. However, progesterone had to be supplemented with estrogen to obtain uterine development in castrates that was

similar to 16 day uteri of pseudopregnant and pregnant animals. Mammary glands removed from castrated pseudopregnant rabbits were indistinguishable from those of pregnancy of comparable stages when progesterone, or estrogen and progesterone, were given through day 16. While the mammary glands could be developed through day 16, they regressed between the 16^{th} and 20^{th} day if only progesterone was continued. Thus, mammary glands responded to progesterone alone in a fashion comparable to the uterus except that the uterus began to fail to respond to progesterone at about the 11^{th} day, whereas the mammary glands began to fail at about the 16^{th} day. When estrogen was given along with progesterone the mammary glands continued to grow and glands were obtained at 21 days after castration which closely resembled those of pregnancy. Mammary gland development in pseudopregnant rabbits is similar to that in pregnant rabbits up to day 16 (Asdell and Salisbury, 1933; Asdell and Hammond, 1933); thereafter, in the absence of conceptuses in the uterus mammary glands regress. Prolonging the life of the corpus luteum of pseudopregnant rabbits from 15-16 days to 27-29 days by hysterectomy does not result in further development of the mammary glands comparable to that found in rabbits with conceptuses in the uterus (Asdell and Hammond, 1933). Apparently both exogenous progesterone and estrogen are needed to mimic the effects that the presence of the conceptus has on accessory sex organs.

Other indications that progesterone and estrogen of ovarian origin are important in endocrine processes associated with gestation come from experiments that investigated the action of oxytocin in the pregnant rabbit. Knaus (1929, 1930) showed that the rabbit's uterus responds to oxytocin <u>in vitro</u> when the animal was in heat, but was

refractory during pseudopregnancy, or 21 days of pregnancy. The non-responsiveness of the uterus to oxytocin was shown to be due to the presence of the corpus luteum and specifically to progesterone. It was also apparent that while progesterone alone made a uterus refractory to oxytocin, estrogen and progesterone together made it more refractory and caused a phenomenon known as relaxation to occur. The relaxation response is characterized by inhibition of existing uterine contractions in the presence of substantial amounts of oxytocin. Relaxation phenomenon can be observed in uteri of pregnant and pseudopregnant rabbits from day six to day eleven. At day 16 relaxation is not quite as marked; however, at this time uteri from pregnant rabbits exhibit more relaxation than uteri from pseudopregnant rabbits which are once again very sensitive to oxytocin. From work with castrated rabbits, Makepeace et al. (1936) showed that crystalline progesterone did not completely replace the action of a normal ovary containing many functional corpora lutea. To produce uteri which respond to oxytocin in the same manner as those from pregnant rabbits it is necessary to give a small amount of estrogen along with progesterone.

The foregoing observations are indicative that in the pregnant rabbit ovarian progesterone and estrogen are associated with mammary and uterine tissue development and regulation of uterine mobility. Follicular estrogen also appears to be required to maintain progesterone secretion by the corpora lutea. More details on the role of follicular estrogens will be presented in the next section in conjunction with regulation of corpus luteum function during gestation.

The function of 20ΩOH-progesterone during gestation is not known. Hilliard et al. (1967) suggested that this compound acts as a positive

feedback agent to prolong and heighten LH discharge to induce ovulation in the mated rabbit. Although 20αOH-progesterone possesses one-third to one-half the activity of progesterone in the Clauberg rabbit bioassay, and one-fifth the activity of progesterone in the Hooker-Forbes mouse bioassay (Zander et al., 1958), it is relatively ineffective in promoting nidation in spayed rabbits (Rennie and Davies, 1965). Wiest et al. (1963) have proposed that the metabolic reduction of progesterone to 20αOH-progesterone serves to reduce the progestational potency of ovarian secretions.

Regulation of Ovarian Steroid Secretion During Gestation in the Rabbit

Introduction. The preceding section documented the function that ovarian steroids serve during gestation in the rabbit. This section documents factors that influence steroid secretion by the female gonad.

Regulation of Luteal Progesterone Secretion by Follicular Estrogen. Follicular involvement in promoting luteal function during gestation in rabbits was indicated by the experiments of Westman (1934) who cauterized follicles of pregnant rabbits at various intervals after mating and observed that the development, as well as the persistence and function of the corpora lutea were adversely affected. Subsequently it was discovered that injections of exogenous estrogen maintained corpora lutea, inhibited parturition of pregnant rabbits (Heckel and Allen, 1939) and sustained pregnancy in hypophysectomized rabbits. However, exogenous estrogen did not sustain pregnancy in the castrate or hypophysectomized castrate rabbit (Greep, 1941; Robson, 1940). Apparently exogenous estrogen is able to sustain pregnancy only if

corpora lutea are present and does so by promoting the corpora lutea to secrete sufficient progesterone to maintain pregnancy. Such action by estrogen is consistent with the observations that exogenous estrogens prolong the functional life span of corpora lutea in pseudopregnant rabbits (Allen and Heckel, 1936) and maintains progesterone secretion by corpora lutea transplanted to the kidney capsule in castrated rabbits (Keyes and Armstrong, 1969). Exogenous estrogen will also maintain progesterone secretion from corpora lutea in ovaries that have had the follicles destroyed by x-irradiation (Keyes and Nalbandov, 1967). A further observation implicating estrogens with luteal function is the recent finding that the corpora lutea of rabbits contains high concentrations of estrogen receptor protein which decline prior to regression of corpora lutea (Lee et al., 1971). Thus, progesterone secretion from luteal tissue during the last half of gestation is dependent upon production of estrogens from the follicles in rabbits.

Regulation of Ovarian Function by the Pituitary. Preceding sections have documented that full development of a progestational uterus and functional mammary glands in the rabbit appears to depend upon progesterone and estrogen secretion from the ovaries. Hypophysectomy, performed at any stage of pregnancy results in atrophy of corpora lutea (Deanesly et al., 1930; Smith and White, 1931; Westman and Jacobsohn, 1937; Firor, 1933) and follicles (Foster et al., 1937; Spies et al., 1968). However, the nature of the pituitary hormone(s) which supports the secretory activity of the ovary during pregnancy has not been resolved.

Injections of antibodies against ovine luteinizing hormone into rabbits between day three and 18 of pregnancy induces regression of

corpora lutea and complete resorption of embryos (Spies and Quadrin, 1967). How LH acts in the intact rabbit to sustain ovarian function is controversial. A single 50 μg injection of LH induces luteal regression in intact, pseudopregnant rabbits (Stormshak and Casida, 1964; Spies et al., 1966). Kilpatrick et al. (1964) found from histological studies of luteal and endometrial tissue that large doses of LH initiated 12 hours after hypophysectomy, partially maintained existing corpora lutea for at least seven days; whereas, if the LH treatment was started at two hours after hypophysectomy, a new crop of corpora lutea resulted and the original corpora lutea degenerated. Following hypophysectomy, LH injections delay atrophy, but do not stimulate steroidogenesis in corpora lutea previously transplanted beneath the kidney capsules; nor does LH alone maintain follicular morphology in the hypophysectomized rabbit (Rennie, 1968). Spies et al. (1968) were able to sustain pregnancy in hypophysectomized rabbits for one week with estrogen or estrogen combined with prolactin and from these and other experiments concluded that subluteolytic amounts of LH combined with FSH were required to maintain estrogen secretion by the follicles. Hilliard et al. (1971) found that follicles sustained on FSH alone failed to synthesize sufficient estrogen to support luteal function in hypophysectomized pseudopregnant rabbits. However, when small doses of LH (65 ± μg administered in saline twice daily/g ovary) were given with the FSH, luteal function was maintained. They suggested that larger doses of LH suppressed estrogen secretion either by luteinizing follicles or depleting cholesterol ester stores which are thought to serve as substrate for progesterone synthesis.

In addition to synergizing with FSH to maintain estrogen secretion by the follicles that is required for progesterone secretion by the corpus luteum, LH is also a specific effector of 20αOH-progesterone secretion by interstitial tissue. This was demonstrated by the experiments of Dorrington and Kilpatrick (1966) in which they measured the amount of 20αOH-progesterone and progesterone secreted into the ovarian venous blood of estrous and hypophysectomized rabbits before and 15 minutes after intravenous injection of LH. Ovarian secretion of 20αOH-progesterone in estrous rabbits increased from an average of 14.4 μg/ovary/hr before injection to 163 μg/ovary/hr after injection. In these same rabbits the level of progesterone rose in response to LH injections from none detectable (1 μg or less/ovary/hr) before injection to an average of 28.1 μg/ovary/hr after injection. In four out of the 11 rabbits no response was observed. Injection of LH into rabbits that were hypophysectomized from three to 56 days stimulated 20αOH-progesterone secretion to rise from less than 1 μg/ovary/hr to an average of 104 μg/ovary/hr. Progesterone secretion in response to LH in these same animals was less marked; it rose from less than 1 μg/ovary /hr to an average of near 1 μg/ovary/hr although in eight out of the 12 rabbits no increase was detectable. Since hypophysectomy produces involution of corpora lutea and atrophy of follicles it was concluded that 20αOH-progesterone secreted by interstitial tissue is independent of both luteal tissue and follicles. Other experiments have also demonstrated this. Rabbits in which the mature follicles have been destroyed by cautery (Hilliard et al., 1963) or x-irradiation (Keyes and Armstrong, 1968) show increased secretion of 20αOH-progesterone in response to LH and comparatively small increases in progesterone.

Corpora lutea transplanted to beneath the kidney capsule do not show acute increased 20αOH-progesterone secretion to intravenous LH injections.

In vitro experiments of Dorrington and Kilpatrick (1969) provide additional evidence that LH is a specific effector of 20αOH-progesterone secretion by interstitial tissue. No significant increase in progesterone synthesis was produced by the addition of 0.1 μg LH/ml to corpora lutea *in vitro* and only a slight increase by 2 μg LH/ml. Similarly, 20αOH-progesterone production by corpora lutea tissue in response to LH was considered less than by whole ovarian tissue. This contrasted with increases in release of 20αOH-progesterone and progesterone from interstitial tissue that was free of corpora lutea in response to LH. Interstitial tissue alone, produced slightly more 20αOH-progesterone than an equal weight of whole ovarian tissue upon stimulation by LH, as would be expected if the corpora lutea are unresponsive to LH.

The 20αOH-progesterone secretion response of interstitial tissue is apparently fairly specific for LH. Purified FSH, prolactin and ACTH do not elicit the response (Hilliard *et al.*, 1968, 1971; Spies *et al.*, 1968). The amount of progesterone secreted by interstitial tissue in response to LH does not appear to exceed the rate of 20αOH-progesterone secretion. Therefore, when progesterone concentrations in ovarian venous blood are equal or are greater than 20αOH-progesterone it would be indicative that corpora lutea are probably functional.

Thus, monitoring 20αOH-progesterone and progesterone concentrations in ovarian venous plasma of rabbits would indicate whether or not the ovary is being stimulated by LH. High 20αOH-progesterone secretion rates would indicate this might be the case. On the other hand, high

progesterone secretion could be interpreted as an indication that ovarian follicles were secreting sufficient estrogen to sustain luteal function.

Regulation of Ovarian Function by the Uterus. The influence of the uterus on ovarian function in the rabbit depends upon the reproductive status of the rabbit when the uterus is removed. Hysterectomy a considerable interval of time before mating (Asdell and Hammond, 1933) or during pseudopregnancy (Siegmund, 1934; Loeb and Smith, 1936; Gillard, 1937; Chu et al., 1946) tends to lengthen the luteal survival time by six to ten days beyond the 15-16 day pseudopregnant period of normal rabbits. Loeb and Smith also emphasized that the rapid involution of the corpus luteum in the normal animal appeared to be an active inflammatory dissolution, whereas in the hysterectomized rabbit the involution was very slow.

Howe (1968) found that a positive relationship, based on corpus luteum weights, exists between the amount of uterine tissue removed and the degree of luteolytic retardation. Luteolytic retardation was not highly significant until about day 18 after ovulation. In contrast, the mean luteal weight was significantly larger in hysterectomized females by day ten after ovulation. Evidently removal of uterine tissue in rabbits causes an increased rate of luteal development, increased maximal luteal weight and retarded luteal regression. Howe suggested that the latter aspect of luteolytic inhibition may simply be the consequence of enhanced luteal development, hence more time needed for luteal regression rather than a direct retardation of luteolysis. Sessums and Murphy (1933) reported that hysterectomy in the rabbit inhibits the development of estrus and causes degenerative changes in

the ovary. Transplantation of the uterine endometrium tended to check the inhibitory and degenerative changes. They thought that the uterine endometrium might elaborate a hormone which influences ovarian activity.

Removal of the uterus during the first half of pregnancy shortens the life span of the corpora lutea of pregnancy to approximately that of pseudopregnancy (Micale, 1940; Greep, 1941), whereas hysterectomy during the second half of pregnancy resulted in regression of corpora lutea to less than half their usual volume within five days (Greep, 1941).

It is generally believed that the corpus luteum is the only part of the reproductive tract which is influenced by the uterus, though a contrary opinion has been expressed by Mishell and Motyloff (1941) and Tenny et al. (1955). These investigators have reported that degeneration of the follicular apparatus and hypertrophy of the interstitial cells are the more usual consequences of removing the uterus in the rabbit. Exogenous estrogen will maintain corpora lutea in the hysterectomized rabbit or in the hysterectomized hypophysectomized rabbits (Greep, 1941).

A conclusion that can be derived from these observations is that during the second half of gestation an influence emanates from the gravid uterus which sustains luteal function in rabbits. The observation that luteal regression occurs when the gravid uterus is removed suggests that the influence of the gravid uterus may be effected by a tropic factor rather than the conceptuses blocking a luteolytic agent from the uterus. The degeneration of follicular tissue observed by Mishell and Motyloff (1941) and Tenny et al. (1955) suggests that perhaps the loss of luteal function following hysterectomy after midgestation in the rabbit may be a consequence of inadequate estrogen secretion

by follicular tissue. Corraborative evidence for this is the observation by Greep (1941) that exogenous estrogen will sustain luteal progesterone secretion in hysterectomized rabbits (Greep, 1941). The exogenous estrogen probably acts directly on the corpora lutea, because it has been shown that exogenous estrogen will sustain progesterone secretion from corpora lutea transplanted to beneath the kidney capsule (Keyes and Armstrong, 1969). If this is the case, then the gravid uterus in rabbits would appear to exert its capacity to sustain ovarian progesterone output by regulating estrogen production from ovarian follicles.

Regulation of Ovarian Function by Deciduoma and Uterine Estrogen Metabolism. Associated with attachment of the blastocyst to the uterine wall of most species is a rapid growth of the uterine mucosa known as the decidual reaction. A similar reaction can be caused by traumatizing the progestational uterus with some foreign material or object in which case the resultant swelling in the uterus is called a deciduoma (Shelesnyak and Krasier, 1963) or deciduomata (Amoroso, 1952).

Production of deciduoma in the rabbit does not appear to lengthen the functional life of corpora lutea in pseudopregnant rabbits (De Feo, 1967; Hammond, 1935; Heckel, 1942). However, uterine trauma plus estradiol are more effective in prolonging luteal function in pseudopregnant rabbits than estradiol alone (Spies et al., 1968). Heckel (1942) observed that a level of estrogen incapable of prolonging the life of corpora lutea in uterine-intact pseudopregnant animals could do so if 75 to 80% of the uterus was removed; whereas, this much removal of uterine tissue in non-estrogen-treated animals did not prolong the life of corpora lutea. Heckel suggested that the endometrium of the pregnant

or traumatized uterus exerts its luteotropic effect by either removing or metabolizing less estrogen than the intact pseudopregnant uterus. The higher titers of estrogen then acted to sustain progesterone secretion by the corpora lutea. Final judgement as to whether or not the uterus exerts a regulatory influence on ovarian endocrine function by metabolizing steroids will have to be postponed until more substantial data are available.

Regulation of Ovarian Function by the Conceptuses. From egg transplantation studies it has been shown that in the rabbit, as in the pig, a minimum number of conceptuses are required to promote and maintain pregnancy (Adams, 1970; Day et al., 1967; Hafez, 1968). When two morulas were transplanted to one uterine horn of a recipient rabbit and both morulas implanted, they were carried to term in 86% of the cases as opposed to only 47% when one morula implanted (Adams, 1970). When one morula was transplanted and implanted in one uterine horn of a recipient doe and three or four morulas were transplanted and implanted in the contralateral horn, all the implanted morulas in the horn by themselves survived to term (Adams, 1970). Hemihysterectomy of the non-pregnant horn or injections of FSH did not maintain pregnancy when a single morula was established in one horn (Adams, 1970). In all of these experiments pregnancy failure occurred most frequently between days 15 and 20 (Adams, 1970. Thus, in the rabbit two conceptuses are apparently required to prevent regression of corpora lutea (Adams, 1970) at the time corresponding to the end of pseudopregnancy.

Rund (1969) conducted experiments to determine if the effects of the conceptus on ovarian endocrine function in the mother could be attributed to chemical substances elaborated by the embryos. His

experiments involved giving injections of supernates of rabbit embryo homogenates to prevent regression of corpora lutea in rabbits made pseudopregnant by intravenous injection of 80 IU human chorionic gonadotropin. On the 12th day after HCG treatment does received intravenous injections of supernantant prepared from either embryonic homogenates or diaphragm muscle homogenate. Injections were continued every four hours for four days. Material for injections was collected from females 12 days pregnant. With regard to collecting embryonic tissue Rund indicated "care was taken to avoid excision of any endometrial material by carefully teasing away uterine tissue with small needle-nosed forceps, although the embryonic-membranes were retrained." What was specifically done with the placenta was not indicated. Rund probably included fetal placenta as part of his embryo preparation.[2] Rund's homogenates were prepared in a minimum amount of saline with a ground glass homogenizer. The homogenate was then spun for 90 minutes at 105,000 X G and the supernant collected. Each pseudopregnant rabbit received 302 to 408 mg of biuret reactive material or about 17 mg per injection. The total dose per animal over the four day period was three to four embryos. On the basis of average dry weights of corpora lutea and progesterone content, the corpora lutea of pseudopregnant does injected with embryo homogenate supernatant were the same as observed

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[2]Hammond (1935) has indicated that up to the 16th day of pregnancy the maternal placenta cannot be removed from the uterus except by cutting with scissors and that up until days 29-32 removing the embryos from the uterus results in fetal placenta pulling away from the maternal placenta. In the course of the present investigations it was observed that the fetal placenta pulls away from the maternal placenta easier at 17 days than at day 21 and that the maternal placenta is more easily freed from the uterus in 21-day pregnant rabbits than 17-day pregnant rabbits.

in 12-day pregnant does. In contrast, corpora lutea of rabbits injected with either pronase-treated embryonic supernatant or diaphragm had less dry weight and progesterone content than 12-day pregnant rabbits or rabbits injected with the embryo homogenate. Rund suggested that the embryonic factor might act in two ways. The first was that since it was reported that LH or LH plus FSH is luteolytic at days 12 to 16 in the pseudopregnant rabbit, embryonic factor might have functioned to check pituitary LH discharge. The second way was that since hysterectomy before day eight of pseudopregnancy prolongs luteal function, embryonic factor may block the action of a luteolytic agent from the uterus. Rund also pointed out that both these mechanisms might work simultaneously.

In 1917 Hammond reported on the effects of the conceptuses on ovarian endocrine function in rabbits in conjunction with mammary gland development. In one of his experiments he removed all the fetuses but left the placentas *in situ* in six does between the 13^{th} and 15^{th} day of pregnancy. Does were killed between the 22^{nd} and 32^{nd} day of pregnancy by which time mammary glands in intact rabbits have developed a characteristic thickening. None of the mammary glands in the operated rabbits developed the characteristic thickening. The sizes of the corpora lutea from does in which the fetuses had been removed resembled those of pseudopregnant and were half the size of corpora lutea measured in control rabbits of the same stage of pregnancy. Evidently the fetuses are necessary between days 13 and 15 of pregnancy if corpora lutea of gestation and complete mammary glands are to develop. Hammond (1917) cited a report by Biedl and Koenigstein (1911) that implantation of placentas into non-pregnant rabbits was without effect on mammary

secretion but that implantation of the fetus resulted in growth of the gland and secretion of milk.

The results of Hammond's (1917) experiments differ from those obtained by Klein (1933), who removed all the fetuses from unilaterally pregnant rabbits on the 17th day of gestation. Five days later, day 22 of gestation, the histological appearance of the uterine mucosa and the responses of uterine muscle to oxytocin were the same as in intact 22-day pregnant rabbits, indicating that the corpus luteum function was sustained in the absence of fetuses. Histological examinations were made of uterine segments that contained the placentas after the fetuses were removed. Klein noticed that in the obplacental region of the uterus that the mucous chorion was invaded by numerous giant cells ("monster cells" of Minot[3]). Schofield (1960) found that when all the fetuses were removed, but the placentas left in situ, at days 24-25 of pregnancy, the response of the uterus to electrical stimuli indicated progesterone dominance was maintained until days 29-30. Uteri from which the placentas as well as the fetuses were removed lost characteristics of progesterone dominance 46-48 hours after surgery (Schofield, 1960). Histological studies of the ovaries from rabbits from which the placentas and fetuses had been removed indicated degeneration of corpora lutea began by 72 hours after the operations. Histological studies were not reported for ovaries from does from which only fetuses had been removed. Thus, removal of fetuses at days 17 or 23-24 of pregnancy does not appear to cause a rapid decline in ovarian output of progesterone in the rabbit.

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[3]Information related to giant cells is presented in the next section.

Chu et al. (1946) determined the survival periods of corpora lutea by laparotomy following extirpation of placentas from uteri of pregnant rabbits between the 11th and 16th days of gestation. The survival periods were also observed for corpora lutea in pregnant rabbits that were hysterectomized, or hysterectomized and placentas autotransplanted to the abdominal cavity between days 13 and 17 of gestation. Placentas were "removed and implanted into the abdominal cavity at the lumbar level. In a few cases additional pieces of placental tissue were implanted under the skin of the abdomen or in the region of the mammary glands." Chu et al. (1946) did not indicate if both the maternal and fetal components of placentas were implanted or just the fetal component. Since at the stage of pregnancy at which the transplants were performed, the maternal component has a tendency to remain in place when the fetal membranes and placenta are pulled upon (Hammond, 1935), it is probable that the transplants involved primarily fetal placenta. Corpora lutea survived an average of ten days after placentas were removed, which was three days longer than the survival time of corpora lutea in ovaries of rabbits that were hysterectomized. Corpora lutea in hysterectomized does bearing autotransplanted placentas were maintained an average of 14.1 days after the operations. That is as long as normal pregnancy. Thus, transplanted placentas appear able to sustain the morphology of corpora lutea following hysterectomy in rabbits; however, direct measures were not made to determine if these corpora lutea continue to secrete progesterone.

The foregoing review suggests that the presence of the placentas, but not the fetuses, are important in sustaining luteal function during gestation in rabbits. Whether or not the maternal placenta is involved

in maintenance of the corpus luteum during pregnancy in the rabbit is not clear.

Giant Cells and Myometrial Glands in the Pregnant Rabbit. In the rabbit at least three types of cells are involved in formation of the deciduoma: 1) uninucleate decidual[4] cells which appear early in pregnancy as perivascular sheaths; 2) much larger vesicular multinucleate cells, derived apparently from the uninucleate ones; and 3) myometrial gland cells which Mossman classified as giant cells[5] of uncertain origin.

Ancel and Bouin (1911) were perhaps the first to report the existence of groups of large cells around capillaries in the antimesometrial uterine musculature of pregnant rabbits. They called the groups of cells the myometrial gland and described the cells as having an epithelioid appearance and that they lay in close proximity to blood vessels between the circular and longitudinal muscle layers. They suggested that the cells formed a gland of internal secretion which took on the functions of the corpus luteum during the second half of pregnancy, controlling the glandular phase of the mammary gland (Bouin and Ancel, 1912a) and also the tolerance of the uterus for the fetus (Bouin and Ancel, 1912b). Fraenkel (1912) verified the presence of the cells in the rabbit but did not accept the functions Ancel and Bouin had suggested--

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[4]Mossman (1937) defines decidual cells in the human endometrium as endometrial connective tissue cells which have become rounded or polyhedral due to the storage of glycogen or lipoids in their cytoplasm.

[5]According to Mossman (1937) a giant cell can be considered to be any large cell occurring in the uterus or fetal membranes whether they be mononuclear or polynuclear and of maternal or fetal origin. Mossman (1937) classified giant cells into three types based on origin: 1) trophoblastic, 2) epithelial (uterine), and 3) giant cells of uncertain origin. The majority of giant cells are probably trophoblastic in origin.

because of the small amount of vascularity in the surrounding tissue and their absence in some species. Mercier (1912) ascribed a phagocytic function to the cells. Hammond (1917) found cells corresponding to those described by Ancel and Bouin. In every case they were found in the region of the uterus opposite the placenta and beneath pustules consisting of giant cells and surrounded by a mass of decidual cells. Hammond thought it possible that the myometrial cells could be trophoblast cells which had wandered to the muscular coat. However, Hammond could not consistently find the cells in every pregnant rabbit and, therefore, did not believe they performed the functions Ancel and Bouin had assigned them. Mossman (1937) also acknowledged the presence of the myometrial gland cells but indicated that there was not sufficient evidence to warrant considering them as an endocrine gland. Mossman considered the myometrial gland cells to be the result of a decidual reaction in the connective tissue of the myometrium.

The cause of the decidual response in pregnant rabbits is generally thought to be the invasion of the trophoblast into the uterus at a time of favorable endocrine conditions (Finn, 1971). During the course of the seventh day of pregnancy the blastocyst becomes free from the zona and has localized trophoblastic multinucleate thickenings which fuse with the uterine epthelium (Boving, 1961). The uterine epithelium breaks down at the point of fusion and multinucleate bodies pass in the uterine mucosa where they increase in size and form giant cells. These giant cells are active phagocytic cells.

Sansom (1927) considers that the pregnant uterus has two kinds of giant cells. The larger and the most conspicuous variety are those derived from the fetal trophoblast, cells which become detached from

the blastocyst about the seventh day of gestation and penetrate into the obplacental mucosa. These cells grow rapidly and attain a size of as much as 0.4 mm in length. They persist until about day 22. Another source of these giant cells is that portion of the trophoblast of the proximal zone of the bilaminar omphalopleure which projects free into the uterine cavity after the attachment of the blastocyst to the placenta folds on the eighth day. The cells proliferate in large numbers off from this trophoblastic fringe, pass into the uterine cavity, penetrate the uterine epithelium of the antimesometrial uterine wall and enter the underlying tissues. This source of giant cells persists until the 16^{th} day of gestation. The second type of giant cells are of maternal origin and are formed by the proliferation of the endothelial lining of the capillaries in the deep placental region. They appear at about the 11^{th} day of gestation and persist until after the 27^{th} day. They never attain a great size and are confined to the mesometrial region.

Whether or not the myometrial gland described by Ancel and Bouin is formed in response to giant cells of fetal origin, or if such giant cells actually make up the myometrial gland cannot be said at present with certainty. Mossman's (1937) observation that there is insufficient evidence to assign an endocrine function to the myometrial gland, as suggested by Ancel and Bouin (1912a), still stands until more data that defines the role of the myometrial gland are available.

Summary of Ovarian Function in the Rabbit. The preceding sections have documented a number of premises with regard to endocrine functions of the maternal ovary during gestation in the rabbit. The following paragraphs summarize this information.

The rabbit ovary is known to secrete two specific species of steroids, 20αOH-progesterone and progesterone. 20αOH-progesterone is secreted primarily from the interstitial gland in response to LH. Progesterone is secreted primarily from the corpora lutea. 20αOH-progesterone from the ovaries is considered to be important in neuro-endocrinological processes enhancing LH release associated with ovulation, but has no known functions during gestation in the rabbit. Progesterone from the ovaries, together with estrogen, has a critical function in conditioning the uterus and stimulating development of the mammary glands. Estrogen, but not pituitary hormones, is required for progesterone secretion by the corpora lutea of pregnant rabbits. There is evidence that the elaboration of estrogens by follicles is dependent upon low levels of pituitary LH. However, LH alone is incapable of sustaining the production of estrogens; prolactin-like or FSH-like stimulation is apparently also required.

The presence of the conceptus in the rabbit induces changes in sex accessory glands suggesting the ovaries secrete estrogens during the last half of pregnancy. These changes include complete development of the mammary glands and conditioning of the uterus. Neither induced decidual reactions nor prolongation of luteal function by hysterectomy at appropriate times are capable of mimmicking the changes in sex accessory gland brought on by the conceptuses. Maintaining pregnancy in castrated rabbits with progesterone alone does not ensure complete development of sex accessory glands, indicating that the effects of the conceptus on accessory glands is mediated by ovarian and not placental estrogen. This would be consistent with the observation that progesterone output by the corpus luteum is dependent upon follicular

estrogens and that it is the conceptuses in the uterus that help sustain follicular estrogen production at an appropriate level to promote progesterone secretion by luteal tissue in the rabbit. Evidently estrogen production by the f llicles during pregnancy in rabbits is dependent upon synergistic influences from both the hypophysis and uterus, since removal of either terminates pregnancy. Other mechanisms to explain the effects of the gravid uterus on ovarian function in rabbits have been proposed.

The effect of the pregnant uterus after midgestation in the rabbit has been proposed to stem from an anti-luteolytic action or an estrogen "sparing" action. The estrogen "sparing" effect of the gravid uterus has not been conclusively shown to function in any species. Luteolytic factors emanating from the gravid uterus at a time beyond the duration of pseudopregnancy would be effectively removed by hysterectomy, yet hysterectomy after midgestation results in rapid involution of corpora lutea, indicating removal of a tropic factor. There are well established precedences for induction of gonadotropin production from the gravid uterus by the conceptus in two groups of animals, horses and primates. Evidence has been presented indicating production of gonadotropins by the conceptuses of some of the small laboratory animals, though it was noted that no specific compounds have been isolated to date. The placentas appear to cause prolonged luteal function although the fetuses have also been implicated in regulation of ovarian function late in gestation in some species.

The experiments of Klein (1933), Chu et al. (1946), and Schofield (1960) indicate the placenta to be the source of a gonadotropic factor which stimulates prolonged secretion of progesterone by the corpora

lutea during pregnancy in rabbits. However, none of their experiments provide definitive proof for the presence of a gonadotropin originating from the placentas of rabbits. The experiments of both Klein (1933) and Schofield (1960) lacked specificity in that an estrogen "sparing" effect could account for their results as well as a gonadotropin could. Chu et al. (1946) relied upon gross observations of ovaries at frequent laparotomies to determine maintenance of luteal function, a process lacking in quantitative character and open to the risk of bias. None of the reports of work with rabbit placentas clearly defined whether they dealt with the entire placenta or the maternal or fetal component; however, it is probable that the fetal component was involved in all cases. The experiments with transplanted embryos (Adams, 1970; Day et al., 1967; Hafez, 1968) indicate that embryos possess the capacity to prolong progesterone secretion by corpora lutea beyond the duration of pseudopregnancy in rabbits, but indicate little about the mechanism whereby this is accomplished. Rund's (1969) experiments give evidence of a positive nature for a gonadotropic substance in rabbit embryos but his findings have not been formally reported in the literature. Hammond's work of 1917 suggested the fetuses were the component of the gravid uterus that stimulated maintenance of luteal function. Ancel and Bouin (1912a) believed that the myometrial gland was responsible for maintenance of luteal function in rabbits. Thus, with the exception of these latter two studies the majority of the studies indicate that the presence of the placentas at midgestation in the primary cause of continued progesterone secretion from the corpora lutea in pregnant rabbits; however, definitive evidence is still lacking. Particularly lacking is information as to which species of steroids is most sensitive

to influences from the conceptus. Information on specific species of steroids is also needed to confirm the previous observations, based primarily on biological end points, that ovarian secretion of progestins declines rapidly following extirpation of the gravid uterus after midgestation.

Summary and Conclusions

The foregoing review of literature has documented a number of points with regard to endocrine functions of the maternal ovary during gestation in the rabbit and some other species. In most viviparous eutherian mammals several questions remain unanswered including 1) which ovarian tissues are subject to influence from the gravid uterus, 2) from which anatomical part of the gravid uterus do influences that regulate the maternal ovary originate, and 3) how long acting are the influences that emanate from the gravid uterus?

The rabbit may be considered as an example of a viviparous species in which the presence of conceptuses in the uterus influences the endocrine processes of the female gonad. This phenomenon in the rabbit can serve as a specific example to be studied for determining how ovarian endocrine function is regulated during gestation. The rabbit is particularly suited to such studies since its ovarian interstitial, luteal, and follicular tissues appear to have some clearly defined functions which can be attributed to the production of 20α-OH-progesterone, progesterone, and estrogen, respectively. Furthermore, the regulation of the production of at least two of these steroids, 20α-OH-progesterone and progesterone, has been partially elucidated and offers a basis from which to conduct and interpret experiments designed

to enhance understanding of regulation of the endocrine processes of the adult female gonad by the conceptus.

Experiments designed to establish the site from which the influence of the gravid uterus originates and the ovarian tissues subject to its influence should incorporate several features. One is the capacity to specifically and accurately monitor ovarian endocrine function by measuring particular species of steroids. The experiments should also be able to test individual or various combinations of the components of the gravid uterus with respect to their influence on ovarian progestin secretions.

Once the site of origin of the stimulus was definitely established it would be desirable to have an assay system that might be used to test cell-free extracts of the tissues in question to confirm the presence of a gonadotropic principle and to be used as a basis for isolation procedures for the principle.

CHAPTER III

MATERIALS AND METHODS

Introduction

Materials and methods used in the experiments may be grouped in four categories: 1) experimental design; 2) experimental protocols; 3) animal husbandry; and 4) isolation, identification, and assay of steroids.

Experimental Design

Objective

The purpose of the experiment was to collect information pertinent to two points. One was to determine the acute effects of removing the entire gravid uterus upon ovarian output of 20αOH-progesterone and progesterone in rabbits at 21 days of gestation. The other was to determine whether the effects of removing the gravid uterus could be attributed to removing particular components of the gravid uterus, namely, the fetuses, or fetuses plus placentas together.

Design

20αOH-progesterone and progesterone concentrations were measured in systemic plasma before and after treatments and ovarian output of 20αOH-progesterone and progesterone from one ovary was measured after

treatments in eight 21-day pregnant rabbits in each of five treatment groups including 1) laparotomy, 2) fetectomy, 3) conceptectomy with removal of the maternal placentas, 4) hysterectomy with samples collected six hours after treatment operations, and 5) hysterectomy with samples collected 24 hours after treatment operations. The experiment yielded 40 observations of pre-treatment and 40 observations of post-treatment concentrations of 20αOH-progesterone and progesterone in systemic plasma. The five treatment group means for pre-treatment and post-treatment systemic plasma concentrations of 20αOH-progesterone and progesterone were subjected to analysis of variance (Snedecor and Cochran, 1967) in order to elucidate significant variance due to treatment regimes. When significant treatment effects were observed, differences between individual treatment means were detected by Newman-Keul's Sequential Range Test (Snedecor and Cochran, 1967). The mean pre-treatment and the mean post-treatment 20αOH-progesterone concentrations in systemic plasma within a single treatment group were compared for differences by Steel and Torrie's (1960) procedures for comparing two means with paired observations in order to determine if particular treatments caused statistically significant changes in progestin concentrations in the systemic plasma of the rabbits within a given treatment group. Ovarian venous output of 20αOH-progesterone was measured by three indices including 1) ng/ml ovarian venous plasma, 2) μg/ovary/hr, and 3) μg/g ovary/hr, and yielded a total of 40 observations for each index. Ovarian venous output of progesterone was measured by four indices including 1) ng/ml ovarian venous plasma, 2) μg/ovary/hr, 3) μg/g ovary/hr, and 4) μg/corpus luteum/hr, and yielded a total of 40 observations for each index. The five treatment

group means of ovarian output of 20αOH-progesterone were subjected to analysis of variance for each index of output (Snedecor and Cochran, 1967). The five treatment group means of ovarian output of progesterone were subjected to analysis of variance for each index of output (Snedecor and Cochran, 1967). When significant treatment effects were observed, differences between individual treatment means were elucidated by Newman-Keul's Sequential Range Test (Snedecor and Cochran, 1967).

The weights, blood flow rates, and number of corpora lutea were recorded for each of the ovaries that were cannulated, resulting in 40 observations for each of the three parameters. The five means of the treatment groups for the same parameter was subjected to analysis of variance for each of the three parameters (Snedecor and Cochran, 1967). When significant differences were observed, differences between individual treatment groups were elucidated by Newman-Keul's Sequential Range Test (Snedecor and Cochran, 1967).

Experimental Protocols

Introduction

When monitoring ovarian endocrine function by measuring 20αOH-progesterone and progesterone concentrations in ovarian venous plasma, the concentrations in ovarian venous plasma should be corrected for the concentrations of the progestins reaching the ovary via the arterial blood. For this purpose concentrations of the progestins in the rabbits' ovarian arterial plasma were estimated by measuring progestin concentrations in systemic blood drawn by cardiac puncture 10-15 minutes before cannulating the ovarian vein.

The concentrations of progestins in plasma from samples of cardiac blood represented systemic plasma concentrations of the progestins. Such systemic plasma concentrations of progestins could be expected to be indicative of the effects that removing components, or the entire gravid uterus had on ovarian endocrine function. Post-treatment systemic plasma concentrations of progestins could be particularly indicative of the effects of treatments on ovarian endocrine function when compared with concentrations of progestins in systemic plasma before the rabbits were subjected to treatments. Furthermore, in laparotomized animals, comparison of pre-treatment with post-treatment systemic plasma concentrations of progestins could provide information on the effects that anesthesia and the trauma of surgery had on ovarian endocrine function. A convenient manner and time for collecting pre-treatment plasma were by cardiac puncture immediately after the rabbits were anesthetized to undergo the surgery associated with their particular treatment. Figure 1 presents the experimental protocol in outline form. Details of the procedures used to assay samples of blood for 20αOH-progesterone and progesterone content are given in the section of this chapter entitled "Isolation, Identification and Assay of Steroids."

Collection of Systemic Blood Samples

A one and one-half inch disposable, thin-walled, 19 or 20 gauge needle attached to a 30 or 50 ml capacity, Luer-Lok tip, glass syringe was used to collect samples of systemic blood by cardiac puncture from the left side of the thoracic cavity at the level of the elbow of the rabbit's foreleg. Heparin (5-10 drops of a 1000 unit per ml solution; approximately 62 mg of Nutritional Biochemical synthetic heparin was

Induction of anesthesia in 21-day pregnant rabbit
(eight per treatment)

↓

Collection of 25 ml pre-treatment systemic blood by cardiac puncture

↓

Replacement of blood with plasma expander via a marginal ear vein

↓

Surgery associated with treatment consisting of one of the following

a. laparotomy (sham hysterectomy)
b. removal of fetuses by hysterotomy
c. removal of fetuses plus placentas by hysterotomy
d. hysterectomy (removal of entire gravid uterus)

↓

Six or 24 hour waiting period

↓

Induction of anesthesia

↓

Collection of 25 ml post-treatment systemic blood by cardiac puncture

↓

Replacement of blood with plasma expander via a marginal ear vein

↓

Collection of ovarian venous blood and replacement
of blood with plasma expander

↓

Collection of ovaries

↓

Termination of rabbit with overdose of anesthetic

Figure 1. Protocol Used to Collect Samples

dissolved in 10 ml isotonic saline) was used as anticoagulant and was in the syringe at the time of cardiac puncture. Immediately following collection samples were cooled in test tubes standing in crushed ice. The volume of the blood collected was recorded. Between two to three minutes after systemic blood samples were collected a volume of plasma expander at 38° C, equal to the volume of blood collected, was injected into the rabbit via a marginal ear vein previously cannulated with a P.E. 50 polyethylene tubing (Intramedic, Clay-Adams, Inc.). Plasma expander consisted of 6% dextran (60,000-90,000 molecular weight, Nutritional Biochemical) dissolved in an isotonic saline solution containing 100 mg percent glucose.

Collection of Ovarian Venous Blood

A P.E. 90 polyethylene tubing was used to cannulate ovarian veins (Hilliard et al., 1963). Tributaries other than those from the ovary were blocked off. Just prior to insertion of the cannula into the ovarian vein 2500 µ heparin was injected into the rabbit via a marginal ear vein. After discarding the initial 10-20 drops, blood was collected for 15 minutes in a 50 ml graduate cylinder standing in crushed ice. At five minutes into the collection, the volume of collected blood was measured and the volume of the 15 minute collection estimated between five and ten minutes into the cannulation. A volume of plasma expander (see Collection of Systemic Blood Samples), equal to the estimated volume of blood that was expected to be collected in 15 minutes from the ovarian vein, was infused into the rabbit via a marginal ear vein. At the end of the 15 minute collection period, the volume of collected blood was measured and the ovary removed to be weighed and have the corpora lutea counted.

Determination of Percent Packed Cell Volume

The percent packed cell volume of blood samples was determined by drawing well-mixed blood from the sample into heparinized capillary tubes which were then sealed at one end with sealing clay (Clay-Adams). The sealed capillary tubes were spun for five minutes in an International micro-capillary centrifuge model MB. The percent packed cell volume in the spun capillary tubes was measured with a circular type microhematocrit reader or a centrimeter ruler.

Separation of Plasma from Blood Cells

Blood samples were spun at 1,500-2,000 rpm for 20 minutes in an eight place swinging bucket #240 head (International) with an International universal centrifuge model UV. An attempt to keep samples cool during centrifugation was made by packing crushed ice around the sample test tube in the swinging bucket. Plasma was lifted from the cells with the aid of a 10 ml pipette and a safety propipette.

Plasma Storage Prior to Processing

Plasma samples were stored at -12°C in one or four ounce round, wide-mouthed, polyethylene bottles (Naglene) that had previously been washed and methanol rinsed.

Anesthesia

Three ml of a 64.8 mg per ml solution of pentobarbital sodium (Haver-Lockhart) was diluted to 5 ml with 38°C isotonic saline in a 5 ml capacity, Luer-Lok tip, glass syringe. Diluted anesthetic was

injected with a 23 gauge disposable needle via a marginal vein until the desired level of anesthesia was obtained. An ether cone was used to maintain an appropriate level of anesthesia. In the event of respiratory failure and when physical stimuli were unsuccessful in reinstating respiration, 0.5 ml injections of doxapram hydrochloride (A. H. Robins Co.) at a concentration of 2 mg per ml were injected via the marginal ear vein. The 2 mg per ml solution of doxapram hydrochloride was prepared by diluting 0.1 ml of a 20 mg per ml stock solution to 1 ml in a 1 ml capacity plastic disposable tuberculin syringe.

Surgery

Surgery was performed while the rabbits were under anesthesia induced by pentobarbital and maintained with an ether cone. Nylon-reinforced, disposable towels or a sheet of transparent plastic were used as drapes. Chlorhexidine solution (Nolvasan, Fort Dodge Laboratories) was used as disinfectant for instruments. Rabbits were kept warm with a heat lamp.

Antibiotics

Following surgery rabbits were given an intra-muscular injection of 100,000 IU penicillin G and 125 mg dihydrostreptomycin (Combiotic, Chas. Pfizer and Co., Inc.).

Animal Husbandry

Rabbits

Sexually mature female rabbits were obtained from either a local supplier or Redwood Game Farms, Salt Lake City. Sexually experienced buck rabbits were obtained from a local supplier. Rabbits were caged in individual metal cages in air-conditioned rooms maintained at approximately 25° C under a light regime of 12 hours light followed by 12 hours of darkness. Bucks and non-pregnant does received 4 oz Purina Rabbit Chow per day. Pregnant does and does in a hormone regime for insemination received 6 oz Purina Rabbit Chow per day. Fresh water was supplied *ad libitum*.

Semen Collection

Semen for use in artificial insemination was collected from healthy bucks using a procedure similar to that used in The Rabbit Genetics Laboratory, Hamilton Station, The Jackson Laboratory, Bar Harbor, Maine (Gregoire, *et al.*, 1958) as demonstrated by Dr. C. Desjardins. This technique was accomplished with an artificial vagina of which the principal components were a rubber director cone (Jensen-Salisbury Labs, Inc.), graduated plastic 15 ml centrifuge tube cut at the 8 ml mark, 3 inch length of automobile radiator hose (i.d. 1 1/2", o.d. 1 7/8"), and number nine and number five rubber stoppers. The temperature of the artificial vagina was adjusted to between 40-55° C with water at approximately 75° C. Four bucks were collected separately and the semen pooled and diluted to a final volume of 10 ml. Diluent for the semen was isotonic saline containing 1000 IU buffered potassium penicillin G

(crystalline) and 100 µg of dihydrostreptomycin (crystalline) per ml. Immediately prior to collection of each ejaculate the artificial vagina was rinsed with approximately 10 ml diluent at 38° C.

Artificial Insemination and Induction of Pregnancy

Artificial insemination of does to induce pregnancy was performed within an hour of semen collection. Diluted semen was held in a 25 ml graduate cylinder standing in a hot water bath at 37.5° C. Approximately 2 ml diluted semen was deposited in the reproductive tract of each doe with the aid of a bent glass tube fitted with a 2 ml capacity heat-cured rubber bulb. Does received one of two hormone regimes. One regime consisted of 0.0625 mg equivalents F.S.H.-P. (Armour-Baldwin Laboratories) injected subcutaneously at 12 hour intervals (twice daily) for three consecutive days. Twelve hours following the last injection of FSH-P the does were artificially inseminated and 2.5 mg equivalents P.L.H. (Armour-Baldwin Laboratories) was injected via the marginal ear vein. A second hormone regime used prior to artificial insemination of does consisted of injecting 25 IU PMSG (Haver-Lockhart Laboratories) subcutaneously for three consecutive days. Twenty-four hours following the last injection of PMSG, 80 IU Chorionic Gonadotropin (Jensen-Salsbery Laboratories) was injected intravenously and the doe artificially inseminated. The hormone regimes were based on data available from Kennelly and Foote (1965) and Mauer et al. (1969).

Isolation, Identification, and Assay of Steroids

Reference Steroids

Testosterone, progesterone, and 20βOH-progesterone were purchased from Steraloids, Inc. 20αOH-progesterone was obtained from The Upjohn Co. through the courtesy of Dr. Gordon W. Duncan. Reference 20α-heptafluorobutyryl-progesterone, 20β-heptafluorobutyryl-progesterone, and heptafluorobutyryl-testosterone were synthesized using the procedures of Nakagawa et al. (1966) and van der Molen et al. (1967). All reference steroids were filtered, recrystallized, and their uncorrected melting points determined before use. Elemental analysis (performed by Galbraith Laboratories) and mass spectrometric analysis (performed in the Biochemistry Department, Oklahoma State University) were carried out on samples of reference 20α-heptafluorobutyryl-progesterone and 20β-heptafluorobutyryl-progesterone.

Isotopically-Labelled Steroids

In order to correct for loss of 20αOH-progesterone and progesterone through the isolation procedures progesterone-1,2-^{3}H, 40-50 Ci/mM, and 20αOH-progesterone-1,2-^{3}H, 40-50 Ci/mM, were purchased from New England Nuclear. The isotopically-labelled steroids were purified on thin-layer chromatography (TLC) in the solvent system cyclohexane:ethylacetate (1:1; v:v). The major radioactive peak was eluted from the silica gel with benzene over water and stored in benzene at 5°C at a concentration of approximately 2×10^{6} dpm/ml. Radiochemical purity of the stored purified isotopically-labelled steroids was checked at appropriate intervals by running aliquots on TLC in unlined tanks in the solvent

system water:methanol:chloroform:toluene (1:20:60:120; v:v:v:v). Radiochemical purity was considered adequate when the only discernible radioactivity between the origin and solvent front was located at the R_f of carrier reference steroid. Instrument settings were such that the radioactivity located at the R_f of the carrier reference steroid produced at least a full scale deflection. Radioactive areas on TLC plates were detected and measured with a Packard Model 7201 radiochromatogram scanner. Radioinert reference steroids were detected on TLC visually with the aid of ultra-violet light.

Solvents

The majority of the solvents were Nanograde (Mallinckrodt Chemical Works) and included: chloroform, dichloromethane, ethyl-acetate, acetone, ethyl-ether, benzene, hexane, toluene, and methanol. Ethyl-ether and dichloromethane were further purified by distillation over glass. Water used in the steroid assay method was distilled twice over glass, washed with ethyl-ether, and finally redistilled over glass. Toluene used in liquid scintillation counting was Spectroquality (Matheson, Coleman, and Bell). Tetrahydrofuran, analytical grade, (Mallinckrodt) was refluxed three hours over sodium hydroxide, distilled once off of fresh sodium hydroxide and then stored over sodium.

Reagents

Sodium hydroxide, reagent grade (Fisher Scientific Co.) and heptafluorobutyric anhydride (PCR, Incorporated) were used without further purification. Liquid scintillation cocktail was prepared by adding 12 g 5-diphenyloxazole (PPO) and 0.12 g 1,4-bis-[5-phenyloxazolyl]-benzene

(POPOP) to 3 l toluene. Fluors were scintillation grade (Packard Instrument Company, Inc.).

Glassware

Glassware was rinsed immediately after use, soaked in detergent, brushed, rinsed in tap water, and placed in chromic acid overnight. Following ten tap water rinses the acid-soaked glassware was soaked in detergent, rinsed again with tap water, and placed in dilute hydrochloric acid. After removal from hydrochloric acid the glassware was rinsed ten times with tap water, ten times with distilled water, and air dried. Shortly before use glassware was rinsed three times with Nanograde methanol and air dried.

Thin-Layer Chromatography

Silica gel (Silicar-TLC-7GF, Mallinckrodt), that was washed with acetic acid and rinsed with water and methanol, was spread to an approximate thickness of 0.25 mm on glass plates. The silica gel coated glass plates were air dried and then activated in an oven at 120° C.

Gas-Liquid Chromatography and Electron Capture Detection

Heptafluorobutyryl derivatives of steroids to be assayed were injected in 2-10 µl benzene into a 1 m X 4 mm id silanized U-shaped glass column that was packed with 3% OV-225 on GAS-CHROM Q 80/100 mesh (Applied Science Laboratories) housed in a Barber-Coleman Selectra Series 5000 gas chromatograph and fitted with a ^{63}Ni Barber-Coleman model 5125-100 electron capture detector. Carrier gas was extra dry

nitrogen (Matheson Gas Products) passing through molecular seive at a flow rate of approximately 120 ml/min with 40 p.s.i. Column temperature was approximately 200° C. The electron capture detector was operated in the DC mode. The detector bath temperature was 240° C. Sensitivity and linearity of detector response to known amounts of heptafluorobutyryl derivatives of testosterone, 20αOH-progesterone and 20βOH-progesterone were determined for each day of data collection with the gas chromatograph. Samples were not processed unless the response of the detector was linear over the range of expected levels of steroids in the samples.

Identification of Compounds Isolated from Rabbit Plasma and Assayed as 20α- and 20β-Heptafluorobutyryl-Progesterone

The principal method employed to verify the identities of the compounds isolated from rabbit plasma was mass spectrometric analyses. These analyses were performed on a LKB-9000 (prototype) mass spectrometer through the courtesy of the Oklahoma State University Mass Spectrometry Laboratory. The operations of the mass spectrometric analyses have been described by Waller (1967). To obtain sufficient masses of the isolated material for such analysis ovarian venous plasma was collected from three 21-day pregnant rabbits and was pooled to give a final total volume of approximately 150 ml which then served as the source for the compounds. Twenty-five ml aliquots of this plasma were pipetted into each of six 90 ml extraction tubes and processed, with two exceptions, in the same manner as for assaying plasma for 20αOH-progesterone and progesterone. The two exceptions were: 1) tritiated 20αOH-progesterone and progesterone were not added to the six extraction

tubes to monitor recovery rates; and 2) material from the six extraction tubes was combined at the thin-layer chromatographic purification of heptafluorobutyration products, to give two pooled samples for mass spectrometric analysis. One pooled sample was comprised of the eluates from silica gel of the sample lanes that was scraped off the plates at the R_f value corresponding to that of reference 20α-heptafluorobutyryl-progesterone run in a separate lane. The other pooled sample was comprised of the eluates from silica gel of the sample lanes that was scraped off the plates at R_f values corresponding to that of reference 20β-heptafluorobutyryl-progesterone run in a separate lane.

Previous experience gained by colleagues (Rhynes, 1971; Goodwin, 1972) with mass spectrometric identification of steroids isolated from plasma indicated that repeating the thin-layer chromatographic purification of heptafluorobutyration products was desirable to reduce background contaminants appearing in the mass spectra of the isolated steroids. Previous experience also indicated that the extent of background contamination appearing in mass spectra of steroids isolated from plasma could be further reduced by subtracting from such spectra background obtained from spectra of silica gel blanks.

For these reasons the thin-layer purification on silica gel in cyclohexane:ethylacetate (140:70; v:v) was repeated and silica gel blank samples were prepared. To prepare silica gel blank samples, silica gel in unused lanes from the plates employed to repeat the chromatographic purification was scraped off at the R_f values for reference 20α- and 20β-heptafluorobutyryl-progesterone. The silica gel from the unused lanes was eluted in the same manner as silica gel from lanes with the material isolated from plasma. Special attention was

given to keeping the amount of silica gel and volumes of solvents used for the silica gel blanks the same as for the samples of material isolated from plasma.

Mass spectra of silica gel blanks for 20α- and 20β-heptafluorobutyryl-progesterone contained no peaks of significant intensity beyond m/e 57. In both spectra the principal peak was at m/e 28. All other peaks had intensities less than 10% of the height of the m/e 28 peaks, with the exception of peaks at m/e 32 which were about 15% of the peaks at m/e 28. Since the mass spectra of silica gel blanks indicated no high molecular weight background contaminants, the mass spectra of compounds isolated from plasma were compared unaltered with mass spectra of reference compounds.

Extraction of Progesterone and 20αOH-Progesterone and Formation of Heptafluorobutyryl Derivatives

Except where formation of monochloroacetyl-esters was changed to making heptafluorobutyryl-esters, extraction and preparation of samples for measuring progesterone and 20αOH-progesterone with electron capture detection following gas-liquid chromatography was similar to the procedures described by Stabenfeldt et al. (1969).

Plasma, harvested from heparinized blood samples, was extracted three times with two volumes of dichloromethane which was then evaporated to dryness under nitrogen. The samples were then reconstituted in 30 ml ethyl-ether and saponified with 5 ml 0.1 N sodium hydroxide. The sodium hydroxide layer was discarded and the ethyl-ether washed twice with 5 ml water. The ethyl-ether was then evaporated under nitrogen and concentrated to the tip of the tube with dichloromethane

for application to TLC plates using benzene. The TLC plate was run in benzene:ethyl-acetate (120:80; v:v) and the silica gel at the R_f values corresponding to those of reference progesterone and 20αOH-progesterone (run in a separate lane) was eluted three times with benzene over eight to ten drops of water. The benzene was evaporated under nitrogen and the eluates containing 20αOH-progesterone were reacted with 0.1 ml 2% heptafluorobutyric acid anhydride in hexane with 1% tetrahydrofuran at 60° C with refluxing for 30 minutes. The eluates containing progesterone were reacted with 20β-hydroxysteroid dehydrogenase in 2 ml 0.15 M phosphate buffer (pH 5.2 - 5.4) containing 1 mg NADH and 2 mg EDTA. The enzymatic conversion of progesterone to 20βOH-progesterone was performed at 37 - 40° C for 1½ - 2 hours. 20βOH-progesterone was extracted from the aqueous reaction mixture with two partions of 1 ml benzene. Benzene of the extraction was evaporated under nitrogen and the residue reacted with heptafluorobutyric acid anhydride as for 20αOH-progesterone. Following heptafluorobutyration the reaction medium was evaporated under nitrogen and the residue concentrated into the tip of the tube with benzene:acetone (1:1; v:v) and applied to TLC plates with benzene. The TLC plates were then run in cychohexane:ethyl-acetate (140:70; v:v) and the silica gel at R_f values corresponding to those reference heptafluorobutyryl derivatives of 20αOH- and 20βOH-progesterone was eluted three times with 1 ml benzene over eight to ten drops water. The benzene was then evaporated under nitrogen and the residue reconstituted in 1 ml benzene, of which 0.2 ml was placed in a liquid scintillation vial. One tenth to six tenths ml of the reconstituted sample was pipetted into a 2 ml gas tube containing a known amount (usually 10 - 30 nanograms) of heptafluorobutyryl-testosterone, the internal standard

used to determine the amount of sample applied to the column from the gas tube.

Monitoring Recovery Rates

Recovery rates for the steroids in samples processed for application to the gas chromatograph column were measured by adding approximately 10,000 cpm (c.a. 100 pg at 35% counting efficiency) high specific activity (> 40 Ci/mM), radiochemically pure (for purification see "Isotopically-Labelled Steroids" above), isotopically-labelled progesterone and 20αOH-progesterone in benzene to extraction tubes before commencement of extraction procedures. At the same time, and in the same manner, an equivalent amount of isotopically-labelled steroid that was placed in each of the extraction tubes, was placed in liquid scintillation vials, with the exception that each scintillation vial received either ^{3}H-progesterone or ^{3}H-20αOH-progesterone, in contrast to the extraction tubes which received both. Benzene of the isotope solution in the extraction tubes was evaporated under nitrogen before addition of the sample. Following elution of heptafluorobutyryl derivatives from TLC a two-tenths aliquot of the sample was placed in a liquid scintillation vial and its radioactivity counted in a Packard liquid scintillation spectrometer (Model 3003). Twelve ml scintillation cocktail was used in each vial. The recovery rate for the steroid in the sample was calculated by correcting the counts recorded from the sample vial for background counts and then multiplying the corrected counts by the reciprocal of the fraction of the sample placed in the scintillation vial. This product was divided by the number of counts (corrected for background) recorded from scintillation vials that

received the same amount of appropriate isotopically-labelled steroid as placed in the sample's extraction tube at the beginning of the assay procedure. The resulting quotient when multiplied by one hundred expressed the recovery rate as a percentage of the original sample.

Calculations Associated with Steroid Assay Procedures

The mass of the parent steroid in a sample was computed from detector recorder tracings using the following formula:

Mass of the Parent Steroid Expressed in Nanograms
= (ISTD)(rSTD)(GLC)(D)(MW)(R)(X)

where:

ISTD = ng internal standard added to the gas tube;

rSTD = area of the peak produced by 10 ng of the compound used for internal standard divided by the area of the peak produced by 10 ng of the derivitized compound being assayed;

GLC = reciprocal of the fraction of the sample placed in the gas tube;

D = dilution factor;

MW = gram molecular weight of the parent steroid being assayed divided by the gram molecular weight of the derivative;

R = reciprocal of recovery; and

X = area of the peak in the sample's GLC tracing produced by the derivatized compound being assayed divided by the peak area in the sample's GLC tracing produced by the compound used as internal standard.

CHAPTER IV

RESULTS

Introduction

Specific objectives of this study were: 1) to determine the acute effects removing the gravid uterus has on ovarian progestin secretion; and 2) to determine which components of the gravid uterus act to regulate progestin secretions in rabbits at 21-days of gestation. To accomplish these ends progesterone and 20αOH-progesterone concentrations were measured in systemic and ovarian venous plasma of 21-day pregnant rabbits that underwent one of the following treatments: 1) laparotomy, 2) fetectomy, 3) conceptectomy with removal of the maternal placentas, 4) hysterectomy with samples collected six hours after treatment operations, and 5) hysterectomy with samples collected 24 hours after treatment operations.

Data were also obtained to validate the progestin assays used to measure ovarian endocrine function. Three approaches were used to accomplish these objectives. One was to study the physicochemical characteristics of the compounds measured to verify their identities. These physicochemical studies involved determination of melting points, elemental analysis, and mass spectrometric analysis of reference compounds as well as mass spectrometric analysis of compounds isolated from rabbit plasma. Another approach used to determine the accuracy and precision of the assays was to perform the assays several times on

samples drawn from a pooled volume of ovarian venous plasma and on 0.5% solutions of bovine serum albumin to which known amounts of the progestins were added. An index of precision was then calculated from the variances among the values for the amounts of progestins measured in the repetitions. The third approach was to scrutinize results obtained from thin-layer and gas chromatographic processes of the isolating procedures for indications of the assays' specificities.

Identification of the Compounds Assayed and the Specificities of the Assays

Electron capture detection following gas-liquid chromatography of heptafluorobutyryl derivatives has been used for assaying progesterone and 20α-OH-progesterone in biological fluids (Challis and Heap, 1969; Exely et al., 1968) and was used in this study to monitor ovarian endocrine function. Confirmation of the specificity of such methods as they were applied in the assay procedures of this study was deemed desirable since heptafluorobutyryl derivates had not been previously used in this laboratory to measure 20α-OH-progesterone and progesterone. Furthermore, while use of heptafluorobutyryl derivatives in conjunction with electron capture detection had been previously reported for measuring 20α-OH-progesterone in rabbit plasma (Exely et al., 1968) there were no such reports for progesterone.

The practical difficulty of isolating greater than microgram amounts of the substances in rabbit plasma that were measured by the assays limited the physicochemical tests that could be conveniently applied. Mass spectrometric analysis provided a feasible physicochemical test that could be applied, even with the small masses of material

available. The mass spectrometric analysis yielded substantial evidence with which to confirm the identities of the compounds in question. Besides providing indications of molecular structure and weight the mass spectra of isolated compounds could be compared with the mass spectra of reference compounds of which sufficient quantities were available to verify identities by melting points and elemental analysis.

Radiochemical Purity and Identity of Isotopically Labelled 20α-OH-Progesterone and Progesterone Used to Monitor Recovery Rates

Lack of radiochemical purity in isotopes used to monitor losses of progestins during isolation procedures would result in lower apparent recovery rates than were actually obtained. To prevent this type of error from occurring in the assay procedures isotopes were purified by thin-layer chromatography on silica gel in a solvent system of cyclohexane:ethyl-acetate (1:1; v:v). In order to avoid contaminating the high specific activity steroids with radioinert steroids during the thin-layer purification, radioinert reference steroids were not used to locate the appropriate R_f at which to elute the purified isotopic steroids. Instead, the appropriate areas to elute were determined with the aid of radiochromatograph scans of the thin-layer plates (scans A, Figures 2 and 3) and the assumption that the principal peak of radioactivity indicated the position of the desired steroid. To verify that the correct area of silica gel had been eluted and to check radiochemical purity, aliquots of the purified isotopic steroids were run on silica gel, with appropriate radioinert reference steroid as carrier, in a solvent system of water:methanol:chloroform:toluene (1:20:60:120;

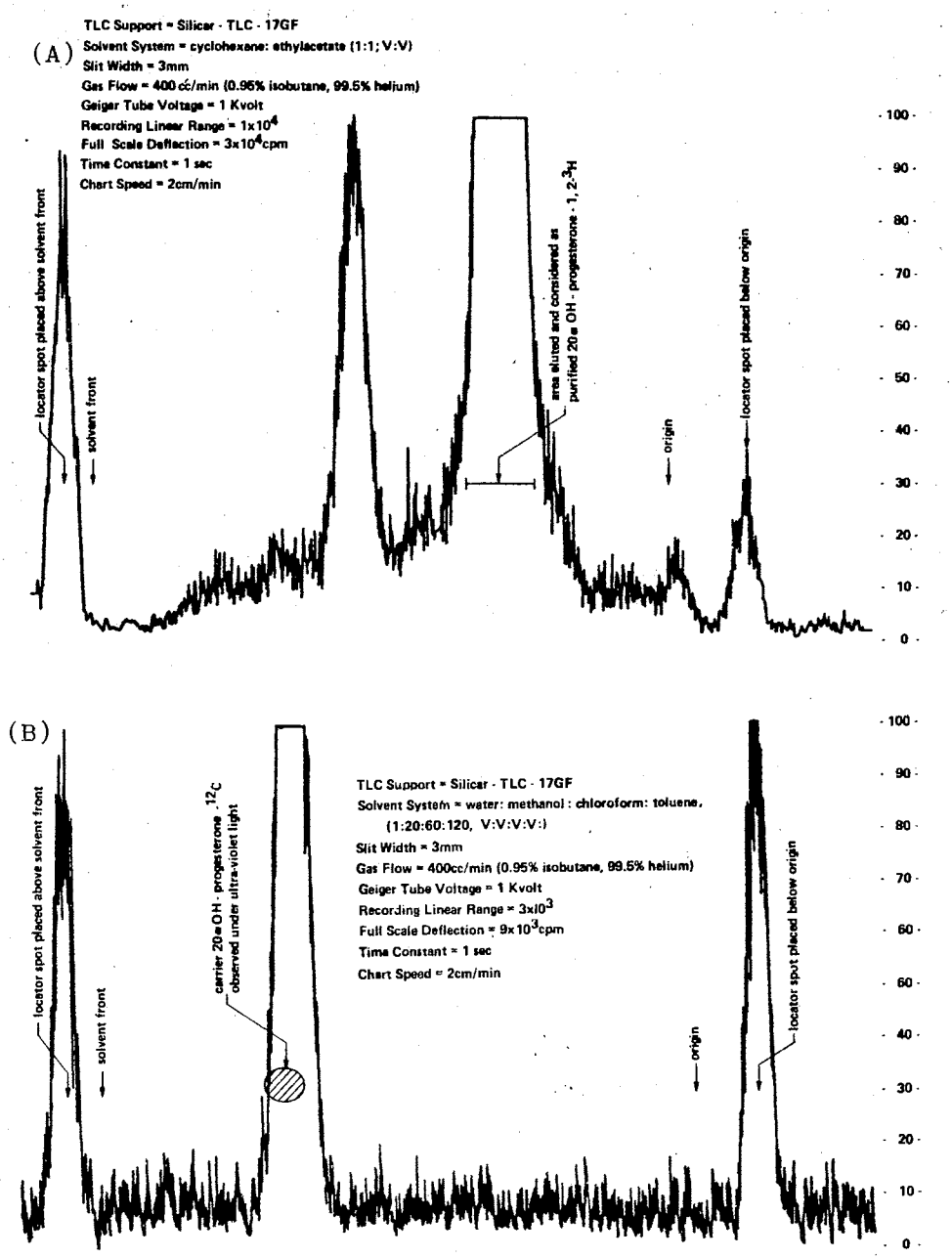

Figure 2. Tracings From Radiochromatograph Scans of 20αOH-Progesterone-1,2-^{3}H Run on TLC Plates. (A) is a scan tracing of the TLC plate on which 20αOH-progesterone-1,2-^{3}H was initially purified in a solvent system of cyclohexane:ethylacetate(1:1; v:v). (B) is a subsequent scan tracing of a TLC plate on which an aliquot of the purified 20αOH-progesterone-1,2-^{3}H was run with radio-inert reference 20αOH-progesterone in a solvent system of water:methanol:chloroform:toluene (1:20:60:120; v:v:v:v) to verify the identity and radiochemical purity of the purified 20αOH-progesterone-1,2-^{3}H.

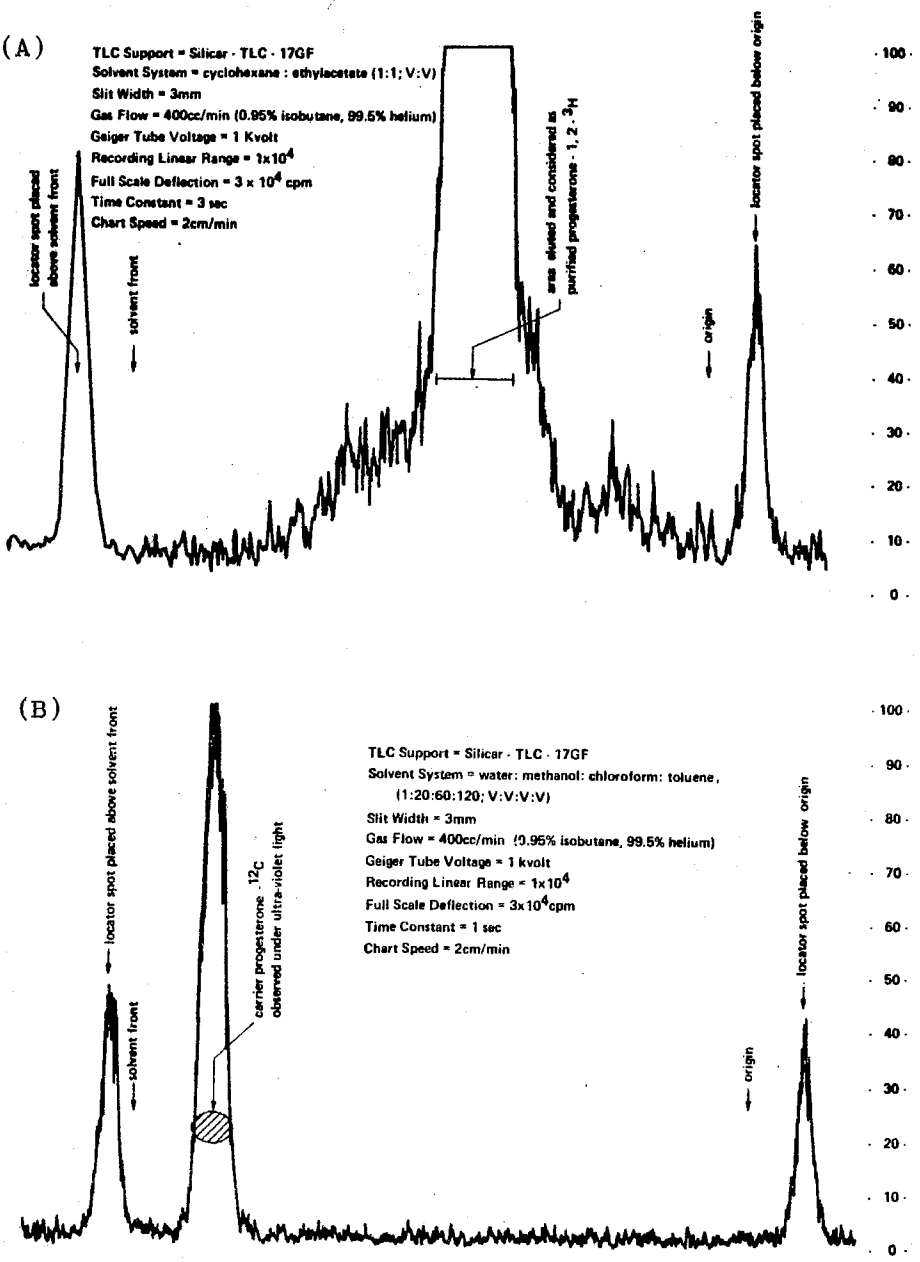

Figure 3. Tracings From Radiochromatograph Scans of Progesterone-1,2-^{3}H Run on TLC Plates. (A) is a scan tracing of the TLC plate on which progesterone-1,2-^{3}H was initially purified in a solvent system of cyclohexane:ethyl-acetate (1:2; v:v). (B) is a subsequent scan tracing of a TLC plate on which an aliquot of the purified progesterone-1,2-^{3}H was run with radioinert reference progesterone in a solvent system of water:methanol:chloroform: toluene (1:20:60:120; v:v:v:v) to verify the identity and radiochemical purity of the purified progesterone-1,2-^{3}H.

v:v:v:v) in unlined tanks. After the thin-layer plates were run the location of the carrier steroids was determined with the aid of ultraviolet light and compared with the location of areas of radioactivity observed in tracings of radiochromatograph scans (scans B, Figures 2 and 3). Such studies on aliquots gave no indications of radiochemical impurities in the purificed isotopes. Location of the radioactivity at the same R_f value as reference carrier steroid was indicative that the radioactivity was associated with the desired compound, particularly since the plates used for purification (scans A, Figures 2 and 3) were run in a solvent system (cyclohexane:ethyl-acetate; 1:1; v:v) which was distinctly different from that used with verification plates (water: methanol:chloroform:toluene; 1:20:60:120; v:v:v:v).

Identities of Reference Steroids

Reference Progesterone. A compound designated as progesterone was purchased from Steraloids, Incorporated. Recrystallization of this compound from methanol gave prism form crystals that had an uncorrected melting range of 127.5-129.0° C. Fieser and Fieser (1959) and Handbook of Chemistry and Physics (1963) give a melting point of 128° C for the α isomer of progesterone.

Reference 20αOH- and 20βOH-Progesterone. Compounds designated as 20αOH-progesterone and 20βOH-progesterone were obtained from the Upjohn Company and Steraloids, Incorporated, respectively. The uncorrected melting range of such 20αOH-progesterone after recrystallization from ethanol was 161.5-162.0° C. The uncorrected melting range of such 20βOH-progesterone after recrystallization from acetone was 169.5-172.0° C. Hayano et al. (1954), Sweat et al. (1958), and van der Molen

et al. (1967) reported melting points for 20αOH-progesterone as 162°C, 162°C, and 167°C, respectively. Wiest (1956) reported a melting point of 172°C for 20βOH-progesterone which in that publication he incorrectly called 20αOH-progesterone (Fieser and Fieser, 1959). Van der Molen et al. (1967) reported a melting point of 174°C for 20βOH-progesterone.

Reference 20α- and 20β-Heptafluorobutyryl-Progesterone. Recrystallized 20αOH- and 20βOH-progesterone was treated as indicated under Materials and Methods to form heptafluorobutyryl derivatives which in the case of 20αOH-progesterone yielded crystals from hexane that had an uncorrected melting range of 138.0-140.5°C. In the case of 20βOH-progesterone crystals were obtained from ethanol and then acetone and had an uncorrected melting range of 113-114°C. Van der Molen et al. (1967) reported melting points of 140°C and 105°C for the monoheptafluorobutyryl derivatives of 20αOH- and 20βOH-progesterone, respectively.

Elemental analysis of the recrystallized heptafluorobutyryl derivatives of 20αOH- and 20βOH-progesterone indicated an elemental composition with 58.44% carbon, 6.13% hydrogen, and 25.73% fluorine for the derivative of 20αOH-progesterone and 58.46% carbon, 6.19% hydrogen, and 25.94% fluorine for the derivative of 20βOH-progesterone. The calculated percent composition of carbon, hydrogen, and fluorine in both 20α- and 20β-monoheptabutyryl derivatives of progesterone is 58.82%, 5.73%, and 26.05%, respectively.

Reference Heptafluorobutyryl-Testosterone. The identify of heptafluorobutyryl-testosterone used in these studies as an internal standard in the assay of 20αOH-progesterone and progesterone with electron capture following gas-liquid chromatography has been discussed (Rhynes, 1971; Goodwin, 1972).

Features of the Isolating Procedures that Provide for the Assays' Specificities

Mass spectrometric analysis (see page 81) established the chemical identity of the compounds isolated from ovarian venous plasma and assayed as 20α- and 20β-heptafluorobutyryl-progesterone. Such identification taken together with some features of the isolating procedures provide unequivocal evidence for the specificities of the assays (Brooks et al., 1970).

The first thin-layer separation of the procedures was run in benzene:ethyl-acetate (120:80; v:v) on silica gel and served not only to partially isolate the sought-after progestins from extraneous material remaining in the samples after CH_2Cl_2 extraction and NaOH saponification, but also served to separate 20αOH-progesterone (R_f 0.30) from progesterone (R_f 0.46).

The ability to separate 20αOH-progesterone from progesterone constituted an important feature of the isolation procedure because once separated, the two steroid progestins could be processed independently of each other for derivative formation, GLC purification, and assaying with ECD. This separation was particularly important since effective separation of either 20αOH- from 20βOH-progesterone or 20α- from 20β-heptafluorobutyryl-progesterone was not achieved by thin-layer chromatography on silica gel, though a variety of solvent systems were tried. However, 20α- and 20β-heptafluorobutyryl-progesterone were partially separated by the gas-liquid chromatographic procedures performed immediately before electron capture detection. Under standard operating conditions (column temperature 205°C, nitrogen carrier flow rate

100 ml/min at 40 psi) the retention time of 20α-heptafluorobutyryl-progesterone on the column (1 m X 4 mm id U-shaped, packed with 3% OV-225 on 80/100 mesh GAS-CHROM Q) was three to four minutes longer than for 20β-heptafluorobutyryl-progesterone (17 min versus 14 min). With the recorder's chart paper speed set at the standard 1 cm/min feed-out rate, a simultaneous application to the column of both 20α- and 20β-heptafluorobutyryl-progesterone would result in a 3 to 4 cm separation of their peaks as they were traced by the recorder. Thus, the presence of 20βOH-progesterone in the samples assayed for 20αOH-progesterone, or vice versa, would be clearly discernible. Peaks indicating such contamination were not observed; their absence attested to the effective separation of 20αOH-progesterone from progesterone by the thin-layer procedure following saponification and also to the specificity of the enzymatic conversion of progesterone to 20βOH-progesterone prior to heptafluorobutyration.

Identity of Compounds Isolated from Rabbit Plasma and Assayed as 20α- and 20β-Heptafluorobutyryl-Progesterone

Mass spectra of the reference heptafluorobutyryl derivatives of 20αOH- and 20βOH-progesterone (Figures 4 and 5) had molecular ion peaks at m/e 512. Intense peaks were observed at m/e 124, 175, 256, 298, and 470. These peaks would be consistent with loss of carbons 2 and 3 with an oxygen and 2 hydrogens (M^+ - 42) producing the intense peaks at m/e 470 and m/e 256. The m/e 256 peak being produced from loss of the heptafluorobutyryl moiety by a McLafferty rearrangement (M^+ - 214 = m/e 298; McLafferty, 1967; Rock, 1971) in addition to the loss of the

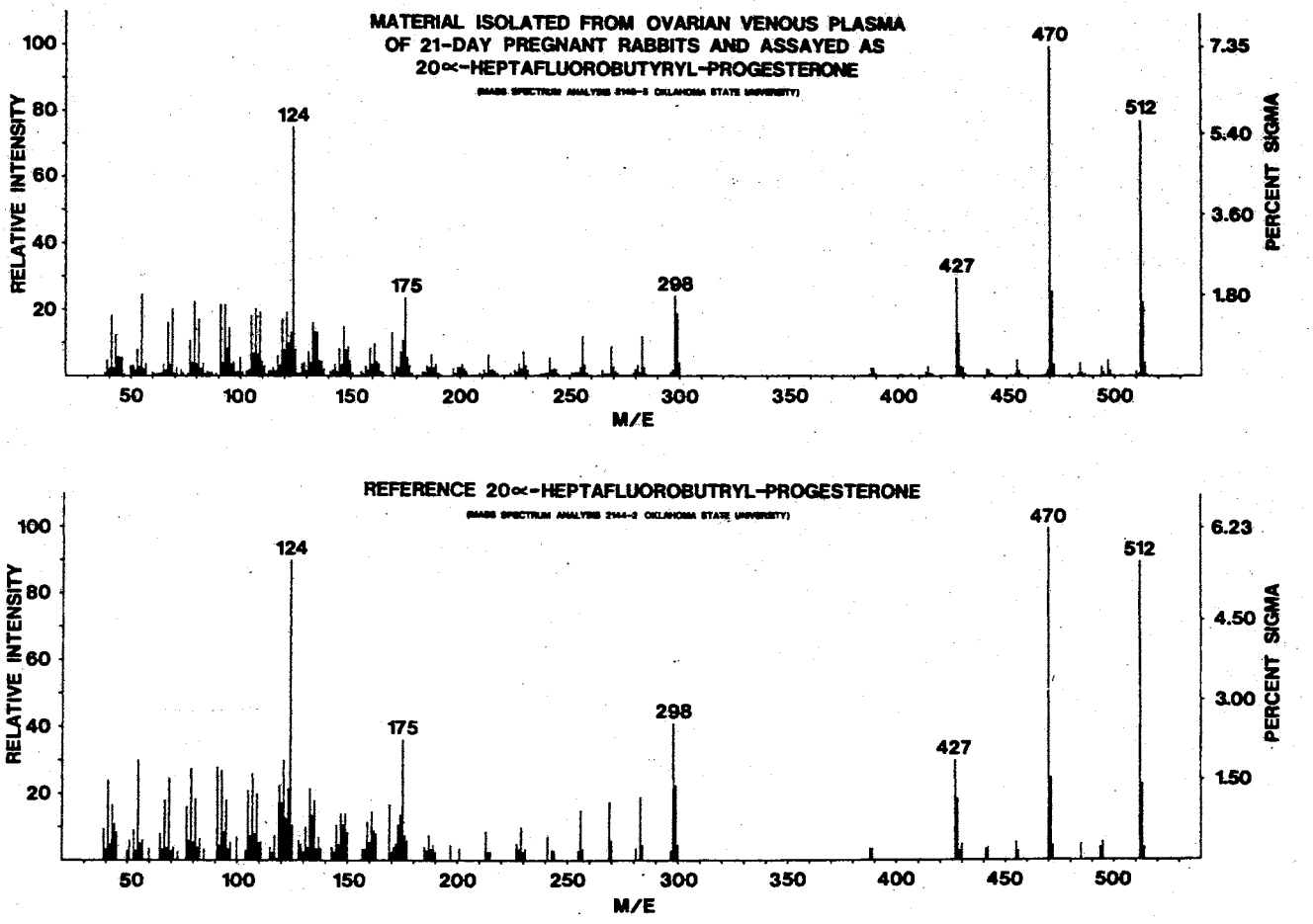

Figure 4. Mass Spectra of Material Isolated From Ovarian Venous Plasma of 21-Day Pregnant Rabbits and Assayed as 20α-Heptafluorobutyryl-Progesterone and Reference 20α-Heptafluorobutyryl-Progesterone

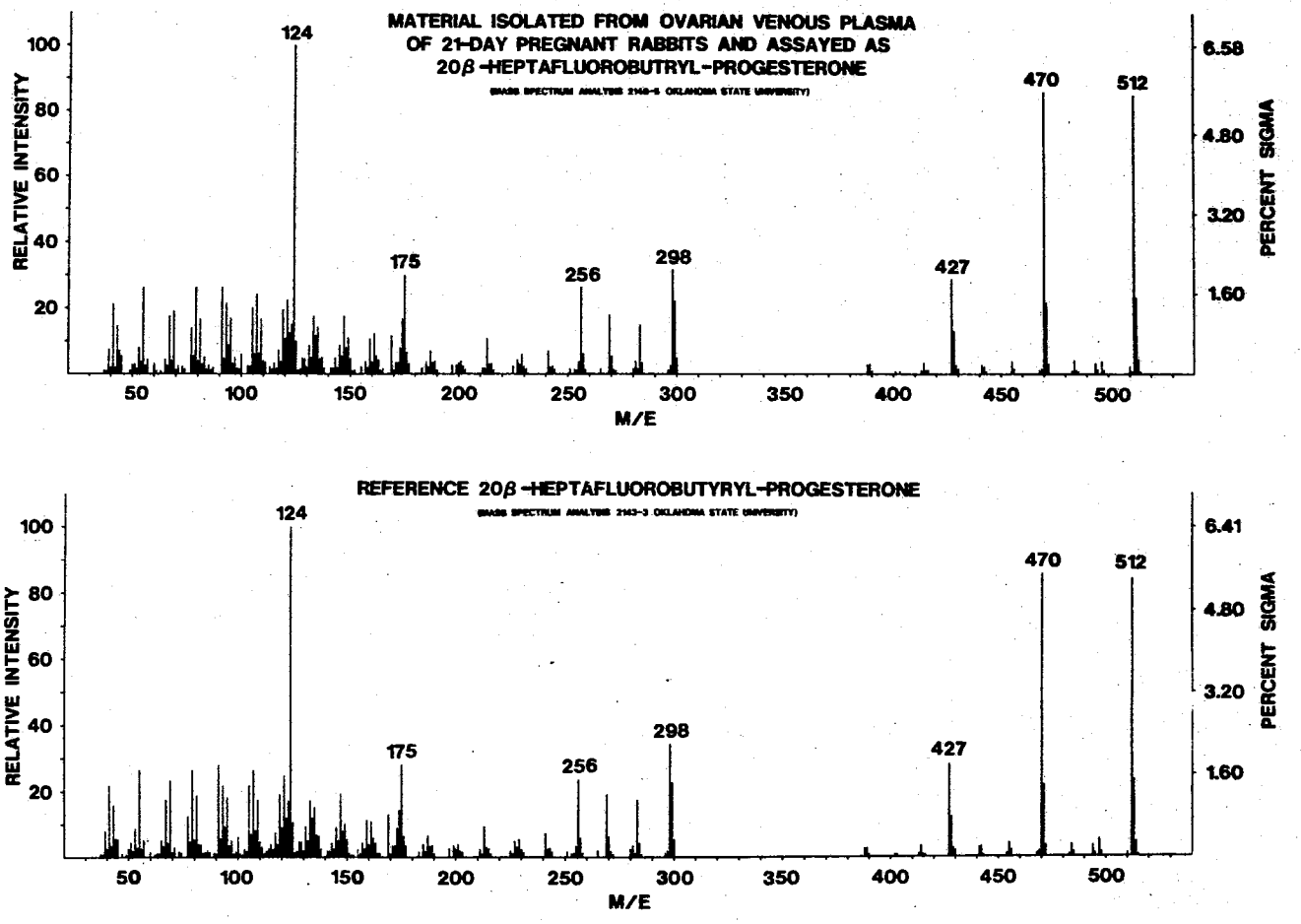

Figure 5. Mass Spectra of Material Isolated From Ovarian Venous Plasma of 21-Day Pregnant Rabbits and Assayed as 20β-Heptafluorobutyryl-Progesterone and Reference 20β-Heptafluorobutyryl-Progesterone

carbon 2 and 3 fragment (298 - 42 = m/e 256). The intense peak at m/e 124 would be produced by a characteristic B ring cleavage for steroids with rearrangement of two hydrogens (Budzikiewiez et al., 1964). Mass spectra of 20β-monochloroacetyl-progesterone by Stabenfeldt et al. (1969) had intense peaks at m/e 124, 175, 256, and 298 which were in agreement with earlier mass spectrometric studies on progesterone and derivatives (Peterson, 1962). Noteworthy is the absence of the large peak at m/e 175 in mass spectra of progesterone (Peterson, 1962) without an ester-carbon chain moiety on carbon 20 indicating a possible atypical ester cleavage effected by the halogenated carbon chain.

The distribution of the peaks with strong intensities and the relative heights of peaks within a mass spectrum are quite similar for the compounds isolated from ovarian venous plasma with respect to the appropriate reference compound. Ionization curves obtained at the time of mass spectrometric analyses are given in Figures 6 and 7 (see Appendix B). Total ionization curves of samples were studied at the time of the mass spectrometric analyses; no indications were found for the presence of more than one principal compound in the samples.

Thus, the chromatographic data from the isolation procedures and the identification of the isolated compounds by mass spectrometric analyses provide uniquivocal evidence for the specificities of the assays (Brooks et al., 1970).

Precision and Accuracy of the Assays for 20αOH-Progesterone and Progesterone

Introduction

The preceding section gave results of physicochemical tests to confirm the specificities of the assays for 20αOH-progesterone and progesterone. In this section results are given for two experiments in which the assays were repeated a number of times to provide indices of how precisely and accurately the assays measured 20αOH-progesterone and progesterone with the routine processing conditions encountered by samples taken from rabbits of the experiments addressing biological questions. No attempt was made at demonstrating the maximum resolving potentials of the assays.

Precision

The first experiment measured 20αOH-progesterone and progesterone in samples taken from a volume of pooled ovarian venous plasma collected from three rabbits. Theoretically each milliliter of the pooled plasma contained the same amount of the progestins. Variation among the respective values for progestin content, as measured by the assays, made possible calculation of indices of precision for the assays. Ovarian venous plasma was used because it represented a fluid that was assayed for progestin content in the biological experiments of the thesis. Two 2.5 ml samples and two 5.0 ml samples, drawn from the pooled volume of plasma, were processed on each of three different dates. This experimental design was used because the samples from rabbits of the experiments addressing biological questions were not all processed at

the same date nor were they of a constant volume. The results of the repeated assays were expressed in nanograms of the progestins per milliliter of plasma (Table I). The percent coefficients of variation were found to be 11.4 and 11.9 for the 20αOH-progesterone and progesterone assays, respectively.

Accuracy

The principal objective of the second experiment was to calculate indices of accuracy for the assays. In this experiment 40, 120, or 360 ng of both progestins were added in benzene to each of duplicate extraction tubes. The benzene was evaporated under nitrogen and the progestins were reconstituted in 5 ml 0.5% bovine serum albumin (Nutritional Biochemicals Corporation). The reconstituted progestins were allowed to equilibrate with the bovine serum albumin under refrigeration for a minimum of 15 minutes following which the bovine serum albumin was assayed for 20αOH-progesterone and progesterone content by the usual procedures. The experiment was replicated on three different dates, giving a total of six measurements for each progestin at each of the three levels of progestin. Results were expressed as total nanograms of progestin measured in the sample (Table II). The indices of accuracies of the assays were calculated from the variation in differences between the values for measured masses of progestins and the masses of progestins assumed to have been added to the tubes. Indices of the precisions of the assays were calculated from the variation among the values for measured masses of progestins. The percent coefficients for accuracies averaged 11.8. The percent coefficients of precision averaged 7.72.

TABLE I

PRECISION OF ASSAYS WHEN MEASURING 20αOH-PROGESTERONE AND PROGESTERONE IN POOLED OVARIAN VENOUS PLASMA FROM PREGNANT RABBITS

Date of Replicate	Volume of Plasma Assayed (ml)	Amounts of Progestins Found	
		20αOH-progesterone (ng/ml)	progesterone (ng/ml)
8·25·71	2.5	480	313
	2.5	552	308
	5.0	438	292
	5.0	406	296
8·30·71	2.5	390	335
	2.5	442	268
	5.0	421	255
	5.0	381	265
9· 6 ·71	2.5	437	347
	2.5	404	350
	5.0	407	332
	5.0	378	363
mean		428	315
precision[a]		11.4%	11.9%

[a]Percent coefficient of variation of observations from the mean for observations calculated according to Ostle (1964).

TABLE II

PRECISION AND ACCURACY OF ASSAYS WHEN MEASURING KNOWN AMOUNTS OF 20αOH-PROGESTERONE AND PROGESTERONE ADDED TO 0.5% BOVINE SERUM ALBUMIN

Steroid Assayed	20αOH-Progesterone			Progesterone		
Mass of Steroid Added (ng)	40	120	360	40	120	360
Number of Observations	5	6	6	5	6	6
	Amounts of Progestins Found					
Mean of Observations (ng)	33.3	119	378	43.7	125	382
Precision[a]	10.3%	11.4%	9.44%	3.75%	5.92%	5.53%
Accuracy[b]	20.5%	11.6%	11.2%	11.0%	7.77%	8.83%

[a]Percent coefficient of variation of observations from the mean for observations. Calculated according to Ostle (1964).

[b]Percent coefficient of variation of observations from the mass of steroid added. This method of expressing accuracy is based upon Ostle's (1964) discussion of precision and accuracy.

Acute Effects of Removing Components, or the Entire Gravid Uterus, on Systemic Plasma Concentrations and Ovarian Output of Progestins

Systemic Plasma Concentrations of 20αOH-Progesterone and Progesterone

20αOH-Progesterone. Average pre-treatment concentrations of 20αOH-progesterone in rabbits that were subsequently laparotomized or had fetuses, fetuses plus placentas, or the entire gravid uterus removed were 8.46, 9.58, 6.79, 7.51, and 10.1 ng/ml, respectively (Table III). The last two values represent two groups of rabbits from which the entire gravid uterus was subsequently removed; however, post-treatment blood was collected from the last group at six hours, instead of 24 hours after the operations. No significant differences were found at the 5% level among the mean pre-treatment systemic 20αOH-progesterone concentrations for the five groups of rabbits with analysis of variance (Table VIII). This indicated some similarity among the groups of rabbits used in the different treatments with regard to systemic levels of 20αOH-progesterone prior to initiating treatments. Average post-treatment concentrations of 20αOH-progesterone in systemic plasma of these same rabbits were 6.31, 7.61, 5.61, 5.62, and 6.84 ng/ml (Table III). There were no significant differences at the 5% level between these five means (Table IX) as determined by analysis of variance. However, it was noted that following treatment operations a decrease in systemic plasma concentrations of 20αOH-progesterone, ranging from 17.4 to 32.3% was observed in all treatment groups (Table III). To test

TABLE III

EFFECTS OF LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS ON 20αOH-PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS

Treatment (Components Removed)	None (Laparotomy)[a]	Fetuses[a]	Fetuses Plus Placentas[a]	Entire Gravid Uterus[a]	Entire Gravid Uterus (6 hr)[b]
Number of Rabbits	8	8	8	8	8
Pre-treatment (ng/ml ± s.e.m.)	8.46 ±2.54	9.58 ±2.21	6.79 ±2.46	7.51 ±0.94	10.1 ±3.69
Post-treatment (ng/ml ± s.e.m.)	6.31 ±1.12	7.61 ±1.37	5.61 ±1.26	5.62 ±1.50	6.84 ±1.63
Percent Change	-25.4%	-20.6%	-17.4%	-25.2%	-32.3%

[a]Post-treatment systemic plasma collected 24 hours after treatment operations.

[b]Post-treatment systemic plasma collected six hours after treatment operations.

whether these decreases were significant within any one treatment group, the mean post-treatment concentration of 20αOH-progesterone was compared to the mean pre-treatment value. The comparisons were performed using paired observations (Table X), since any rabbit in a treatment provided both a pre- and post-treatment observation. The decline due to operation, anesthesia, and manipulation was not significant at the 5% level in any of the treatments (Table X). These observations and the lack of significant differences found by analysis of variance for post-treatment 20αOH-progesterone concentrations in different treatment groups (Table IX) indicate that 20αOH-progesterone concentrations in systemic plasma were not significantly affected by any of the treatments.

Progesterone. Laparotomized rabbits and rabbits that had fetuses, fetuses plus placentas, or the entire gravid uterus removed had average pre-treatment concentrations of progesterone of 10.6, 12.2, 5.66, 11.4, and 14.2 ng/ml, respectively (Table IV). No significant differences were found at the 5% level between the means for pre-treatment systemic plasma concentrations of the five groups of rabbits with analysis of variance (Table XI). Average post-treatment concentrations of progesterone in systemic plasma of these same rabbits were 10.1, 11.1, 2.30, 1.34, and 16.8 ng/ml, respectively (Table IV). Analysis of variance indicated differences ($P < .01$) between these means (Table XII). Newman-Keul's Sequential Range Test (Table XIII) showed that the differences were between rabbits from which blood was collected 24 hours after either the entire gravid uterus, or the fetuses plus placentas, were removed and rabbits from which blood was collected six hours after removing the entire gravid uterus.

TABLE IV

EFFECTS OF LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS ON PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS

Treatment (Components Removed)	None (Laparotomy)[a]	Fetuses[a]	Fetuses Plus Placentas[a]	Entire Gravid Uterus[a]	Entire Gravid Uterus (6 hr)[b]
Number of Rabbits	8	8	8	8	8
Pre-Treatment (ng/ml ± s.e.m.)	10.62 ±3.48	12.25 ±1.25	5.66 ±0.76	11.36 ±3.07	14.18 ±2.43
Post-Treatment (ng/ml ± s.e.m.)	10.10 ±3.10	11.06 ±1.65	2.30 ±0.75	1.34 ±0.42	16.80 ±2.73
Percent Change	-4.81%	-9.75%	-59.4%	-88.6%	+18.4%

[a]Post-treatment systemic plasma collected 24 hours after treatment operations.

[b]Post-treatment systemic plasma collected six hours after treatment operations.

Table IV shows that the treatment operations were followed by changes in mean systemic progesterone concentrations ranging from a 88.6% decrease to a 18.4% increase. To test whether these changes were significant within any one treatment group, the mean post-treatment concentration of progesterone was compared to the mean pre-treatment value. Because any rabbit in a treatment provided both pre- and post-treatment observations the comparisons were performed using paired observations (Table XIV). Neither the 4.81% nor 9.75% decreases in laparotomized rabbits and rabbits that had only fetuses removed, respectively, was significant at the 5% level, indicating that systemic plasma progesterone concentrations were not significantly affected by these treatments. In contrast, the 59.4% decrease that occurred in rabbits with fetuses plus placentas removed was highly significant ($P < .01$). The 88.6% decrease that occurred when blood was collected 24 hours after removing the entire gravid uterus was significant at the 5% level. The 18.4% increase that occurred when blood was collected six hours after removing the entire gravid uterus was highly significant ($P < .01$). These comparisons indicate that 24 hours after laparotomies were performed, or the fetuses removed, no significant changes in systemic concentrations of progesterone were observed. However, 24 hours after removing fetuses plus placentas or the entire gravid uterus, significant decreases in systemic concentrations of progesterone occurred and that six hours after removing the entire gravid uterus a significant increase was observed. Taken together these results suggest alterations in progesterone but not 20αOH-progesterone secretion by the rabbit ovaries due to removal of placentas. Since these changes in progesterone concentrations could be due to altered metabolism rather than

biosynthesis and secretion we quantified progestin secretions from rabbit ovaries by measuring progestin concentration in ovarian venous effluent as well as rate of blood flow through the ovary.

Weights, Blood Flow Rates, and Number of Corpora Lutea of Cannulated Ovaries

The mean weights for the cannulated ovaries in the different treatments ranged between 439 and 531 mg (Table V). No significant differences ($P > .25$) were found among the means of ovarian weights by analysis of variance (Table XV). The mean rates of blood flow for the cannulated ovaries in the different treatments ranged between 3.82 and 6.02 ml/g ovary/min (Table V). No significant differences ($P > .25$) were found among the means for blood flow by analysis of variance (Table XVI). The mean number of corpora lutea for the cannulated ovaries in the different treatments ranged between 4.1 and 8.2 (Table V). Significant differences ($P < .01$) were found among the means for number of corpora lutea by analysis of variance (Table XVII). Application of Newman-Keul's Sequential Range Test (Table XVIII) indicated that all means were similar except that the average number of corpora lutea was significantly ($P < .01$) higher in rabbit ovaries obtained from animals in which the entire gravid uterus was removed six hours before collecting ovarian venous blood when compared to ovaries in which fetuses plus placentas were removed. Thus, there were no significant differences ($P > .25$) in ovarian weight and blood flow through the ovaries between the groups. This coupled with the observation that there were no differences among groups with regard to pre-treatment systemic plasma concentrations of 20αOH-progesterone and progesterone suggests that the

TABLE V

MEAN WEIGHTS, BLOOD FLOW RATES, AND NUMBERS OF CORPORA LUTEA OF THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	None (Laparotomy)[a]	Fetuses[a]	Fetuses Plus Placentas[a]	Entire Gravid Uterus (6 hr)[b]	Entire Gravid Uterus[a]
Number of Rabbits	8	8	8	8	8
Mean Weight (mg ± s.e.m.)	528 ±73	463 ±31	439 ±63	496 ±61	531 ±58
Blood Flow (ml/g ovary/min ± s.e.m.)	3.92 ±0.60	4.71 ±0.70	5.33 ±0.89	3.82 ±0.86	6.02 ±1.04
Number of Corpora Lutea (± s.e.m.)	6.5 ±2.4	6.5 ±0.6	4.1 ±0.6	8.2 ±0.7	7.6 ±0.8

[a]Ovarian venous blood collected 24 hours after treatment operation.

[b]Ovarian venous blood collected six hours after treatment operation.

animals randomly allotted to the five treatment groups were from one homogenous population.

20αOH-Progesterone and Progesterone Output by Cannulated Ovaries

20αOH-Progesterone. Output of 20αOH-progesterone from cannulated ovaries was measured by three indices: concentration of 20αOH-progesterone in ovarian venous plasma, secretion rate per ovary, and secretion rate per gram ovary (Table VI).

Analysis of variance (Table XIX) revealed no differences at the 5% level between the means of the different treatments for 20αOH-progesterone concentrations in ovarian venous plasma, for ovarian 20αOH-progesterone output expressed as μg/ovary/hr, or for ovarian 20αOH-progesterone output expressed as μg/g ovary/hr (Table XXI), indicating lack of significant effect of treatments on ovarian 20αOH-progesterone output.

Progesterone. Output of progesterone from the cannulated ovaries was measured by four indices: concentration of progesterone in ovarian venous plasma, secretion rate per ovary, secretion rate per gram ovary, and secretion rate per corpus luteum (Table VII).

Analysis of variance of concentrations of progesterone in the venous plasma of cannulated ovaries indicated significant differences ($P < .01$) due to the effects of treatments between the means of the treatment groups (Table XXII). Newman-Keul's Sequential Range Test (Table XXIII) indicated progesterone concentrations in the ovarian venous plasma of cannulated ovaries was significantly lower ($P < .05$) in rabbits that had either the entire gravid uterus or fetuses plus

TABLE VI

20αOH-PROGESTERONE OUTPUT FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	None (Laparotomy)[a]	Fetuses[a]	Fetuses Plus Placentas[a]	Entire Gravid Uterus[a]	Entire Gravid Uterus[b] (6 hr)
Number of Rabbits	8	8	8	8	8
Index of Ovarian 20αOH-Progesterone Output					
ng/ml ovarian venous plasma ± s.e.m.	317.4 ±52.6	352.7 ±108.5	190.3 ±55.5	77.6 ±17.3	343.8 ±104.9
μg/ovary/hr ± s.e.m.	28.5 ±4.6	19.5 ±3.6	19.9 ±7.0	10.4 ±2.2	16.5 ±3.4
μg/g ovary/hr ± s.e.m.	59.3 ±9.8	39.9 ±6.8	39.6 ±10.9	21.0 ±5.3	41.0 ±7.2

[a]Ovarian venous blood collected 24 hours after treatment operations.

[b]Ovarian venous blood collected six hours after treatment operations.

TABLE VII

PROGESTERONE OUTPUT FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	None (Laparotomy)[a]	Fetuses[a]	Fetuses Plus Placentas[a]	Entire Gravid Uterus[a]	Entire Gravid Uterus (6 hr)[b]
Number of Rabbits	8	8	8	8	8
Index of Ovarian Progesterone Output					
ng/ml ovarian venous plasma ± s.e.m.	358.7 ±76.3	421.1 ±116.7	49.7 ±10.1	50.0 ±16.5	477.3 ±90.7
µg/ovary/hr ± s.e.m.	29.2 ±7.2	41.1 ±7.1	4.2 ±0.7	6.6 ±2.2	39.0 ±5.8
µg/g ovary/hr ± s.e.m.	52.0 ±5.5	78.2 ±12.9	11.2 ±2.4	13.3 ±1.4	83.6 ±13.0
µg/corpus luteum/hr ± s.e.m.	4.42 ±0.56	6.39 ±0.98	1.26 ±0.30	0.93 ±0.32	4.94 ±0.85

[a]Ovarian venous blood collected 24 hours after treatment operations.

[b]Ovarian venous blood collected six hours after treatment operations.

placentas removed 24 hours before collection of ovarian venous blood than in laparotomized, fetectomized rabbits, or rabbits from which ovarian venous blood was collected six hours after hysterectomy.

Analysis of variance indicated significant differences ($P < .01$) due to the effects of treatments between the five means for progesterone output from cannulated ovaries expressed as μg/ovary/hr (Table XXIV). Newman-Keul's Sequential Range Test (Table XXV) indicated that the mean progesterone output from cannulated ovaries expressed as μg/ovary/hr was significantly lower ($P < .05$) in rabbits that were collected 24 hours after the entire gravid uterus, or fetuses plus placentas were removed, than from laparotomized or fetectomized rabbits or rabbits from which the entire gravid uterus was extirpated six hours before collection of samples of ovarian venous blood.

Analysis of variance indicated significant differences ($P < .01$) due to the effects of treatments between the five means for progesterone output from cannulated ovaries expressed as μg/g ovary/hr (Table XXVI). Newman-Keul's Sequential Range Test (Table XXVII) indicated that when progesterone output from cannulated ovaries was expressed as μg/g ovary/hr, output from ovaries of rabbits that had either the entire gravid uterus or the fetuses plus placentas removed 24 hours before ovarian blood collection, was significantly ($P < .01$) lower than from laparotomized rabbits, fetectomized rabbits, and rabbits from which ovarian venous blood was collected six hours after hysterectomy. The same significant differences were found among the means of the five treatment groups when ovarian progesterone output was expressed as μg/corpus luteum/hr and subjected to analysis of variance (Table XXVIII) and Newman-Keul's Sequential Range Test (Table XXIX).

Thus, by all four of the indices used to measure ovarian output, progesterone output was lower in rabbits from which ovarian venous blood was collected 24 hours after removing the entire gravid uterus, or fetuses plus placentas, than in rabbits from which ovarian venous blood was collected 24 hours after laparotomy, fetectomy, or rabbits from which ovarian venous blood was collected six hours after hysterectomy. No significant differences resulting from treatments were found in ovarian progesterone output, as measured by the four indices, between rabbits from which ovarian venous blood was collected six hours after removing the entire gravid uterus, or 24 hours after removing the fetuses, or laparotomy.

Taken together the observations on 20αOH-progesterone and progesterone output from the ovaries cannulated in the five treatment groups indicate that continued ovarian progesterone output at day 21 of gestation in rabbits is dependent upon the presence of placentas, but not fetuses. In contrast, continued ovarian output of 20αOH-progesterone in 21-day pregnant rabbits is not dependent upon the presence of the gravid uterus. Progesterone output fails between six and 24 hours after removing the influence of the gravid uterus, indicating a relatively short half life for the influence and an acute dependence of ovarian progesterone output for this influence.

The low progesterone output from the ovaries cannulated in rabbits 24 hours after hysterectomy or removal of the placentas indicates that the low post-treatment concentrations of progesterone in systemic plasma observed in these treatments was caused by decreased ovarian output of progesterone rather than altered systemic metabolism of progesterone.

CHAPTER V

DISCUSSION

Introduction

The present experiment was designed to examine the regulatory influence of the gravid uterus on the endocrine functions of the female gonad by monitoring concentrations of specific species of hormonal steroids in systemic and ovarian venous blood after surgically altering the status of pregnancy at 21 days of gestation in rabbits. Answers were sought to the following questions: What are the acute effect(s) of removing the gravid uterus, at a time beyond the duration of pseudopregnancy, on ovarian endocrine function? From what anatomical part of the gravid uterus does the influence that sustains ovarian progestin secretions beyond the duration of pseudopregnancy originate? Prior to obtaining answers to these questions it was imperative to establish that the hormone assay procedures were specific, accurate, and precise. Once the hormone assays were established as valid, the effects of removing the gravid uterus upon systemic concentrations and ovarian output of 20αOH-progesterone and progesterone were examined six and 24 hours post-operatively. Observations were also made on the effects of fetectomy or removal of the fetuses plus placentas upon systemic concentrations and ovarian output of 20αOH-progesterone and progesterone 24 hours post-operatively. Such observations on specific progestins

have not been previously reported in the literature and, thus, yield new insight into the regulatory influences that the conceptus exerts on the adult female gonad.

The first part of the discussion is directed at the validation of our methods for collecting data. The second portion takes up the answers to the biological questions.

Assay Procedures

Precision and Accuracy

Electron capture detection following gas-liquid chromatography provided the means for measuring submicrogram amounts of progestins encountered in the biological samples obtained in the present study. Coefficients of precision and accuracy with this assay method as performed by this investigator ranged between 3.75 and 20.5 percent when measuring progestins added to water containing 0.5 percent bovine serum albumin. These coefficients of precision and accuracy are approximately the same as values reported by Resko (1971) for measuring the chloroacetate derivative of testosterone via gas-liquid chromatography equipped with electron capture detection. Resko reported coefficients of accuracy of 25 percent when measuring 5 and 10 ng of testosterone added to plasma obtained from immature male rhesus monkeys and a coefficient of precision of 11.7 percent when measuring concentrations of testosterone in aliquots from the same pool of plasma obtained from adult male rhesus monkeys. Stabenfeldt et al. (1969) reported coefficients of precision of from 2.4 to 3.3 percent when measuring progesterone added to water containing 0.75 percent bovine serum albumin

using the chloroacetate derivative with electron capture detection. Thus, the assay techniques used in the present experiment allowed measurement of submicrogram amounts of steroidal hormones in biological samples with acceptable reliability. However, to establish that the assay systems were unequivocally specific for the progestins in question, evidence of the structural identities of the compounds measured was obtained.

Confirmation of the Chemical Structures of the Compounds Measured by the Assay Systems

Confirmation of the chemical structures of the compounds measured by the assay system were obtained by mass spectrometric analysis. The mass spectra of the compounds isolated from rabbit plasma and assayed as 20α- and 20β-heptafluorobutyryl-progesterone bore a strong resemblance to mass spectra of the reference 20α- and 20β-heptafluorobutyryl-progesterone. Complete spectra of these compounds were not found in the literature. Van der Molen et al. (1967) reported a base peak at m/e 512, the molecular ion for 20β-heptafluorobutyryl-progesterone. The base peak for reference 20β-heptafluorobutyryl-progesterone in the present studies was at m/e 124; however, the relative intensity of the molecular ion peak at m/e 512 was large (85 percent). The difference between the location of the base peak reported by van der Molen et al. (1967) and the present studies can be accounted for by differences in mass spectrometric analysis techniques.

The 113-114°C melting range for 20β-heptafluorobutyryl-progesterone found in the present studies is 9°C higher than the 105°C reported by van der Molen et al. (1967). It is not possible to explain this

variance since the melting point of 172°C given by Fieser and Fieser (1959) for 20βOH-progesterone agreed with the melting range of (169.5 - 172.0°C) of precursor 20βOH-progesterone used in the present studies and the elemental composition of the product of heptafluorobutyration was in close agreement with the calculated values for 20β-heptafluorobutyryl-progesterone.

Corroborative evidence for the identities of the compounds measured were the observations that the concentrations of the compounds assayed as 20αOH-progesterone and progesterone were found to be 14-50 and 35-40 times greater, respectively, in ovarian venous plasma than in systemic plasma, indicating the compounds to be of ovarian origin.

Thus, mass spectrometric analysis confirmed the occurrence of 20αOH-progesterone and progesterone in ovarian venous plasma of rabbits as previously reported by Mikhail et al. (1961) and Simmer et al. (1963), and when combined with the chromatographic evidence available from the isolation procedures provided uniquivocal evidence for the specificity of the assay systems. With the validity of the progestin assay systems established the next question of a technical nature was whether or not surgical protocols could be followed on pregnant rabbits without inducing artifactual changes in progestin output.

Effects of Surgical Protocols Common to All Ovarian Progestin Secretion Treatments

The surgical procedures used in the present experiment encompassed anesthesia with pentobarbital and ether, trauma of entering the abdominal cavity, cardiac punctures, and replacing blood with dextran-saline plasma expander. Samples of systemic and ovarian blood collected from

the laparotomized 21-day pregnant rabbits during the course of the experiment afforded the opportunity to evaluate the effects of these procedures on the endocrine processes of the experimental animals with respect to progestins and to compare the levels found for progestins in the present study with those observed by previous workers using the rabbit.

The average systemic plasma concentrations of 20α-OH-progesterone and progesterone in eight rabbits declined from pre-treatment values of 8.46 and 10.6 ng/ml, respectively, to post-operative values of 6.31 and 10.1 ng/ml, respectively, 24 hours after laparotomy. These declines were not statistically significant indicating that the trauma of surgery was well tolerated by the rabbits with respect to progestin output. Ovarian output of progestins from the cannulated ovaries of the laparotomized rabbits were 59.3 ± 9.84 and 52.0 ± 5.46 μg/g ovary/hr for 20α-OH-progesterone and progesterone, respectively. These figures agree with values of 66 ± 23 and 35 ± μg/g ovary/hr for 20α-OH-progesterone and progesterone observed by Hilliard et al. (1968) in seven normal rabbits between 21 - 25 days of gestation. Corroborative evidence indicating that trauma of surgical procedures did not have detrimental effects on ovarian output of progestins was provided by the observations that high ovarian progesterone output occurred for as long as six hours after removing the entire gravid uterus and that 20α-OH-progesterone output continued for as long as 24 hours post-operatively.

The foregoing discussion indicates that the assay system and surgical procedures were compatable with collecting meaningful results on the effects of the conceptus upon endocrine functions of the female gonad. Since the present experiment was a completely randomized design

it was critical to show that the experimental animals represented a single population and that effects between treatment groups were not confounded with animals of various populations. Measurements were made on several parameters (pre-treatment systemic plasma concentrations of 20αOH-progesterone and progesterone and the weights, blood flow rates, and number of corpora lutea of the cannulated ovaries) in an effort to determine the similarity of the experimental animals with regard to ovarian function prior to initiation of treatments.

Pre-Treatment Systemic Plasma Concentrations of Progestins and the Weights, Blood Flow Rates, and Number of Corpora Lutea of Cannulated Ovaries Among the Rabbits in the Five Treatment Groups

Systemic Plasma Concentrations of Progestins Prior to Initiation of Treatments

Analysis of variance indicated that pre-treatment systemic plasma progestin concentrations in the five treatment groups of the experiment were similar. Thus, the rabbits were essentially similar with regard to systemic plasma concentrations of progestins at the outset of the experiment.

Weights, Blood Flow Rates, and Numbers of Corpora Lutea in the Cannulated Ovaries

The mean weights of ovaries for the five treatments ranged between 439 and 531 mg which agrees with ovarian weights of 657 ± 75 mg observed by Hilliard *et al.* (1968) in rabbits between days 21-25 of pregnancy.

Analysis of variance indicated that averages for weights of the ovaries cannulated in the five treatment groups were similar ($P > .25$).

Blood flow rates reported by Mikhail et al. (1961) were about 50 ml blood/ovary/hr which is similar to the 40 - 50 ml blood/ovary/hr reported by Okano et al. (1966). Their blood flow rates were low compared to the 120 - 240 ml blood/ovary/hr reported by Hilliard et al. (1968) and the 80 - 160 ml blood/ovary/hr routinely obtained in the present studies. Analysis of variance indicated that averages for blood flow rates of the ovaries cannulated in the five treatment groups in the present experiment were similar ($P > .10$).

The average number of corpora lutea observed per cannulated ovary in the present studies ranged between 4.1 and 8.2 for the different treatments and tended to be somewhat higher than the approximately 4.8 average observed by Keyes and Armstrong (1968) in a group of six rabbits. Possibly the hormone regime associated with artificial insemination in our experiments could account for the higher numbers of corpora lutea observed in the present studies.

Thus, ovarian blood flow rates, weights, and numbers of corpora lutea were relatively uniform among the five treatments with the exception of the difference of numbers of corpora lutea observed between rabbits from which ovarian venous blood was collected 24 hours after removing the fetuses plus placentas and the rabbits from which ovarian venous blood was collected six hours after hysterectomy.

The similarities between the different groups of rabbits insured that post-treatment effects were probably not due to some pre-existing differences among the groups.

One of the questions this study was intended to answer was, what are the acute effect(s) of removing the gravid uterus, at a time beyond the duration of pseudopregnancy, on ovarian endocrine function? The other was from what part of the gravid uterus does the influence that sustains ovarian progestin secretions beyond the duration of pseudopregnancy originate? The answers to these two questions were sought in a single experiment.

Effects of Removing Components, or the Entire Gravid Uterus, on Systemic Plasma Concentrations and Ovarian Output of Progestins

Acute Effects of Removing the Entire Gravid Uterus on Systemic Plasma Concentrations and Ovarian Output of Progestins

Previous investigators obtained indirect evidence supporting the concept that ovarian progestin output declines following extirpation of the gravid uterus after midpregnancy in the rabbit. Klein (1933) noted a decline in progestin dominance five days after removing the gravid uterus on day 17 of pregnancy. His results were based on changes in the glands of the sterile uterine horn following extirpation of the contralateral gravid horn and the response of the sterile horn to oxytocin. Greep (1941) found a decline in the volume of corpora lutea five days after hysterectomy at day 20 of gestation in rabbits. Schofield (1960) demonstrated that uteri in rabbits at 24 - 25 days of gestation lost characteristics of progesterone dominance with respect to oxytocin stimulation 46 - 48 hours after removing placentas along with the fetuses

from the uterus. However, these indirect measures of ovarian endocrine function provide only a broad indication of the changes that occur in ovarian endocrine function. There is a time lag between such indirect endpoints and the changes occurring in ovarian endocrine function. Moreover, such end points provide only indirect circumstantial evidence insufficient to develop a unified theory of the role of the gravid uterus on endocrine functions of the ovary.

The present study with direct monitoring of specific progestins indicated that systemic plasma concentrations and ovarian output of 20αOH-progesterone were not significantly affected six and 24 hours after extirpation of the entire gravid uterus. In contrast, systemic plasma concentrations and ovarian output of progesterone declined significantly between six and 24 hours following extirpation of the gravid uterus in 21-day pregnant rabbits. These represent the first results showing that ovarian output of a specific steroidal hormone, progesterone, in pregnant rabbits, is acutely dependent upon the presence of influences emanating from the gravid uterus and that this influence of the gravid uterus has a life span of less than 24 hours.

Thus, in the rabbit the gravid uterus sustains ovarian output of progesterone, but not 20αOH-progesterone. The next part of the discussion pertains to that portion of the experiment designed to resolve from where in the gravid uterus this influence originates.

Effect of Particular Components of the Gravid Uterus on Systemic Concentrations and Ovarian Output of Progestins

Twenty-four hours after either the entire gravid uterus, or the fetuses plus placentas, were removed from 21-day pregnant rabbits, systemic concentrations and ovarian progesterone output declined significantly. In contrast, when the fetuses alone were removed, but the placentas were left *in situ*, systemic plasma concentrations and ovarian output of progesterone did not decline significantly, indicating that the placentas, but not the fetuses were the principal component of the gravid uterus responsible for maintaining progesterone output from 21-day pregnant rabbits. It is also possible to conclude that the uterus alone is not able to maintain luteal function which indicates that the myometrial gland does not possess the capacity to regulate luteal endocrine function as Ancel and Bouin (1912b) proposed. If the gravid uterus exerts a luteotropic influence on the ovary by "sparing" the metabolism of estrogen (Heckel, 1942) the metabolic effect is acutely dependent upon the presence of the conceptus in the uterus. The results of this study indicated that it is improbable that the estrogen "sparing" theory is the mechanism by which the corpus luteum is maintained during the second half of pregnancy in the rabbit.

The present experiment showed that ovarian output of progesterone is acutely dependent upon the presence of the placentas. Since during pregnancy the corpus luteum is the principal site of progesterone output in the rabbit (Hilliard *et al.*, 1968), the principal tissue affected by the presence of placentas appears to be the luteal tissue. The

endocrine function of the interstitial gland does not appear to be acutely dependent upon influences of the gravid uterus since significant effects resulting from treatments were not found for concentrations in systemic plasma and ovarian output of 20αOH-progesterone.

Results of the present study do not elucidate the number of steps intervening between the placentas and corpus luteum maintenance but do prove conclusively the existence of such a link. The influence of the placentas on ovarian function could be exerted directly on the corpus luteum or indirectly via the follicles, since exogenous estrogen will maintain the volumes of corpora lutea in hysterectomized rabbits at day 20 of gestation (Greep, 1941).

Whether the placentas must be attached to the uterus to exert their influence of sustaining progesterone output was not indicated by the present experiments. Kirby (1965) successfully transplanted placentas in mice to a number of sites in the abdominal cavity. Courrier (1941) succeeded in establishing ectopic pregnancies in the rabbit which indicated either induction of ectopic pregnancies or transplantation of placental tissue coupled with hysterectomy might be a feasible means of observing the effects of the conceptus on endocrine functions of the female gonad in the absence of the uterus. The recent advent of assay procedures capable of detecting subnanogram amounts of estrogens in biological sample (Korenman et al., 1970; Mikhail et al., 1970) renders possible studies on the influence of the conceptus on follicular tissue. Such studies would further characterize regulatory influences on the endocrine functions of the female gonad and would constitute appropriate sequels to the present studies.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Regulation of endocrine functions of the female gonad during gestation was studied by combining sensitive physicochemical techniques for monitoring hormonal steroids with surgical procedures for altering the status of pregnancy in 21-day pregnant rabbits. Specific objectives of the study were to determine the acute effect(s) that removing the gravid uterus, at a time beyond the duration of pseudopregnancy, had on ovarian endocrine function and to determine from what anatomical part of the gravid uterus the influence that sustains ovarian progestin secretions beyond the duration of pseudopregnancy originates. To these ends 40, 21-day pregnant rabbits were subjected to either laparotomy, removal of the fetuses, removal of the fetuses plus the placentas, or removal of the entire gravid uterus. Eight rabbits were allotted at random to each of the surgical procedures with the exception that 16 rabbits were allotted to the treatment in which the entire gravid uterus was removed. Twenty-five ml systemic blood samples were drawn by cardiac puncture immediately prior to performing the surgical procedures. A second 25 ml sample of systemic blood was collected from all rabbits 24 hours after the operations, except eight rabbits from which the second systemic sample of blood was collected six hours after removing the entire gravid uterus. Immediately following collection of the second systemic blood sample, a 15 minute collection of ovarian

venous blood was obtained from all rabbits. The volume of the sample of ovarian venous blood was noted. The ovary from which the venous blood had been collected was removed from the rabbit, weighed, and the number of corpora lutea in it counted. The plasma of each blood sample was analyzed for 20αOH-progesterone and progesterone content. The progestins were assayed as heptafluorobutyrate derivatives by electron capture detection following gas-liquid chromatography. The specificities of the assay systems were established by chromatographic isolation procedures and mass spectrometric analysis. Coefficients of precision and accuracy of the assays ranged between 3.75 and 20.5 percent.

Prior to surgical procedures there were no significant differences ($P > .25$) between the means for systemic plasma concentrations of progestins which ranged between 6.79 and 10.1 ng/ml for 20αOH-progesterone and between 5.66 and 14.2 ng/ml for progesterone. Significant ($P < .05$) decreases in systemic plasma concentrations of progesterone occurred within 24 hours after removing the gravid uterus, or removing the fetuses plus placentas, but not six hours after removing the gravid uterus. No significant decreases at the 5% level occurred in systemic plasma concentrations of 20αOH-progesterone following any of the surgical procedures. These results indicate that the groups of rabbits were similar with regard to progestins in the systemic plasma prior to altering the status of pregnancy by surgical intervention. Since plasma 20αOH-progesterone concentrations were not significantly decreased by removing any of the components of the gravid uterus, the interstitial gland of the rabbit ovary probably functions independently from the influences of the gravid uterus. On the other hand progesterone levels in systemic plasma declined between six and 24 hours in the rabbits from

which the placentas were removed either by extirpating the entire gravid uterus or by hysterotomy. Thus, ovarian output of progesterone, and probably luteal function, appeared acutely dependent upon a luteotropic influence from the placentas at 21 days of gestation in the rabbit. Direct monitoring of ovarian endocrine functions by measuring 20αOH-progesterone and progesterone in ovarian venous plasma confirmed the observations made on systemic plasma concentrations.

No significant differences due to treatments were found at the 5% level among the means for ovarian output of 20αOH-progesterone in the groups subjected to the various surgical and sample collecting protocols. Ovarian output of 20αOH-progesterone among treatments ranged between 77.6 and 352 ng/ml ovarian venous plasma, 10.4 and 28.5 μg/ovary/hr, and 21.0 and 59.3 μg/g ovary/hr. Ovaries from which blood was collected 24 hours after removing the entire gravid uterus, or the fetuses plus placentas, had significantly lower ($P < .05$) progesterone output than ovaries from which blood was collected 24 hours after laparotomy, or removing the fetuses, or six hours after removing the entire gravid uterus. These differences were present whether ovarian output of progesterone was expressed as ng/ml ovarian venous plasma, μg/ovary/hr, μg/g ovary/hr, or μg/corpus luteum/hr. With these units of ovarian output of progesterone, the mean values among treatments ranged between 49.7 and 477, 4.22 and 41.1, 0.93 and 6.39, and 11.2 and 83.6, respectively. No significant differences ($P > .25$) were found among the mean weights, that ranged between 439 and 531 mg, for the ovaries from which blood was collected, or among the mean blood flow rates, that ranged between 3.82 and 6.02 ml/g ovary/hr, for the ovaries from which blood was collected, indicating that the differences in ovarian progesterone

output were probably due to surgically induced alterations in influences emanating from the gravid uterus rather than inherent differences in the ovaries.

Taken together the results of the present study indicate that endocrine functions of the maternal gonad in the rabbit are more acutely dependent upon influences emanating from the gravid uterus than previously indicated by the work of Klein (1933), Greep (1941), and Schofield (1960). 20αOH-progesterone output from the ovaries of 21-day pregnant rabbits does not appear to be acutely dependent upon influences from the gravid uterus. However, the present results clearly indicate that ovarian secretion of progesterone at day 21 of gestation in rabbits is linked to the presence of the placentas. Whether this is a direct effect of the placentas on luteal tissue or is possibly mediated by the follicles, or both, as suggested by the work of Keyes and Nalbandov (1967), and Keyes and Armstrong (1968) has not been determined and merits further study. Whether or not the placentas must be attached to the uterus as a condition for exerting their influence on ovarian output has not been resolved in the rabbit and requires further study before definite conclusions may be reached.

The final conclusions based on the present study are: 1) progesterone, but not 20αOH-progesterone, secretion from ovaries in 21-day pregnant rabbits is dependent upon influences emanating from the gravid uterus; 2) the influence of the gravid uterus on ovarian output of progesterone has a life span of less than 24 hours; and 3) the component of the gravid uterus responsible for maintenance of ovarian secretion of progesterone is the placenta.

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APPENDIX A

TABLE VIII

ANALYSIS OF VARIANCE OF 20αOH-PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS BEFORE LAPAROTOMY OR COMPONENTS OF THE GRAVID UTERUS WERE REMOVED

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	60.88	15.22	0.30
Error	35	1778.11	51.09	
Total	39	1849.99		

TABLE IX

ANALYSIS OF VARIANCE OF 20αOH-PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS AFTER LAPAROTOMY OR COMPONENTS OF THE GRAVID UTERUS WERE REMOVED

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	17.38	4.34	0.28
Error	35	544.31	15.55	
Total	39	561.69		

TABLE X

COMPARISONS[a] OF MEAN SYSTEMIC PLASMA CONCENTRATIONS OF 20αOH-PROGESTERONE BEFORE AND AFTER LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	None (Laparotomy)[b]	Fetuses[b]	Fetuses Plus Placentas[b]	Entire Gravid Uterus[b]	Entire Gravid Uterus (6 hr)[c]
$\bar{d}_{X_1-X_2}$	2.15	1.97	1.18	1.89	3.26
$s_{\bar{d}_{X_1-X_2}}$	1.23	1.34	1.39	1.25	2.09
$t^d = \frac{\bar{d}_{X_1-X_2}}{s_{\bar{d}_{X_1-X_2}}}$	1.75	1.47	0.857	1.52	1.56
Degrees of Freedom	7	7	7	7	7

[a]Calculated as comparison of sample means with paired observations according to Steel and Torrie (1960), pp. 78-79, where $\bar{d}_{X_1-X_2}$ is the average difference between the means of pre- (X_1) and post-treatment (X_2) samples and s is the standard deviation.

[b]Post-treatment systemic plasma collected 24 hours after treatment operations.

[c]Post-treatment systemic plasma collected six hours after treatment operations.

[d]Tabulated $t_{.01}$ for seven degrees of freedom and a two-tailed test is 3.50; tabulated $t_{.05}$ for seven degrees of freedom and a two-tailed test is 2.36.

TABLE XI

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS BEFORE LAPAROTOMY OR COMPONENTS OF THE GRAVID UTERUS WERE REMOVED

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	322.51	80.63	1.70
Error	35	1659.64	47.42	
Total	39	1982.15		

TABLE XII

ANALYSIS OF VARIANCE OF PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS AFTER LAPAROTOMY OR COMPONENTS OF THE GRAVID UTERUS WERE REMOVED

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	1340.82	335.22	10.19[a]
Error	35	1551.33	32.90	
Total	39	2892.15		

[a] $P < .01$

TABLE XIII

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO PROGESTERONE CONCENTRATIONS IN SYSTEMIC PLASMA OF 21-DAY PREGNANT RABBITS AFTER LAPAROTOMY OR COMPONENTS OF THE GRAVID UTERUS WERE REMOVED

Treatment (Components Removed)	Entire Gravid Uterus[b]	Fetuses Plus Placentas[b]	None (Laparotomy)[b]	Fetuses[b]	Entire Gravid Uterus (6 hr)[c]
Mean	1.34	2.30	10.1	11.0	16.8
Value of $Q_{.01}$		3.85	4.42	4.75	4.99
Value of $Q_{.05}$		2.88	3.46	3.81	4.07
(35 degrees of freedom)					
Value of $D_{.01}$ (solid line)		11.04	12.68	13.62	14.31
Value of $D_{.05}$ (dashed line)		8.26	9.92	10.93	11.67
(pooled $s_{\bar{x}} = 2.87$)					

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Post-treatment systemic plasma collected 24 hours after treatment operations.

[c]Post-treatment systemic plasma collected six hours after treatment operations.

TABLE XIV

COMPARISONS[a] OF MEAN SYSTEMIC PLASMA CONCENTRATIONS OF PROGESTERONE BEFORE AND AFTER LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	None (Laparotomy)[b]	Fetuses[b]	Fetuses Plus Placentas[b]	Entire Gravid Uterus[b]	Entire Gravid Uterus (6 hr)[c]
$\bar{d}_{X_1-X_2}$	0.52	1.19	3.36	10.1	2.62
$s_{\bar{d}_{X_1-X_2}}$	0.55	0.89	0.79	2.90	0.68
$t^{d} = \frac{\bar{d}_{X_1-X_2}}{s_{\bar{d}_{X_1-X_2}}}$	0.93	1.33	4.23[e]	3.34[f]	3.86[e]
Degrees of Freedom	7	7	7	7	7

[a]Calculated as comparison of sample means with paired observations according to Steel and Torrie (1960), pp. 78-79, where $\bar{d}_{X_1-X_2}$ is the average difference between the means of pre- (X_1) and post-treatment (X_2) samples and s is the standard deviation.

[b]Post-treatment systemic plasma collected 24 hours after treatment operations.

[c]Post-treatment systemic plasma collected six hours after treatment operations.

[d]Tabulated $t_{.01}$ for 7 degrees of freedom and a two-tailed test is 3.50; tabulated $t_{.05}$ for 7 degrees of freedom and a two-tailed test is 2.36.

[e]$P < .01$

[f]$P < .05$

TABLE XV

ANALYSIS OF VARIANCE OF THE WEIGHTS OF THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	5.01	1.25	0.44
Error	35	98.54	2.82	
Total	39			

TABLE XVI

ANALYSIS OF VARIANCE OF THE BLOOD FLOW RATES OF THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	36.99	9.25	1.78
Error	35	182.33	5.21	
Total	39	219.53		

TABLE XVII

ANALYSIS OF VARIANCE OF THE NUMBER OF CORPORA LUTEA IN THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	78.64	19.65	4.39[a]
Error	35	157.01	4.48	
Total	39	235.65		

[a] $P < .01$

TABLE XVIII

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO THE NUMBER OF CORPORA LUTEA IN THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	Fetuses Plus Placentas[b]	None (Laparotomy)[b]	Fetuses[b]	Entire Gravid Uterus[b]	Entire Gravid Uterus (6 hr)[c]
Mean	4.1	6.5	6.5	7.6	8.2
Value of $Q_{.01}$		3.85	4.41	4.75	4.99
Value of $Q_{.05}$		2.88	3.46	3.81	4.07
(35 degrees of freedom)					
Value of $D_{.01}$ (solid line)		2.88	3.30	3.55	3.74
Value of $D_{.05}$ (dashed line)		2.17	2.59	2.85	3.05
(pooled $s_{\bar{x}} = 0.748$)					

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Ovarian venous blood collected 24 hours after treatment operations.

[c]Ovarian venous blood collected six hours after treatment operations.

TABLE XIX

ANALYSIS OF VARIANCE OF THE 20αOH-PROGESTERONE CONCENTRATIONS IN VENOUS PLASMA FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	4,543	1135.0	2.47
Error	35	16,072	459.2	
Total	39	20,615		

TABLE XX

ANALYSIS OF VARIANCE OF THE 20αOH-PROGESTERONE OUTPUT (μg/OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	1,386	346.4	2.16
Error	35	5,595	159.8	
Total	39	6,981		

TABLE XXI

ANALYSIS OF VARIANCE OF THE 20αOH-PROGESTERONE OUTPUT (μg/g OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	15,735	3934	2.58
Error	35	53,215	1520	
Total	39	68,950		

TABLE XXII

ANALYSIS OF VARIANCE OF THE PROGESTERONE CONCENTRATIONS IN VENOUS PLASMA FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	13,653	3,413	7.66[a]
Error	35	15,604	445.8	
Total	39	29,257		

[a] $P < .01$

TABLE XXIII

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO THE PROGESTERONE CONCENTRATIONS IN VENOUS PLASMA FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	Fetuses Plus Placentas[b]	Entire Gravid Uterus[b]	None (Laparotomy)[b]	Fetuses[b]	Entire Gravid Uterus (6 hr)[c]
Mean ng/ml plasma	49.7	50.0	358	421	477
Value of $Q_{.01}$		3.85	4.41	4.75	4.99
Value of $Q_{.05}$		2.88	3.46	3.81	4.07
(35 degrees of freedom)					
Value of $D_{.01}$ (solid line)		287	329	354	372
Value of $D_{.05}$ (dashed line)		215	258	284	304
(pooled $s_{\bar{x}} = 74.6$)					

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Ovarian venous blood collected 24 hours after treatment operations.

[c]Ovarian venous blood collected six hours after treatment operations.

TABLE XXIV

ANALYSIS OF VARIANCE OF THE PROGESTERONE OUTPUT (μg/OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	9,896	2,474	10.92[a]
Error	35	7,929	226.5	
Total	39	17,826		

[a] $P < .01$

TABLE XXV

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO THE PROGESTERONE OUTPUT (μg/OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	Fetuses Plus Placentas[b]	Entire Gravid Uterus[b]	None (Laparotomy)[b]	Entire Gravid Uterus (6 hr)[c]	Fetuses[b]
Mean (μg/ovary/hr)	4.22	6.64	29.2	39.0	41.1
Value of $Q_{.01}$		3.85	4.41	4.75	4.99
Value of $Q_{.05}$		2.88	3.46	3.81	4.07
(35 degrees of freedom)					
Value of $D_{.01}$ (solid line)		20.48	23.46	25.27	26.55
Value of $D_{.05}$ (dashed line)		15.32	18.41	20.27	21.65
(pooled $s_{\bar{x}} = 5.321$)					

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Ovarian venous blood collected 24 hours after treatment operations.

[c]Ovarian venous blood collected six hours after treatment operations.

TABLE XXVI

ANALYSIS OF VARIANCE OF THE PROGESTERONE OUTPUT (μg/g OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	105,654	26,413	16.0[a]
Error	35	57,802	1,652	
Total	39	163,455		

[a] $P < .01$

TABLE XXVII

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO THE PROGESTERONE OUTPUT (μg/g OVARY/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	Fetuses Plus Placentas[b]	Entire Gravid Uterus[b]	None (Laparotomy)[b]	Fetuses[b]	Entire Gravid Uterus (6 hr)[c]
Mean (μg/g ovary/hr)	11.2	13.3	52.0	78.2	83.6
Values of $Q_{.01}$ (35 degrees of freedom)		3.85	4.41	4.75	4.99
Value of $D_{.01}$ (pooled $s_{\bar{x}} = 8.62$)		33.2	38.0	41.0	43.0

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Ovarian venous blood collected 24 hours after treatment operations.

[c]Ovarian venous blood collected six hours after treatment operations.

TABLE XXVIII

ANALYSIS OF VARIANCE OF THE PROGESTERONE OUTPUT (μg/CORPUS LUTEUM/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Treatment	4	50,824	12,706	13.02[a]
Error	35	34,008	976	
Total	39	84,833		

[a] $p < .01$

TABLE XXIX

NEWMAN-KEUL'S SEQUENTIAL RANGE TEST[a] APPLIED TO THE PROGESTERONE OUTPUT (μg/CORPUS LUTEUM/HR) FROM THE OVARIES CANNULATED IN 21-DAY PREGNANT RABBITS FOLLOWING LAPAROTOMY OR REMOVING COMPONENTS OF THE GRAVID UTERUS

Treatment (Components Removed)	Entire Gravid Uterus[b]	Fetuses Plus Placentas[b]	None (Laparotomy)[b]	Entire Gravid Uterus (6 hr)[c]	Fetuses[b]
Mean (μg/corpus luteum/hr)	0.931	1.26	4.42	4.94	6.39
Value of $Q_{.01}$ (35 degrees of freedom)		3.85	4.41	4.75	4.99
Value of $D_{.01}$ (pooled $s_{\bar{x}} = 0.662$)		2.55	2.921	3.15	3.30

[a]Snedecor and Cochran (1967), pp. 273-274.

[b]Ovarian venous blood collected 24 hours after treatment operations.

[c]Ovarian venous blood collected six hours after treatment operations.

APPENDIX B

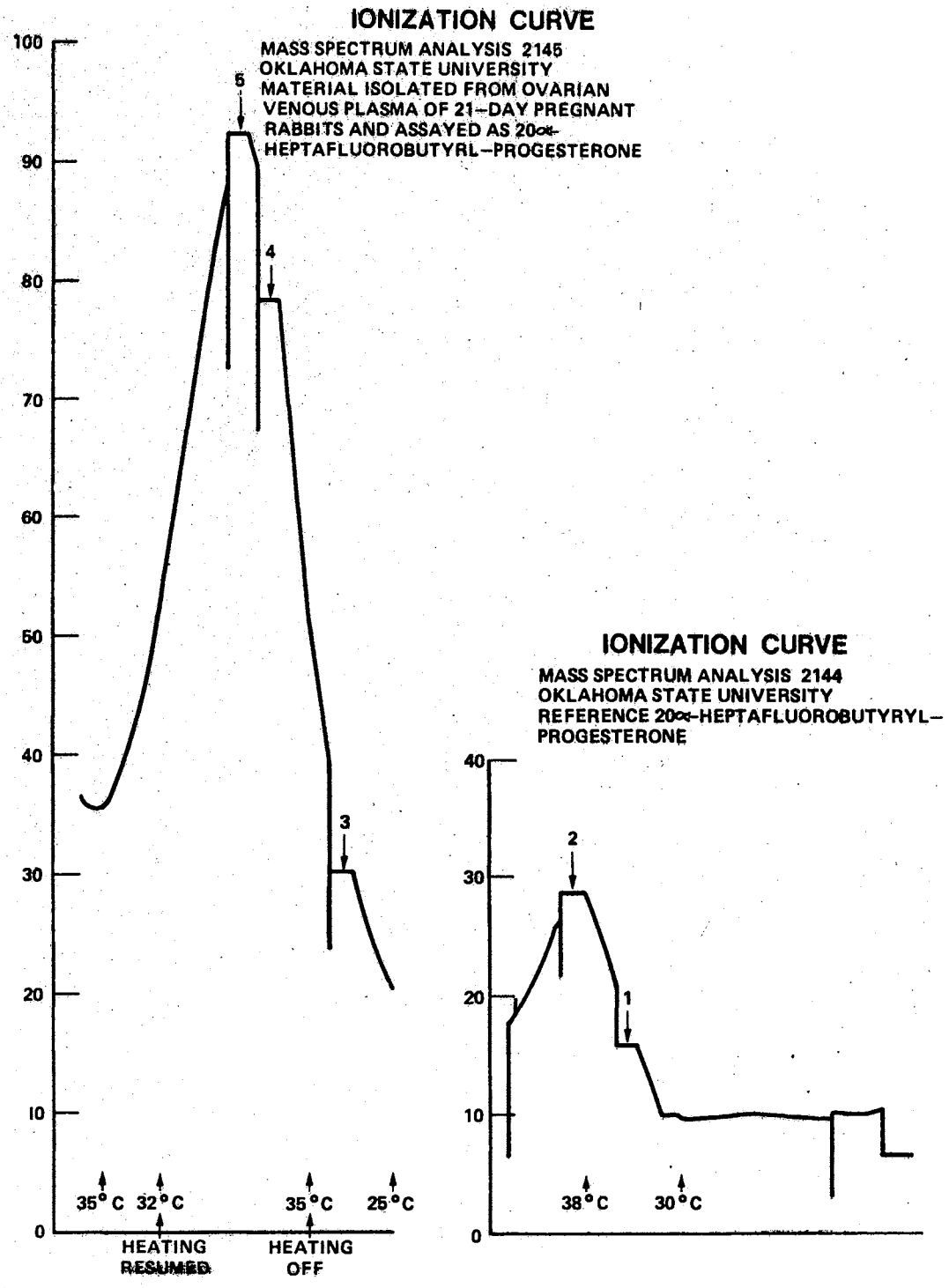

Figure 6. Mass Spectrometer Ionization Curves of Reference 20α-Heptafluorobutyryl-Progesterone and Material Isolated From Ovarian Venous Plasma. (Arrows 2 and 5 indicate the points at which scans were taken that were subsequently used to compose Figure 4.)

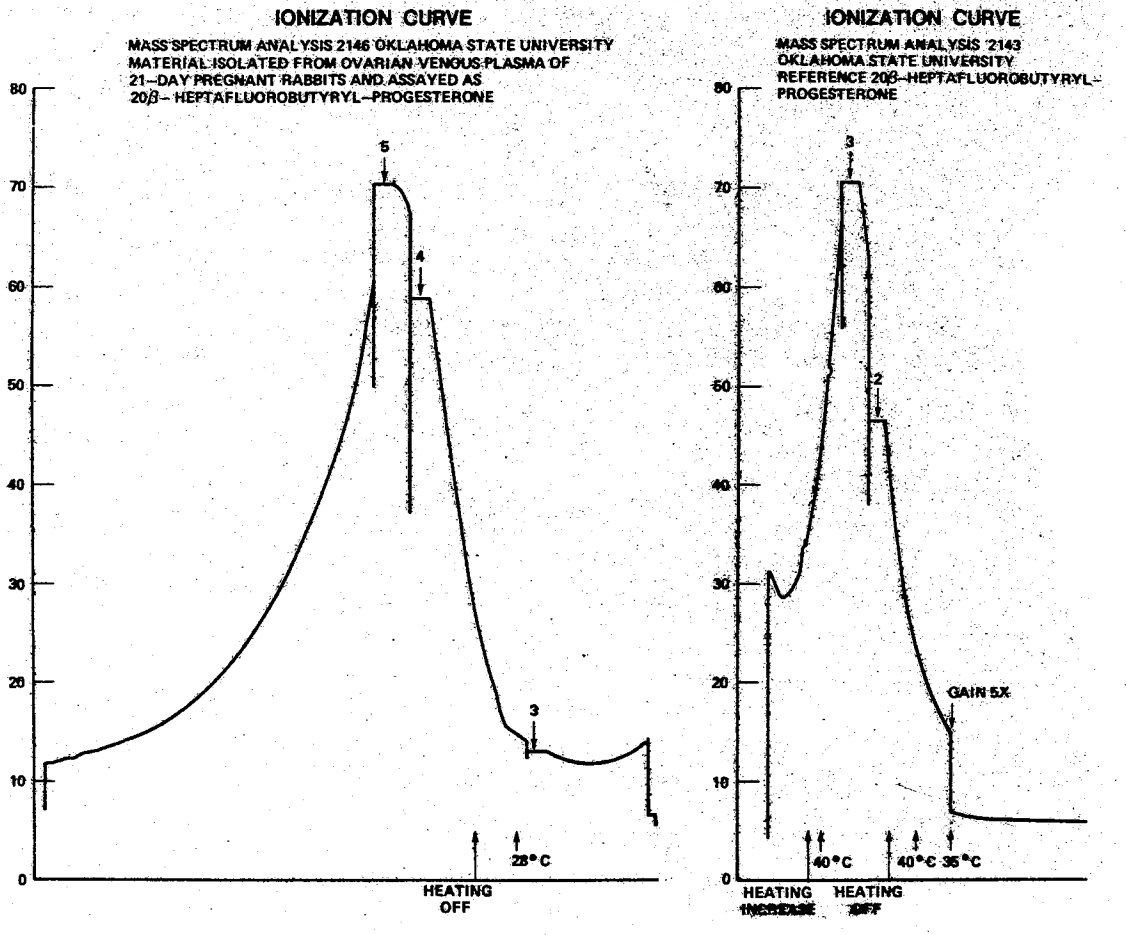

Figure 7. Mass Spectrometer Ionization Curves of Reference 20β-Heptafluorobutyryl-Progesterone and Material Isolated From Ovarian Venous Plasma. (Arrows 2 and 5 indicate the points at which scans were taken that were subsequently used to compose Figure 5,)

VITA

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Doctor of Philosophy

Thesis: ACUTE EFFECTS OF REMOVING COMPONENTS, OR THE ENTIRE GRAVID UTERUS, ON SYSTEMIC PLASMA CONCENTRATIONS AND OVARIAN OUTPUT OF 20αOH-PROGESTERONE AND PROGESTERONE IN 21-DAY PREGNANT RABBITS

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